STUDIES ON GONADAL STEROIDS IN RELATION TO MATURATION IN THE JAPANESE QUAIL

by

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ABSTRACT

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Plasma levels of testosterone (T), oestradiol-17β (E_2-17β) and progesterone (P) were studied using immuno-assays in relation to age, sexual maturity and to Vitamin D_3 metabolism of male and female Japanese Quail. Quail raised on 8L:16D (SD) remained sexually immature while quail on 16L:8D (LD), 24LL or d70TLD became sexually mature. Female birds came into lay around d45 or around the 29th long day in quail transferred from SD to LD at d70 (d70TLD). Plasma steroid levels were dependent on the sexual maturity with lowest levels found in sexually immature quail. Once the time of egg-laying had been reached plasma E_2-17β and P levels were higher in females than in males while T levels were higher in males. In quail bled sequentially during sexual maturation plasma P and E_2-17β levels in females rose prior to the onset of lay. T levels in male quail started to rise at the time of rapid testicular development. The steroid levels observed help to explain the changes in Vitamin D_3 metabolism seen with sexual maturation and the differences between the sexes.

Diurnal changes in plasma steroid levels were studied in laying females and adult males. P levels in females peaked around the time of the expected LH surge when an ovulation occurred but no peak was seen on days when no ovulation occurred. E_2-17β levels were lowest around the onset of light in both ovulating and non- ovulating females and during the night in the males. Plasma T in males were maximal in the 7th h of light. It is proposed that as in chickens P is the hormone most likely to initiate the ovulatory surge of LH. The timing of ovulatory events would seem to be triggered by the onset of light in quail.

An histochemical study was performed on the 5 yolk-filled follicles from laying quail to locate areas containing the enzymes 3β-Hydroxy-steroid dehydrogenase (3β-HSD) and 17β-HSD (both involved in steroido-genesis). Granulosa cells from the largest follicles showed most 3β-HSD while 17β-HSD was located within the thecal cells of medium-sized follicles. These sites parallel the postulated sites of steroidogenesis observed in chicken follicles.

A study on hCG-stimulated steroid secretion from dispersed testicular cells and from semi-purified Leydig cells with age showed that T was produced by both cellular incubates while E_2-17β was not produced by the Leydig cells. There was a change in responsiveness with age with most E_2-17β secreted in immature quail while T secretion increased with sexual maturation and then decreased.

Lastly, the effect of administration of either E_2-17β, oestradiol-17α, oestrone (E_1) or oestriol on oviduct weight, plasma calcium and on Vitamin D_3 metabolic enzymes was studied in immature female quail. All 4 oestrogens increased oviduct weight. E_2-17β and E_1 also increased plasma calcium and stimulated the renal 25-hydroxycholecalciferol-1α-hydroxylase enzyme. The possible involvement of oestrogens other than E_2-17β in regulating Vitamin D_3 metabolism **in vivo** is discussed.

To my mum, dad and 'big' sister.

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C O N T E N T S

LIST OF FIGURES

Page

LIST OF TABLES

ABBREVIATIONS

ATP	Adenosine Triphosphate
BW	Body Weight
c'AMP	cyclic adenosine-3'-5'-monophosphate
°C	Degrees Centigrade
CC	Cholecalciferol, Vitamin D_3
Ci	Curies (1 Ci = 2.2 x 10^{12} dpm)
Ca^{2+}	ionic calcium
d	days
dpm	disintegrations per minute
DHCC	Dihydroxycholecalciferol
FSH	Follicle Stimulating Hormone
FSH-RH	FSH-Releasing Hormone
Fn	Numbering of avian yolk-filled follicles in order of decreasing size (F1 being the next to ovulate)
g	grams
h	hour
HCC	Hydroxycholecalciferol
hCG	Human Chorionic Gonadotrophin
^3H	Tritium
1α-hydroxylase	Renal 25HCC-1α-hydroxylase enzyme
24-hydroxylase	Renal 25HCC-24R-hydroxylase enzyme
25-hydroxylase	Hepatic CC-25-hydroxylase enzyme
I.U	International Unit
l	Litre
LD	Long Photoperiods
LH	Luteinizing Hormone
LH-RH	LH-Releasing Hormone
M	Molar
min	minute
ml	Millilitre
mg	Milligram
μ	(prefix)-micro-x10^{-6}
n	number in group
ng	nanogram - x 10^{-9}g
NL	Normal Light
p	(prefix) - pico - x 10^{-12}
RIA	Radioimmunoassay
RL	Reverse Light
RNA	Ribonucleic Acid
rpm	revolutions per minute
S.E.M.	Standard Error of the Mean
SD	Short Photoperiods
xL:yD	x hours of light followed by y hours of dark.

CHAPTER 1 - REVIEW OF RELEVANT LITERATURE

1.1. Developmental Patterns

1.1.1 Japanese Quail (Coturnix coturnix japonica): A Photostimulated Species

As in many avian species from temperate zones the gonadal activity of the Japanese Quail (Coturnix coturnix japonica) is controlled with considerable precision within the annual cycle by daylength (Farner, 1961). Long daily photoperiods (>12 hours light/24 hours) cause the uniform growth of testes and ovaries to full reproductive function whereas no development occurs in birds held on photoperiods of 6 hours light/24 hours (Follett and Farner, 1966). This effect has been shown to occur both in laboratory conditions where the daylength is held constant, or under natural conditions where the testes develop as the daylength increases and then regress as the daylength shortens below the critical daylength (Follett and Maung, 1978).

While gonadal development is affected by the length of the photoperiod, somatic development is not: quail maintained on either short or long photoperiods reach full adult weight at the same age. Female quail raised on short photoperiods weigh less than their sexually mature counterparts due to the extra weight associated with the ovary, oviduct and calcified eggs in the sexually mature female. Plumage however, is unaffected by daylength and all birds obtain their adult plumage between 21 and 28 days after hatching.

It has been reported that the testes of Japanese Quail mature under short photoperiods after 10 to 12 weeks (Oishi, 1978: Oishi and Konishi, 1978) provided that the

temperature is maintained at 25°-27°C. Follett and Farner (1966) maintained their quail at 19°-20°C and Oishi (1978) suggests this is why no sexual development occurred in their birds. Quail used in this thesis, when maintained under short photoperiods at 20°C did not mature sexually.

1.1.2 Testicular Development

The essential sex organs of male birds are the paired testes which are housed in the body cavity (unlike most mammals), just ventral to the anterior end of the kidneys, the left testis often being larger than the right. Each testis is surrounded by a fibrous coat, the tunica albuginea and internally they consist of a mass of convoluted seminiferous tubules which are, unlike mammals, anastomotic and not restricted by septae. The seminiferous tubules are lined by the germinal epithelium consisting of developing germ cells and Sertoli cells. The interstitial space between the tubules contains blood capillaries, lymphatics and interstitial or Leydig cells. The Leydig and Sertoli cells are the cells which secrete the steroid hormones released from the testes.

When the male is photostimulated, i.e. when somatically mature quail are transferred to long photoperiods, the combined testicular weight increases from 8mg. to 3000mg. within 3 weeks (Lofts, Follett and Murton, 1970). The interstitial cells are often compressed into tight concentrations as a result of the expansion of the tubules, while in a regressed testis the interstitial tissue is more conspicious. A detailed review of avian testicular development has been reported by Lofts and Murton (1973).

The control and development of testicular function by gonadotrophic hormones together with the sites of secretion of gonadal steroid hormones will be discussed later in

1.1.3 Ovarian Development

In most species of birds, the left ovary becomes the dominant and functional sex organ, while the right ovary regresses. The ovary is positioned close to the kidneys and supplied with blood by the ovarian artery, usually a branch of the left renolumbar artery. As in the male, the ovary increases in size when the bird is photostimulated. In the quiescent state the ovary is a compact, flattened organ with small follicles, giving the surface a granular appearance. As it develops under photostimulation, the follicles become distended and bulge out from the surface of the ovary. The largest follicles become suspended from the surface by a narrow isthmus of tissue, the pedicle. The surface of the mature follicles are highly vascularised, except for a narrow band, the stigma. It is at the stigma that the follicle will rupture. Follicular development occurs from the many thousands of oocytes present, of which a very small percentage ever mature fully and the majority undergo atresia while still in an early stage of maturation (Lofts and Murton, 1973).

Deposition of yolk into the follicles is an integral part of their development. When the follicles are small they accumulate little fatty material but some white yolk is deposited a few weeks prior to maturity. A rapid growth phase occurs over approximately 7 days, which ends in ovulation, when accumulation of the yellow yolk occurs (Gilbert, 1972). After ovulation, the ruptured follicle regresses, disintegrates and becomes cleared by phagocytic action. The post ovulatory follicle is still active during the first 24h of its life. In chickens its removal delays the oviposition of the egg derived from it (Rothchild and Fraps, 1944). Unlike mammals no persistent corpora lutea

are formed.

The ovary of a sexually mature female bird is composed of a hierarchy of follicles. Each follicle consists of an outer thecal cell layer and an inner granulosa cell layer. The follicle, due to ovulate next is the largest in the ovary and is designated F1 or C1. The remaining yellow yolk-filled follicles are numbered F2, F3, F4 according to decreasing size until all yolky follicles have been numbered (normally five yolky follicles exist in quail ovaries) and only the small white follicles remain.

Follicular maturation is controlled by gonadotrophic hormones and the feedback effects of gonadal steroids. The factors involved in the control of the egg-laying cycle are discussed in Chapter 1.4.

1.1.4 Gonadal Steroid Hormones Found in Avian Species and their plasma levels during sexual maturation.

The principal avian ovarian hormones are oestrogens and progesterone while androgens are the major testicular steroid hormones. In birds, as in mammals, testosterone appears to be the most important androgen. Structural formulas of some of the major avian gonadal steroid hormones are shown in Figure 1.1

A) The Female

Levels of gonadal steroid hormones in the plasma of laying birds have been reported frequently, and are reviewed in a later section (Chapter 1.4). However, there are few reports on the levels of plasma steroids in birds prior to puberty. Most of these reports concentrate on plasma steroid levels in the prepubertal chicken.

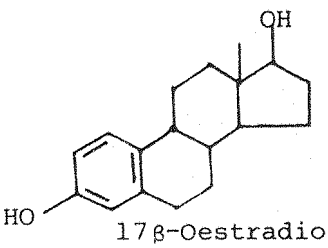
Figure 1.1

Structural Formulae of Some of the Major Avian Gonadal Steroid Hormones

A-D:- Oestrogens
E-F:- Androgens
G:- Progesterone

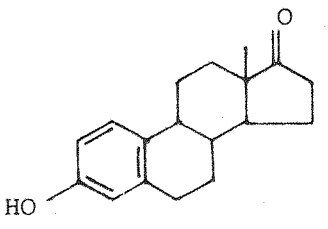
The principal ovarian hormones are the oestrogens and progesterone while the androgens are the major testicular hormones.

A)



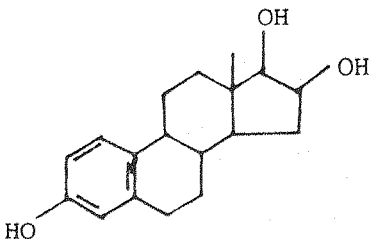
17β-Oestradiol

B)



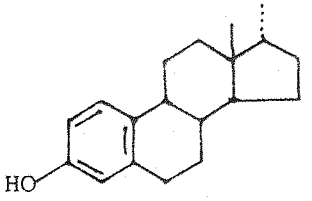
Oestrone

C)



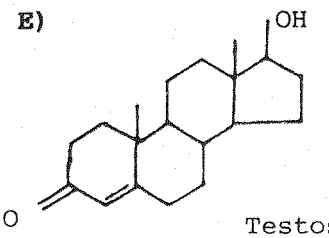
Oestriol

D)



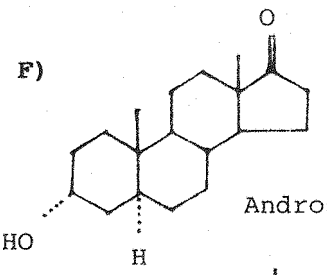
17α-Oestradiol

E)



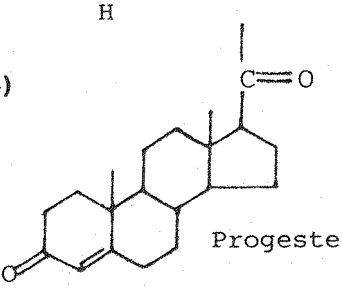
Testosterone

F)



Androsterone

G)



Progesterone

The principal oestrogens occurring in the urine, blood and ovaries of birds are oestrone, oestradiol-17β and oestradiol-17α. Urine from non-laying hens was found to contain less oestrone than from laying hens (Common, Ainsworth, Hertelendy and Mathur, 1965), with rising urinary levels of oestrone occurring from 2 weeks prior to laying. Less oestradiol-17β was detected in urine from non-laying hens than from laying hens (Hertelendy, Taylor, Mathur and Common, 1965), with urinary levels of oestradiol-17β always lower than urinary oestrone levels. Radioactive oestradiol-17α was found in hen's urine together with oestradiol-17β and oestrone after injections of either ^{14}C-labelled oestradiol-17β or ^{14}C-labelled oestrone, (Common, Mathur, Mulay and Henneberry, 1969). The percentage of the total phenolic steroids in the urine as oestradiol-17β was higher and the percentage as oestrone and oestradiol-17α was lower when the hen was not in lay compared with when she was. These authors also showed that irrespective of the radioactive oestrogen injected, the major radioactive steroids in the urine were oestradiol-17β, oestrone and oestradiol-17α, in that order, in the non-laying hen. This order changed in the laying hen to oestrone and oestradiol-17β or oestradiol-17α. Present also in the laying hen's urine are oestriol and 16,17-epiestriol (Mathur and Common, 1967).

O'Grady and Heald (1965) reported that the plasma of laying hens contained oestrone and oestradiol-17β, based on determinations using a double isotope dilution technique. The presence of oestradiol-17β and oestrone in the plasma of laying hens was also reported by Peterson and Common (1972) who used a radioimmunoassay (RIA) for oestradiol which measured principally oestradiol-17β, but also measured oestrone. Chan and Common (1974) identified radioactive oestradiol-17α as well as radioactive oestradiol-17β in the plasma of laying hens after ^{14}C- labelled oestrone had been injected **in vivo**. Thus they showed that oestradiol-17α is

also a major phenolic steroid in the laying hen.

Few reports contain details of plasma oestrogen levels with sexual maturation. Plasma levels of oestradiol-17β in immature chickens (Schrocksnadel, Bator and Frick, 1973) or in immature turkey hens (Wineland and Wentworth, 1975) were found to be lower compared with those of the laying hens when oestradiol was measured by RIA. Plasma oestrogen levels in pullets and in the domestic hen have also been reported to show an increase during maturation, reaching a maximum just prior to the onset of lay (Peterson and Webster, 1974; Senior, 1974). Plasma oestrogen levels decreased once laying had been established, (Senior 1974; Wineland and Wentworth, 1975). Péczely, Pethes and Rudas (1980) showed that sexually immature quail raised on 6L:18D had lower oestradiol levels than those which were sexually mature, raised on 18L:6D.

Apart from the role which oestrogens may play in the growth and development of the ovarian follicles and possibly in the release of the hormone initiating ovulation (discussed later in Chapter 1.4), they are also involved in other metabolic changes. Endogenous oestrogens are responsible for the increase in plasma lipids seen during sexual maturation in quail (Nirmalan and George, 1972) and in chickens (Balnave, 1971). Tamoxifen, an anti-oestrogen, if given with oestradiol benzoate to female quail, inhibits the oestradiol induced increase in fatty acid levels (Pageaux, Laugier, Duperray, Pacheco and Brard, 1980). Oestrogens also stimulate hepatic protein synthesis: they induce the formation of vitellin, a phospholipoprotein, only found in mature females, which binds calcium forming a non-diffusable protein-calcium complex (Griminger, 1976). Oestrogens also stimulate the synthesis of most of the proteins of albumen, in the oviduct (Gilbert, 1972). Oestrogens have also been implicated in the regulation of Vitamin D_3 metabolism, and hence in calcium homeostasis in the laying bird (reviewed in

Chapter 1.2). In addition oestrogens stimulate the growth and development of the oviduct, (see Chapter 1.1.5 for review).

Progesterone is the other principal gonadal steroid hormone in the female bird. It is this hormone which is thought to induce the release of the ovulating hormone (discussed later in Chapter 1.4). Early reports showed that progesterone was present both in the plasma of the laying hen (Fraps, Hooker and Forbes, 1948) and in the plasma of the non-laying hen (Fraps, Hooker and Forbes, 1949). Levels of plasma progesterone, determined by RIA (Furr, 1973), showed that plasma progesterone levels were higher in the laying hen (3.13ng/ml) compared to those of the non-laying (0.35ng/ml), moulting, ovariectomised or hypophysectomised hen. Plasma progesterone levels in maturing hens have since been shown to rise until the 15th week of age and then fall only rising again at the time of the first egg (Muray, Pethes and Péczely, 1980).

Testosterone has also been found in the plasma of female birds. However, no significant changes in the concentration of testosterone during sexual maturation or between laying and non-laying hens have been found (Schrocksnadel, Bator and Frick, 1971: Muray et al, 1980). The plasma concentrations of other androgens possibly formed in the ovaries have not yet been investigated.

The relative contributions which these hormones play in the development of the oviduct and in ovulation are discussed elsewhere in this thesis.

B) The Male

The major gonadal steroid hormones in the male are androgens, in particular testosterone. There are two important pathways for the synthesis of testosterone shown

Figure 1.2 - Testicular Steroid Biosynthesis

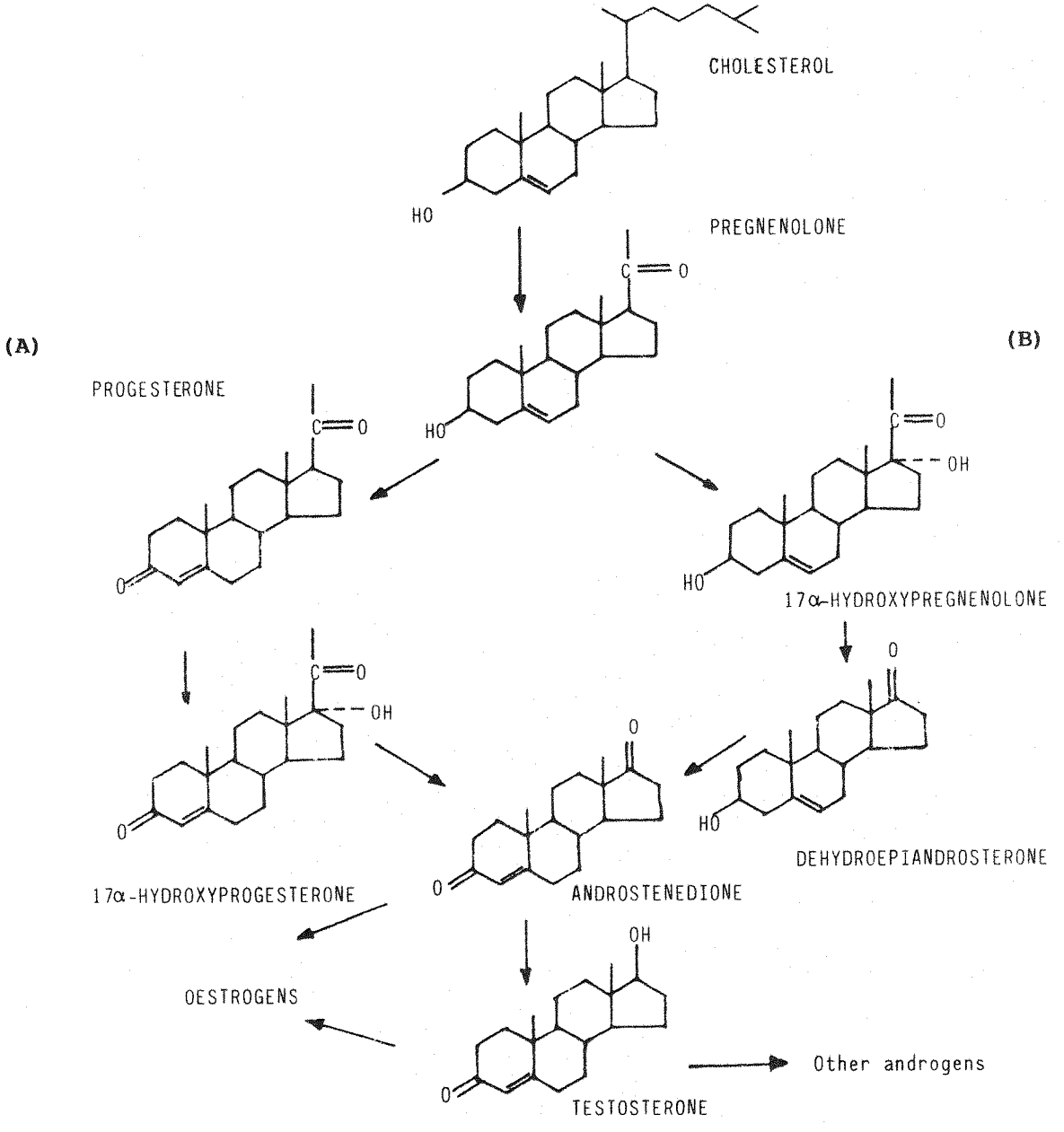
The two major biosynthetic pathways by which testosterone is synthesised:-

(A) The so-called 4-ane pathway through progesterone and

(B) The so-called 5-ane pathway through 17α-hydroxypregnenolone.

In each case, cholesterol is the principal precursor.

Testosterone is the major gonadal steroid hormone in the male bird. Both the above pathways exist in the hen and quail as all the intermediate steroids in the two pathways have been shown to be produced by the testis (see text for references).



in Figure 1.2. All the intermediate steroids in the two pathways have been shown to be produced by the testes of chickens (Nakamura and Tanabe, 1972; Galli, Irusta and Wasserman, 1973; Guichard, Cedard and Haffen, 1973) and quail (Guichard, Cedard, Haffen and Scheib, 1973).

Plasma levels of testosterone, measured using a competitive protein binding method, have been shown to be higher in the sexually mature cock than in the immature bird (Schrocksnadel, Bator and Frick, 1971). Testosterone levels of mature two year old cocks were also found to be about three times higher than those of laying hens of the same age. Plasma testosterone levels in Japanese Quail have been shown to increase slowly throughout maturation (Follett and Maung, 1978). The speed and magnitude of the testosterone increase was dependent on the length of the photoperiod on which the quail were raised. Thus, a slower rate of increase was seen in birds raised on 12L:12D to that seen in birds raised on 20L:4D. In birds raised on natural daylengths a decline in plasma testosterone levels preceded the regression of the testes. A similar decline in plasma testosterone levels prior to gonadal regression has been observed in the Peking duck (Jallageas and Assenmacher, 1974). Dehydroepiandrosterone and androstenedione have also been identified in the plasma of male pigeons (Rivarola, Snipes and Migeon, 1968).

The presence of the female sex hormones, progesterone and oestrogens, in male plasma is less well documented. The testes do produce these steroid hormones and some reports show that they are present within the peripheral plasma. Progesterone is present in the peripheral plasma of mature intact (0.44ng/ml) and castrated (0.16ng/ml) cockerels (Furr, 1973) suggesting that the adrenal gland may also contribute to the levels of reproductive steroids. These progesterone levels are both lower than those seen in laying-hens (3.13ng/ml) but the levels in intact cockerels

are similar to those of non-laying hens. Changes in plasma progesterone levels in male birds during sexual maturation has not been reported previously.

Plasma levels of oestradiol, measured by RIA, in the mature cockerel (6-7pg/ml) are higher than those found in the prepubertal cockerel of 4 months (1.0pg/ml) (Schrocknadel, Bator and Frick, 1973), thus indicating that plasma oestradiol levels in cockerels increase during maturation. Plasma oestradiol levels in the mature cockerel as well as in the 4 month old cockerel were lower than those seen in female hens of the same ages. The presence of other oestrogens within the plasma of male birds has not been reported.

1.1.5 Development of the Oviduct: Dependence on Gonadal Steroids

It is the left oviduct, like the left ovary, which becomes the functional organ in birds. The oviduct remains small in immature birds and increases in size as maturity approaches. The oviduct is attached to the body wall by dorsal and ventral ligaments and consists of five distinct areas, namely: the infundibulum, magnum, isthmus, shell gland or uterus and the vagina. Figure 1.3. shows an oviduct from a sexually mature female. The extruded ovum together with the yolk is received from the ovary via the funnel-like infundibulum (1). It is fertilised in the upper thin-walled neck of the oviduct before passing onto the magnum (2), the largest single portion of the oviduct. It is in the magnum where most of the albumen, the egg-white proteins, are synthesised and deposited around the egg. The egg next passes into a less glandular region, the isthmus (3), where the two shell membranes are secreted. These are 'cemented' together with albumin. The calcareous shell, together with the pigment, is added in the wider

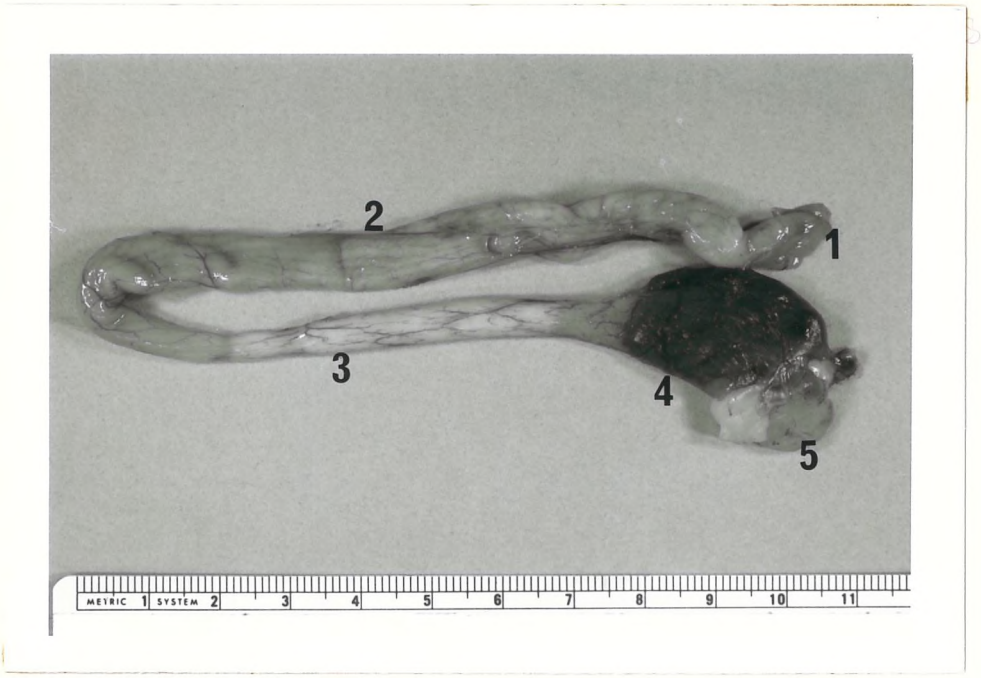


Figure 1.3

The Oviduct of the Laying Japanese Quail

The regions of the oviduct are marked:-

1). The infundibulum

2). The magnum

3). The isthmus

4). The shell-gland or uterus

5). The vagina

shell-gland (4) where the shell is secreted in the form of insoluble calcium salts. In chickens the pigment is evenly distributed throughout the shell during the last 5h before the egg is laid (Warren and Conrad, 1942) while in the quail egg, a deeply pigmented shell, the pigment is deposited 3½h before the egg is laid (Woodward and Mather, 1964). The vagina (5) plays no part in the formation of the egg but may be involved in the final expulsion of the egg from the oviduct at oviposition.

It has been known for some time that sex hormones, in particular the oestrogens, can stimulate the growth of the oviduct in avian species (Witschi and Fugo, 1940; Dorfman and Dorfman, 1948). In contrast testosterone, if injected alone, does not stimulate an increase in oviduct weight but if oestrogens are injected concomitantly then a synergistic effect on oviduct growth is seen (Yu and Marquardt, 1973). Studies on immature oviduct of quail have shown that high doses of oestradiol benzoate (1mg/kg Body weight (BW)) have less effect in increasing oviduct weight in hypophysectomised birds compared with control birds (Laugier, Sandoz, Brard and Sonnerschien, 1978). Administration of either adrenocorticotrophic hormone (ACTH) or corticosterone to the hypophysectomised birds restored the oestrogen induced increase in oviduct weight (Laugier, Sonnerschien and Brard, 1980). This suggests that the pituitary (via ACTH and corticosterone) may have a synergistic effect with oestrogens on the oviduct. Responses to low doses of oestradiol (0.1mg/kg BW) were however unaffected by hypophysectomy, ACTH or corticosterone administration. These results suggest that high doses of oestrogens are more effective in sensitising the oviduct to the synergistic effects of corticosterone.

Sex hormones have also been shown to be involved in stimulating ovalbumin and avidin production (two of the egg-white proteins). It is still unclear which steroids

are involved since Brant and Nalbandov (1956) showed that ovalbumin production could be stimulated by combinations of oestradiol and progesterone or oestradiol and testosterone, while O'Malley, McGuire and Korenman (1967) showed that the synthetic oestrogen, diethylstilbestrol, injected alone, would cause an increase in ovalbumin production. Avidin formation has been shown to be stimulated by stilbestrol plus progesterone **in vivo** (Hertz, Fraps and Sebrell, 1943) while O'Malley (1967) showed that progesterone alone could cause an increase in avidin production **in vitro**.

Steroid hormones therefore do play a role in the growth and development of the oviduct. Oestrogens appear to be the most potent stimulators of oviduct growth and their presence is also required for full glandular and protein synthetic development. Progesterone and testosterone can also stimulate oviduct growth and progesterone is required for avidin production **in vivo**. The development of the oviduct is therefore dependent on the steroids secreted by the developing ovary.

1.2 Vitamin D_3 and Metabolites

1.2.1 Introduction

Vitamin D_3 or cholecalciferol (CC) is a sterol which requires metabolic modification in order to become hormonally active. Its function is to ensure that levels of calcium within the plasma are maintained. A physiological process which alters the calcium requirement of the animal may therefore affect Vitamin D_3 metabolism. One such process is observed within any species whose reproductive cycle involves calcification of an egg-shell prior to oviposition.

1.2.2 Metabolism of Vitamin D_3

Cholecalciferol is produced from dehydrocholesterol in the skin by the action of ultraviolet radiation. Some cholecalciferol can also be obtained from dietary sources. The metabolism of cholecalciferol is a necessary step in the regulation of calcium homeostasis, since cholecalciferol itself is inactive in regulating plasma calcium levels. Cholecalciferol is first hydroxylated at C_{25} in the liver to produce 25-hydroxycholecalciferol (25HCC) (Ponchon, Kennan and DeLuca, 1969). After the hepatic hydroxylation 25HCC is transported to the kidney where it is further hydroxylated at C_1 to form 1,25 dihydroxycholecalciferol (1,25DHCC) (Fraser and Kodicek 1970; Norman, Midgett, Myrtle and Nowicki, 1971; Holick, Schnoes, DeLuca, Suda and Cousins, 1971).

In addition to the 25HCC-1α-hydroxylation of the 25HCC the kidney also catalyses a 25HCC-24R-hydroxylation, producing 24,25DHCC (Holick, Schnoes, DeLuca, Gray, Boyle and Suda, 1972). 24,25DHCC can be further metabolised to form

Figure 1.4

The Pathway of Cholecalciferol Metabolism

Showing the conversion of CC to 25HCC in the liver by the enzyme cholecalciferol-25-hydroxylase and then in the kidney to the active metabolite 1,25DHCC by the enzyme 25-hydroxycholecalciferol-1α-hydroxylase. The sites of action of 1,25DHCC are also shown. 25HCC is also metabolised in the kidney to 24,25DHCC by the enzyme 25-hydroxycholecalciferol-24R-hydroxylase, to 25,26DHCC by 25-hydroxycholecalciferol-26-hydroxylase and to 25,26DHCC by 25-hydroxycholecalciferol-26-hydroxylase. 1,25DHCC and 24,25DHCC are further metabolised in the kidney to 1,24,25THCC, the first inactivation step for 1,25DHCC.

Production of 1,25DHCC is stimulated by Parathyroid hormone (PTH) and low plasma calcium (Ca^{2+}) and phosphorous (P) while production of 24,25DHCC occurs in the presence of normal plasma calcium and phosphorous levels.

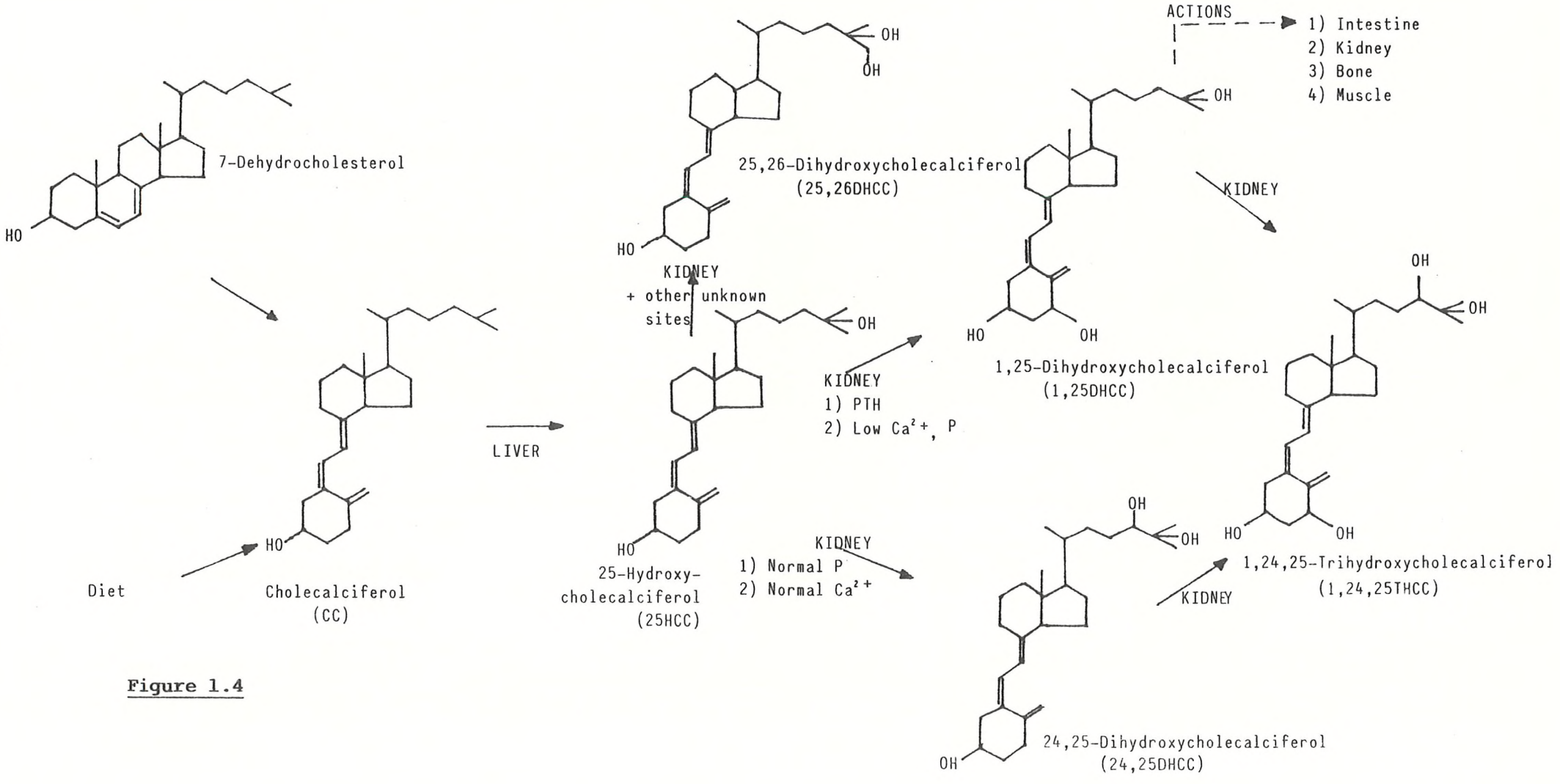

7-Dehydrocholesterol
HO
Diet
HO
Cholecalciferol
(CC)
LIVER
OH
OH
25,26-Dihydroxycholecalciferol
(25,26DHCC)
HO
KIDNEY
+ other unknown
sites
OH
HO
25-Hydroxy-
cholecalciferol
(25HCC)
KIDNEY
1) PTH
2) Low Ca2+, P
KIDNEY
1) Normal P
2) Normal Ca2+
ACTIONS
1) Intestine
2) Kidney
3) Bone
4) Muscle
OH
KIDNEY
HO
OH
1,25-Dihydroxycholecalciferol
(1,25DHCC)
OH
OH
OH
HO
OH
1,24,25-Trihydroxycholecalciferol
(1,24,25THCC)
KIDNEY
OH
OH
24,25-Dihydroxycholecalciferol
(24,25DHCC)

Figure 1.4

the more polar metabolite, 1,24,25 trihydroxycholecalciferol (1,24,25THCC) (Holick, Kleiner-Bossaller, Schnoes, Kasten, Boyle and DeLuca, 1973). Suda, DeLuca, Schnoes, Tanaka and Holick (1970) isolated and identified yet another Vitamin D_3 metabolite, 25,26 dihydroxycholecaliferol (25,26DHCC), from the plasma of pigs. This metabolite was found to be formed by kidney homogenates from chicks fed on Vitamin D_3 - supplemented diets and was not present in Vitamin D_3 - deficient chicks, (Tanaka, Shepard, DeLuca and Schnoes, 1978). The pathways involved in cholecalciferol metabolism are summarised in Figure 1.4.

1.2.3 The Actions of Vitamin D_3 and Metabolites

In vivo work on nephrectomised animals showed that injections of 25HCC did not stimulate intestinal calcium transport (Boyle, Miravet, Gray, Holick, and DeLuca, 1972) phosphate transport (Chen, Castillo, Korycka-Dahl and DeLuca, 1974) or mobilise calcium from bone (Holick, Garabedian and DeLuca, 1972). Yet when 1,25DHCC was injected into the same animals all three responses occurred. Intact animals showed similar responses to 1,25DHCC (calcium transport: - Myrtle and Norman, 1971; Holick, Suda, Tanaka and DeLuca, 1971: phosphate transport:- Wasserman and Taylor, 1973: bone metabolism :- Raisz, Trummel, Holick and DeLuca, 1972; Reynolds, Holick and DeLuca, 1973), implying that 1,25DHCC or a further metabolite is the active form of Vitamin D_3. Evidence now suggests that while stimulation of intestinal calcium transport only requires 1,25DHCC, bone resorption i.e. calcium mobilisation from bone, requires the presence of Parathyroid Hormone (PTH) as well as 1,25DHCC, since a reduction in bone calcium mobilisation is seen in thyroparathyroidectomised rats (Garabedian, Tanaka, Holick and DeLuca, 1974).

The kidney plays an important role in the control of calcium and phosphorus homeostasis. Vitamin D_3 and its

metabolites have been shown to exert an effect on kidney function by increasing the renal absorption of phosphorus (Puschett, Moranz and Kurnick, 1972), while other workers have shown Vitamin D_3 metabolites do not improve renal absorption of phosphorus directly (Steele, Engle, Lorenc, Dudgeon and DeLuca, 1975). However, it seems that Vitamin D_3 and its metabolites do increase renal absorption of calcium (Sutton, Harris, Wong and Dirks, 1977). It is unclear whether PTH is involved in the renal effects of Vitamin D_3.

A role for Vitamin D_3 has also been postulated in the calcium and phosphate homeostasis of muscle. Calcium binding to the myofilaments is enhanced in the presence of cholecalciferol (Kodicek, 1973) while intracellular accumulation of phosphate by muscle is stimulated by 25HCC (Birge and Haddad, 1975). The actual mechanism of this action of Vitamin D_3 is still unclear.

1.2.4 Regulation of Vitamin D_3 Metabolism

A) Regulation of Cholecalciferol-25-hydroxylation

Cholecalciferol is converted to 25HCC primarily in the liver. Studies on the regulation of the CC-25-hydroxylation have caused some controversy over whether the cholecalciferol-25-hydroxylase (25-hydroxylase) is controlled through product inhibition. Injections of cholecalciferol into Vitamin D_3-deficient chicks results in a reduction in the activity of the 25-hydroxylase with less 25HCC produced compared to birds not receiving the injection (Bhattacharyya and DeLuca, 1974). However, high oral doses of Vitamin D_3 can increase circulating levels of 25HCC (Haddad and Stamp, 1974). It does appear that there is some form of regulation occurring within the liver which depends on plasma 25HCC levels (for review see Holick and Clark, 1978).

Recently oestrogens have been shown to increase the production of 25HCC by the liver of Japanese Quail (Nicholson, Akhtar and Taylor, 1979). It is unclear whether this stimulation is brought about by an increase in the activity of the 25-hydroxylase or by a decrease in the activity of enzymes responsible for the degradation of cholecalciferol or 25HCC. These actions of oestrogens may be of physiological significance in relation to the high requirement in laying birds for 1,25DHCC in order to maintain sufficient calcium for egg-shell calcification.

B) Regulation of the Hydroxylation of 25HCC in the Kidney

Perhaps the most significant step in the regulation of cholecalaferol metabolism is the 1α-hydroxylation of 25HCC in the kidney. Many factors have been implicated in regulating 1,25DHCC production, some also regulate the 24R-hydroxylation of 25HCC.

Factors proposed which regulate the hydroxylation of 25HCC are dietary calcium (Boyle, Gray and DeLuca, 1971), dietary phosphorus (Tanaka and DeLuca, 1973; Sommerville, Swaminathan and Care, 1978), circulating PTH levels (Garbedian, Holick, DeLuca and Boyle, 1972; Fraser and Kodicek, 1973), Vitamin D_3 and its metabolites (Boyle et al, 1971; Galante, Colston, Evans, Byfield, Matthews and MacIntyre, 1973), Gonadal hormones (Kenny, 1976), prolactin (Spanos, Pike, Haussler, Colston, Evans, Goldner, McCain and MacIntyre, 1976), growth hormone (Spanos, Barrett, MacIntyre, Pike, Safilian and Haussler, 1978), insulin and glucocorticoids. Many factors have therefore been implicated but still more work has to be done in order to fully understand the regulation of the renal hydroxylation of 25HCC.

1.2.5 Gonadal Hormones and Vitamin D_3 Metabolism in Birds

A possible role for gonadal hormones in calcium metabolism within normal avian reproductive activity has been recognised for some time. Calcium and phosphorus absorption are greatly enhanced in birds just prior to the onset of lay and during egg shell formation (Taylor and Moore, 1954; Taylor and Kirkly, 1967; Hurwitz, Bar and Cohen, 1973). The absorbed minerals are stored as medullary bone and an injection of oestrogens and androgens will result in an increase in the formation of medullary bone. Both oestrogen and androgen must be given in combination since oestrogens alone cause birds to become hypercalcaemic yet they are unable to retain calcium (Common, Rutledge and Hale, 1948). The enhancement of intestinal calcium absorption at the onset of lay, which is maintained during egg production, is accompanied by an increase in calcium-binding protein within the duodenum (Bar and Hurwitz, 1972).

As mentioned earlier calcium absorption from the intestine is stimulated by a metabolite of Vitamin D_3, 1,25DHCC. It has been suggested by several workers that the changes in calcium absorption, seen in avian species at the onset of lay, can be correlated with the changes in the activity of the hepatic and renal hydroxylases involved in Vitamin D_3 metabolism. This has resulted in sex hormones being implicated as possible regulators of Vitamin D_3 metabolism. Kenny (1976) studied the physiological changes in the metabolism of 25HCC during the reproductive cycle of the Japanese Quail. He found that when the requirement for calcium was high, as occurs when an egg is present in the oviduct, the kidney produces mainly 1,25DHCC, while when calcium requirement is low, the main metabolite was 24,25DHCC. He concluded that the physiological state of

the bird had an endogenous control over 1,25DHCC production and that this occurred prior to the calcification of the egg. Sedrani and Taylor (1977) also found that 1,25DHCC production predominates in birds with an egg in the oviduct but found that birds with an empty oviduct showed wide variations in the proportion of 1,25DHCC produced, the percentage declining with increased length of time since last oviposition. Sedrani (1979) showed that production of 1,25DHCC in female Japanese Quail increases with the approach of sexual maturity and production of 24,25DHCC decreases, while in the male bird biosynthesis of 1,25DHCC decreases with age and the activity of the 25HCC-1α-hydroxylase (1α-hydroxylase) is completely suppressed at sexual maturity.

The work of Kenny suggested that gonadal hormones may be involved directly or indirectly in the control of the renal 1α-hydroxylase, however the effect these hormones exert on regulating Vitamin D_3 metabolism is still unclear. DeLuca and co-workers (Tanaka, Castillo and DeLuca, 1976; Castillo, Tanaka, DeLuca and Sunde, 1977) showed that oestrogen administration into mature male Japanese Quail resulted in an increase in 1α-hydroxylase activity and in a suppression of the 25HCC-24R-hydroxylase (24-hydroxylase). This effect was no longer seen in castrated male chickens, immature male quail or immature male chickens until testosterone and oestradiol were injected simultaneously, when a similar pattern of response was found. This conflicts with the work of Baksi and Kenny (1977) who found that oestrogens alone could cause an increase in 1,25DHCC production if injected into either immature male or female birds, and with Pike et al (Pike, Spanos, Colston, MacIntyre and Haussler, 1978) who reported that oestrogens alone can stimulate the 1α-hydroxylase and showed a potentiation of the effect when either testosterone or progesterone was also injected, despite testosterone and progesterone being unstimulatory if injected alone.

Sedrani (1979) injected immature Japanese Quail with either oestradiol dipropionate, testosterone propionate or parathyroid extract (PTE) and also with combinations of these three hormones. He found, as had Baksi and Kenny (1977), that oestradiol dipropionate stimulated the 1α-hydroxylase enzyme, since the production of 1,25DHCC from kidney homogenates incubated **in vitro** was greater than in controls, and that oestrogens inhibited the production of the 24,25DHCC. Testosterone propionate injections resulted in an inhibition of the 1α-hydroxylase while the 24-hydroxylase remained unaffected. PTE stimulated the 1α-hydroxylase and the inhibition seen with testosterone propionate was reversed if PTE was injected with testosterone propionate. Unlike Pike et al (1978), Sedrani did not see an increased response to oestrogens when testosterone propionate was injected simultaneously but saw a slight inhibition of the oestrogen response. This inhibition was overcome when PTE, oestradiol dipropionate and testosterone propionate were all injected together when an even greater stimulation of the 1α-hydroxylase was seen compared to that seen when PTE and oestrogens or PTE and testosterone were injected together.

Baksi and Kenny (1978) showed that ovariectomy in birds on a normal calcium diet caused a decrease in the 1α-hydroxylase activity, and that this inhibition could be reversed by oestradiol injection and to a lesser extent by progesterone. Testosterone injected into intact females resulted in an inhibition of the 1α-hydroxylase and a stimulation in the 24-hydroxylase. This regulatory effect of sex hormones on renal 25HCC hydroxylases was no longer seen if birds were raised on a low calcium diet.

In Summary: Oestrogens, androgens and progestins have been shown to affect Vitamin D_3 metabolism both at the hydroxylation of cholecalciferol in the liver (Nicholson et

al, 1979) and at the hydroxylation of the 25HCC in the kidney. The mechanism of action of gonadal hormones in the regulation of Vitamin D_3 metabolism is still unclear, but it seems that PTH may be also involved in their action. It seems likely that, in avian species, gonadal hormones play an important role in ensuring plasma calcium levels are maintained for complete egg-shell calcification and that they have their effect through regulating Vitamin D_3 metabolism. It was the work of Kenny (1976) and Sedrani (1979) on age dependent changes of Vitamin D_3 metabolism which led to the investigation of changes in plasma levels of oestradiol, testosterone and progesterone during maturation reported in this thesis.

1.3 The Gonadotrophins: Luteinizing Hormone and Follicle Stimulating Hormone

1.3.1 Regulation of Gonadal Functions by Gonadotrophins

Gonadal functions in avian species appear to be controlled, as in mammals, by two distinct gonadotrophic hormones, namely follicle stimulating hormone (FSH) and luteinizing hormone (LH).

The site and control of gonadotrophin release is in the hypothalamus-pituitary axis in the brain. LH and FSH as well as other pituitary hormones: adrenocorticotrophic hormone, prolactin, growth hormone and thyroid stimulating hormone, are released from the anterior pituitary gland. Their secretion is controlled by specific releasing hormones originating in the hypothalamus and by the direct feedback effects of steroids secreted from the target organs into the peripheral blood circulation. The hypothalamus and pituitary are outside the blood-brain barrier and so hormones in the peripheral blood circulation can exert a direct effect on these tissues. On reaching the pituitary the steroids in the peripheral blood circulation may either modify the responsiveness of the pituitary to the releasing hormones or stimulate (positive feedback) or inhibit (negative feedback) the release of the gonadotrophic hormones directly. The releasing hormones, LH- and FSH- releasing hormones (LH-RH and FSH-RH) are carried from the median eminence of the hypothalamus in the blood capillaries of the hypophyseal-hypothalamic portal system to the pituitary. Secretion of LH-RH and FSH-RH by the hypothalamus depends on stimuli from higher centres within the brain and the feedback control of gonadal steroids. As on the pituitary feedback of gonadal steroids onto the hypothalamus may be a negative or positive feedback control.

Purified samples of the two avian gonadotrophic hormones, LH and FSH, have been separated from the chicken pituitary gland (Stockell-Hartree and Cunningham, 1969; Scanes and Follett, 1972) and their specificity established by biological assays (Furr and Cunningham, 1970). Two distinct areas in the hypothalamus have been shown, using specific lesions, to be responsible for the regulation of secretion of FSH and LH in both male and female Japanese Quail (Stetson, 1972a, b). The FSH release system is located in the outer dorsal region of the infundibular nuclear region. Lesions in this area result in the regression of the ovary and oviduct in the female and regression of the seminiferous tubules in the male. The LH release system is located in the posterior region of the infundibular nucleus and lesions in this area prevent ovulation in the females and cause a regression of the interstitial cells in the males.

In the male FSH is responsible, probably together with testosterone, for regulating spermatogenesis and testicular growth (Brown, Baylé, Scanes and Follett, 1975; Brown and Follett, 1977), while LH is the primary steroidogenic hormone causing increased testosterone synthesis by, and secretion from, the interstitial Leydig cells (Maung and Follett, 1977). In the female, FSH promotes the growth and development of the follicles and oviduct while LH induces ovulation (Fraps, 1965). In the female, both FSH and LH are probably required to achieve the full steroid potential of the ovary.

1.3.2 Gonadotrophin Levels: Dependence of Sexual Maturity

The content of gonadotrophin in the pituitary is influenced by the state of sexual maturity of the bird. Thus photostimulation, by long daily photoperiods, of

gonadal growth in quail resulted in elevated pituitary levels of the gonadotrophins as determined by ^{32}Phosphorus uptake by the testes of young chickens following LH or FSH injection (Follett and Farner, 1966b) or by measuring the increase in testicular weight in quail injected with pituitary homogenates (Tanaka, Mathur, Wilson and McFarland, 1965). The gonadotrophin-releasing activity in the hypothalamus also increased with photostimulation. Returning Japanese Quail to short daily photoperiods caused a fall in hypothalamic gonadotrophin-releasing activity and in the pituitary levels of gonadotrophins (Follett, 1970). Growth and the onset of sexual maturity in chickens were also accompanied by an increase in pituitary gonadotrophin levels (Breneman, 1955).

These findings correspond well with plasma LH levels measured in photosensitive birds, raised on different length photoperiods, throughout maturation. Changes in plasma FSH and LH, immediately after a change to long photoperiods, in somatically mature male Japanese Quail have been well documented (Follett, 1976; Gledhill and Follett, 1976; Follett, Davies and Gledhill, 1977; Follett and Maung, 1978). Both plasma FSH and LH increased after a change from short to long photoperiods. LH levels increased after 20h of photostimulation of at least 15h light, while FSH levels rose more slowly (Follett, Davies and Gledhill, 1977). The increased FSH levels in males correlates with increased testicular growth, FSH levels having increased to a maximum after 7-9 days of photostimulation and then decreased again as the testes mature. The rise in FSH was not seen if birds raised and maintained on 8L:16D (8 hours light followed by 16 hours dark) were transferred to 12L:12D and no testicular growth was seen either (Follett and Maung, 1978).

In the chicken, plasma LH levels rose in both male and female birds from hatching, with males having higher levels

than females (Sharp, 1975). LH levels remained high in the males, after a transitory fall, while LH fell and remained low in the females prior to the onset of egg-laying. These changes are probably due to a changing balance between the negative feedback effects of increasing levels of sex steroids, changes in the sensitivity of the hypothalamo-hypophyseal complex to sex steroids and changes in the responsiveness of the pituitary to LH-RH. Cunningham, Bonney, Furr and Onuora (1973) showed that the responsiveness of the pituitary of the domestic fowl to synthetic LH-releasing factor (LH-RF) decreased during maturation. A dose of LH-RF found to cause LH release in immature hens of 11 weeks failed to elicit a response in birds aged 21 weeks possibly because the 'potentiating' effect of LH-RH was developing, as seen in adult mammals.

Gledhill and Follett (1976), using a RIA for LH, found that in Japanese Quail there was little diurnal variation in plasma LH or FSH in birds raised under short or long photoperiods but found that LH was released in a pulsatile fashion (6-10 pulses/day in the male, 4-8 pulses/day in the female). This led to large variations in LH levels between birds and between serial samples taken from the same bird. However other workers using bioassays for LH, have shown that a diurnal rhythm does occur in the pituitary gonadotrophin content of Japanese Quail on a stimulatory photoperiod (Tanaka, Wilson, Mather and MacFarland, 1966; Follett and Sharp 1969; Bacon, Cherms, MacDonald and McShane, 1969), with the highest levels being found during the day and the lowest at night. This was not seen when birds were raised on the non-stimulatory photoperiod 8L:16D (Tanaka, Wilson, Mather and MacFarland, 1966). While the presence of diurnal variations in plasma and pituitary LH in the quail remains unclear diurnal changes do appear to be present in the hen. Maturing cockerels show a peak level of plasma LH immediately after the onset of darkness with the lowest level about 12h later (Bolton, Chadwick, Chapman,

Hall and Scanes, 1974). This pattern was also seen in both sexes of the maturing domestic fowl (Scanes, Chadwick, Sharp and Bolton, 1978) and in mature egg-laying and non-egg-laying hens (Williams and Sharp, 1978). The relationship between the diurnal variation of LH in the laying bird and ovulation will be discussed in Chapter 1.4.3.

In Summary: Levels of pituitary and plasma LH and FSH depend on the state of sexual maturity of the bird. When the bird matures LH and FSH levels increase thus stimulating the growth and development of the gonads. If photo-stimulated birds are subjected to non-stimulatory photo-periods then levels of LH and FSH fall and gonadal regression occurs.

1.3.3 Steroidogenesis in the Male

A) Sites of steroidogenesis

Steroidogenesis in the testis involves the pathway from cholesterol through pregnenolone and/or progesterone to androgens and oestrogens as already shown in Figure 1.2. Most research has centred on testicular function and steroid biosynthesis in mammalian tissues although some work has been done in avian species. In immature mammals the major testicular androgens formed are the androstane metabolites, rather than testosterone which is the main steroid secreted by the adult testis (Moger, 1977; Purvis, Claasen and Hansson, 1978; Moger, 1979). Oestrogens have also been shown to be secreted by the rat testis (De Jong, Hey and van der Molen, 1973).

Using collagenase to disperse the testicular cells, the cellular sites of steroid production have been elucidated. Treatment with the enzyme, collagenase, enables the heavier

seminiferous tubules to be separated from the interstitial Leydig cells and **in vitro** studies on steroid production by the different testicular components to be carried out. It has been shown that testosterone is produced by the Leydig cells **in vitro** and not by the seminiferous tubules, while the site of oestradiol production is less clear and some controversy still exists. Experiments on mammalian testicular tissue have stimulated the idea that the Sertoli cells are the main source of testicular oestradiol (De Jong, Hey and van der Molen, 1974; Dorrington, Fritz and Armstrong, 1978; Rommerts, Kruger-Sewnarain, van Woekom-Blik, Grootegoed and van der Molen, 1978) while other workers have failed to confirm that oestradiol is secreted mainly by the Sertoli cells (Steinberger, Tcholakian and Steinberger, 1979; Canick, Makris, Gunsalis and Ryan, 1979) and have shown that oestradiol is secreted by the Leydig cells **in vitro** (Valladares and Payne, 1979a; van der Molen, Brinkmann, De Jong and Rommerts, 1981).

Much of the controversy in the contribution which the Sertoli cells make to testicular oestrogen production, lies in the techniques used to measure oestradiol production and in the strain of rat used. van der Molen and co-workers measured oestradiol by radioimmunoassay in Wistar Rats while Steinberger et al (1979), Canick et al (1979) and Valladares and Payne (1979a) measured oestradiol by conversion of ^{3}H-testosterone to ^{3}H-oestradiol in Spague-Dawley rats. Steinberger et al (1979) found steroids in Sertoli cell incubations which cochromatographed with oestrogens after incubation with ^{3}H-testosterone, but the oestrogen would not recrystallise to constant specific activity with oestradiol-17β and they suggested that radioimmunoassays overestimate the oestradiol-17β production. Recently, Rommerts, De Jong, Grootegoed and van der Molen (1980) measured oestradiol production from Sertoli cells by both methods and found that both techniques gave similar results, with the Sertoli cells being capable of secreting oestra-

diol-17β . It now seems likely that both Sertoli and Leydig cells can aromatise androgens to oestrogens. The capacity of the Leydig cells to synthesise oestradiol-17β suggests that the Sertoli cells are not obligatory for the production of oestradiol for the regulation of oestradiol-dependent reactions in Leydig cells, in adult rats.

The cellular site of testosterone synthesis in quail is in the Leydig cells (Maung and Follett, 1977). Secretion and cellular site of synthesis of oestrogens from avian testes has not previously been reported. Studies reported in this thesis show that like mammals, the avian testes can secrete oestradiol and that this oestradiol is secreted from cells contained within the seminiferous tubules rather than from the interstitial Leydig cells.

B) Control of Steroidogenesis by Gonadotrophic Hormones

Testosterone and oestradiol production and secretion from the testes can be stimulated by the actions of the gonadotrophic hormones. Testosterone production and secretion from the testes, in particular from the Leydig cells, is elevated after administration of LH or human chorionic gonadotrophin (hCG), a gonadotrophic hormone with LH-like activity, **in vivo** (De Jong et al, 1973) and after **in vitro** addition of LH and hCG to isolated Leydig cells (De Jong, Hey and van der Molen, 1974). De Jong et al (1973) also found an increase in testicular venous oestradiol levels after acute administration of hCG **in vivo** to adult rats while FSH was ineffective. In contrast FSH has been shown to stimulate oestradiol production **in vitro** in Sertoli cells from immature rats (Dorrington and Armstong, 1975; Dorrington, Fritz and Armstrong, 1978; Pomerantz, 1979). hCG also stimulates the increased aromatisation of androgens to oestrogens **in vitro** after either **in vivo** injections of hCG (Canick, Makris, Gunsalis

and Ryan, 1979) or incubations with hCG (Valladares and Payne, 1979a), although in these studies the highest activity of aromatising enzymes was found in the Leydig cells. The effect of age on the response of testicular cells to gonadotrophins will be discussed later.

C) Mechanism of Action of Gonadotrophic Hormones

Much work has now been done to elucidate the mechanism of action of the control of testicular steroidogenesis by gonadotrophins. The most important point of control in the pathway leading to testosterone synthesis is at the mitochondrial cholesterol side chain cleavage activity. This reaction involves the cytochrome P450 system, and results in increased pregnenolone production in the mitochondria of Leydig cells. It is stimulated by LH or hCG administration (Van der Vusse, Kalkman and van der Molen, 1975).

For LH or hCG to exert an effect on steroidogenesis they must first bind to specific membrane receptors on cells within the testis or ovary. Receptor sites for LH and hCG in the testis and ovary have been identified and characterised by binding studies with labelled gonadotrophins, usually using ^{125}I-labelled hCG. Receptors for LH/hCG have been demonstrated in the Leydig cells of the rat testis, (De Kretser, Catt, Burger and Smith 1969; Catt, Dufau and Tsuruhara, 1971), with no binding to the seminiferous tubules (Catt, Tsuruhara, Mendelson, Kelelslegers and Dufau, 1974). The relationship between hormone binding and target cell activation was examined during the uptake of labelled hCG by the rat testis interstitial cells. It was found that increased binding of hormone to receptor sites occurred over a wide range of hCG concentrations, which greatly exceeded those necessary for maximum testosterone production (Catt and Dufau, 1973). Approximately 50% of Leydig cell receptors are coupled to the enzyme adenylate

cyclase (Catt and Dufau, 1978)

There is much evidence indicating that polypeptide hormones activate the membrane bound adenylate cyclase and thus increase intracellular levels of adenosine 3',5'-monophosphate (c'AMP), which acts as an intracellular secondary messenger mediating the actions of the gonadotrophins (Sutherland, Oye and Butcher, 1965; Sutherland and Robison, 1966). Examples of hormones which are thought to have their physiological effect in this way are: catecholamines on heart, liver, skeletal muscle and fat: glucagon on the liver: adrenocorticotrophic hormone (ACTH) on adrenal cortex: vasopressin on toad bladder and LH on the corpus luteum (Sutherland and Robison, 1966). LH action on steroidogenesis in the rat testis also appears to be via c'AMP as the intracellular mediator since intracellular c'AMP levels are elevated after addition of LH (Rommerts, Cooke and van der Molen, 1974; Mendelson, Dufau and Catt, 1975). The intracellular c'AMP levels are regulated by the activities of at least two enzymes. Its formation from adenosine triphosphate (ATP) is catalysed by adenylate cyclase whilst its breakdown to 5'-adenosine monophosphate (5'-AMP) is regulated by a specific c'AMP phosphodiesterase.

LH stimulates the membrane bound adenylate cyclase thus elevating intracellular c'AMP levels. Addition of a phosphodiesterase inhibitor, such as theophylline, often enhances the effects of the exogenous hormone and, if this enhancement does occur, it is often used as circumstantial evidence that the hormone exerts its effect via adenylate cyclase. The obligatory role for c'AMP in testicular steroidogenesis is less certain since several investigations have shown that there is a marked discrepancy between the amounts of LH required to stimulate testosterone synthesis and intracellular c'AMP levels. More LH was required to produce a detectable increase in intracellular c'AMP levels than was needed to stimulate testosterone production (Catt

and Dufau, 1973). Also, the time course of the two events are not parallel. This suggests that at low levels of LH, either c'AMP is not involved in the intracellular action of the hormone or that small changes in c'AMP levels were not being detected. The latter may be the case since increased testosterone release occurred on the addition of the phosphodiesterase inhibitor, theophylline, although there was no detectable change in intracellular c'AMP levels (Catt and Dufau, 1973). However, in the Leydig cells, one must be clear that the role of c'AMP as the only intracellular mediator of the LH-stimulated steroidogenesis remains controversial.

It is postulated that intracellular c'AMP binds to a specific c'AMP receptor protein which exists as a regulatory complex with protein phosphokinase. Once bound the protein kinase is released and becomes activated. This c'AMP dependent protein phosphokinase then utilises ATP for the phosphorylation of proteins within the cell. ACTH stimulation of steroidogenesis has been shown to involve this binding of c'AMP to the phosphokinase with resultant phosphorylations of proteins within the cell (Garren, Gill, Masui and Walton, 1971; Gill, 1972). LH also stimulates a rise in intracellular levels of an active protein kinase within rat Leydig cells; these increased levels of protein kinase paralleled testosterone production, but lower amounts of LH were required to stimulate maximum testosterone production than were required for maximum stimulation of protein kinase activity (Cooke, Lindh and Janszen, 1976).

Studies on the action of ACTH in the adrenal gland have shown that c'AMP may not be the only mediator in ACTH-induced steroidogenesis and that ionic calcium (Ca^{2+}) may also be involved as a secondary messenger. Ca^{2+} uptake by cells paralleled the increased intracellular c'AMP production after hormone action (Ramussen, 1970). Ca^{2+} has also been shown to have a direct effect on isolated rat adrenal

mitochondria in stimulating pregnenolone formation from these organelles (Simpson, Waters, Williams-Smith, 1975). These workers suggest that the mitochondrial uptake of Ca^{2+} enhances the substrate binding to the cytochrome P450 involved in the cholesterol side chain cleavage and thus increases the availability of substrate for pregnenolone production. Ca^{2+} is also required for ACTH-stimulation of adenylate cyclase and possibly for ACTH-R interaction (for review on Ca^{2+} and ACTH action on adrenal cells, see Schulster, Burstein and Cooke, 1976). A role for Ca^{2+} in gonadal steroidogenesis could therefore exist as it does in adrenal steroidogenesis and c'AMP may not be the only secondary mediator within these cells. Other cyclic nucleotides may also be involved as secondary messengers such as cyclic guanosine monophosphate although no direct evidence for its involvement has as yet been found.

Activation of cholesterol esterase is mediated by c'AMP and protein kinase resulting in increased levels of free cholesterol, the precursor for steroidogenesis, within the lipid droplet stores. Free cholesterol, together with cholesterol taken up by the cells from the blood, is then probably transported to the mitochondrial cholesterol side chain cleavage complex by a cholesterol binding protein (Schulster et al, 1976), where it is converted to pregnenolone.

Protein synthesis is also involved in LH-stimulated testosterone production since, in the presence of labelled amino acids, specific proteins become labelled after the addition of LH to isolated Leydig cells (Janszen, Cooke and van der Molen, 1977). Furthermore, the addition of cycloheximide (a protein synthesis inhibitor, preventing the translation of the transfer ribonucleic acid [tRNA]) results in a decrease in LH-stimulated testosterone production to control levels (Cooke, Janszen, Clotscher and van der Molen, 1975). Protein synthesis may be involved in the long-term

control of testosterone production rather than the short-term control since increased **de novo** protein synthesis was only seen 2h after the addition of LH. The protein also had a half-life of 30 minutes while increased testosterone production could be measured after 5-10 minutes. LH-induced protein synthesis would also seem to be mediated through c'AMP since the addition of the analogue dibutyryl c'AMP stimulated synthesis of the same specific protein (Janszen, Cooke, van Driel and van der Molen, 1978).

LH-induced protein synthesis was also inhibited by Actinomycin D (a protein synthesis inhibitor, preventing transcription of messenger RNA (mRNA) from deoxyribonucleic acid (DNA)) if it was added at the start of the incubation, while Actinomycin D had no effect when added 5h after the start of the incubation. Early addition of Actinomycin D therefore prevented the synthesis of mRNA coding for the LH-induced protein (Janszen et al, 1978). It seems likely that LH-induced protein synthesis is acting through c'AMP to increase mRNA levels within the cell by increasing the transcription of mRNA from DNA. A summary of the mechanism of action of LH on Leydig cell production of testosterone is shown in Figure 1.5.

Less research has been done on the mechanisms by which FSH stimulates oestrogen secretion from the Sertoli cells and its role in inducing spermatogenesis. Receptors for FSH have been shown to be in the seminiferous tubule fractions, with the Sertoli cell as the primary target, while no binding occurred in the intestitial cell fraction (Means and Huckins, 1974). This interaction resulted in the stimulation of membrane-bound adenylate cyclase. Dorrington, Fritz, and Armstrong (1978) showed that the FSH-stimulated oestrogen production was paralleled by an increase in intracellular c'AMP levels. Addition of dibutyryl c'AMP **in vitro** stimulated both the incorporation of ^{3}H -Leucine into protein and increased the production of

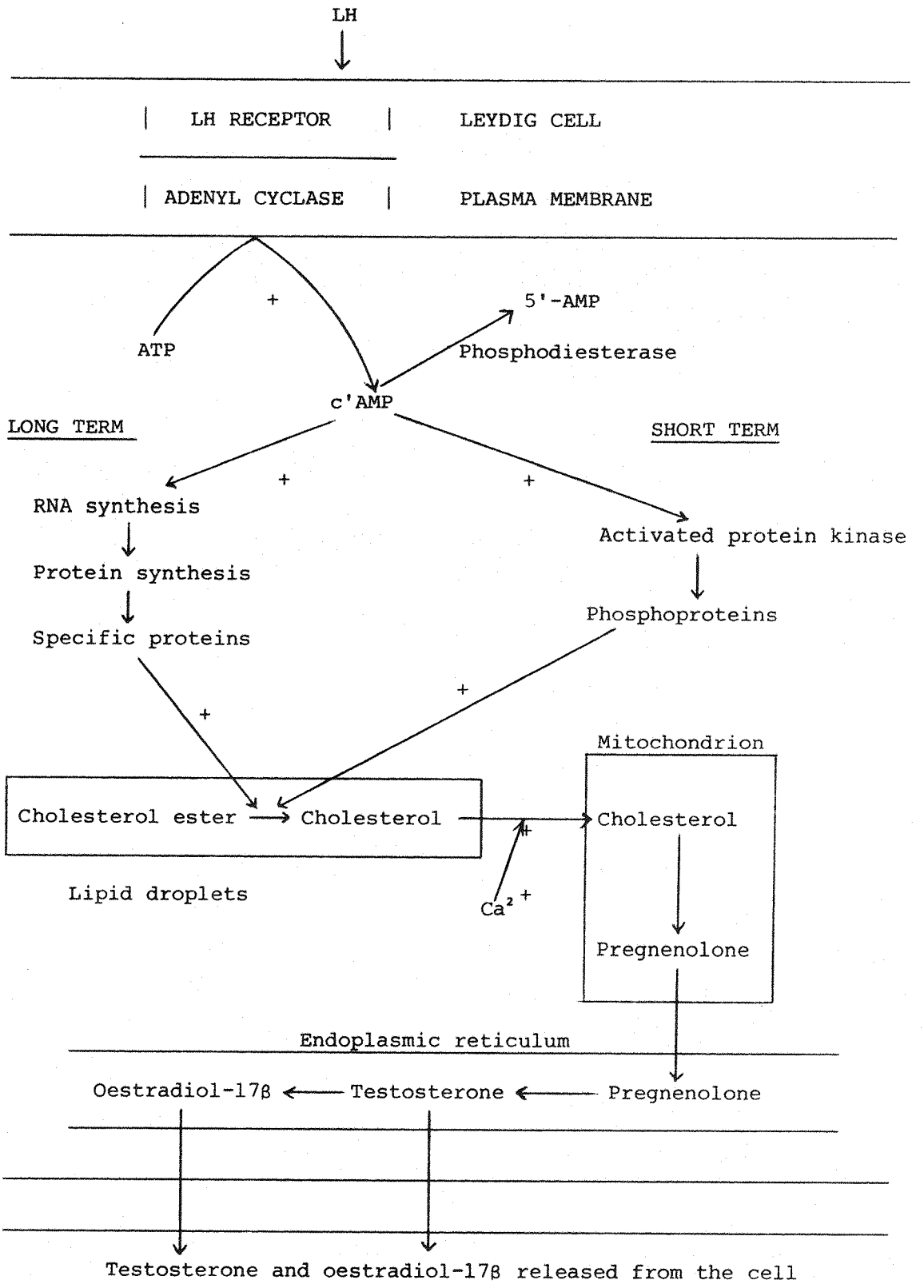
Figure 1.5

A Simplified Model for the Mechanism of Action of LH on Rat Testicular Leydig Cells

LH binds to its receptor on the cell membrane stimulating adenyl cyclase. Intracellular c'AMP levels rise and results in activiation of a specific protein kinase which causes the phosphorylation of proteins within the cell (short term control). These phosphorylated proteins alter the rate of cholesterol metabolism by elevating substrate levels. Protein synthesis is also stimulated by c'AMP (long term control) by increasing the transcription of mRNA coding for the LH-induced protein. Thus resulting in an increase in steroidogenesis. Ca^{2+} and/or c'GMP may also be involved as secondary messengers in the stimulation of steroidogensis. Pregnenolone produced in the mitochondria is converted to testosterone and other steroids on the endoplasmic reticulum.

+ = stimulation of the process.

Figure 1.5


LH
LH RECEPTOR
LEYDIG CELL
ADENYL CYCLASE
PLASMA MEMBRANE
ATP
+
5'-AMP
Phosphodiesterase
c'AMP
LONG TERM
SHORT TERM
+
+
RNA synthesis
Protein synthesis
Specific proteins
Activated protein kinase
Phosphoproteins
+
+
Mitochondrion
Cholesterol ester
Cholesterol
Cholesterol
Lipid droplets
Ca2+
Pregnenolone
Endoplasmic reticulum
Oestradiol-17β
Testosterone
Pregnenolone
Testosterone and oestradiol-17β released from the cell

androgen-binding protein (ABP) from seminiferous tubule cells. This increase in ABP was also shown by Rommerts et al (1980). Oestradiol production was inhibited if puromycin (a protein synthesis inhibitor preventing complete translation) was added up to 2h after the addition of FSH or testosterone but after 4h the addition of puromycin had no effect. FSH therefore seems to stimulate **de novo** protein synthesis of enzymes involved in oestrogen production.

These results suggest that FSH has a similar mechanism of action on steroidogenesis in the Sertoli cells as LH has on steroidogenesis in the Leydig cells, but oestradiol is only formed when testosterone is present, i.e FSH action affects the aromatase enzyme and not the cholesterol side chain cleavage enzyme.

The mechanisms by which trophic hormones stimulate steroidogenesis in mammalian testes have been well documented, as discussed above, while the mode and sites of action of the trophic hormones in birds has only recently come under investigation. Brown et al (1975) showed that injections of chicken LH into hypophysectomised quail led to the differentiation of the Leydig cells while FSH stimulated testicular growth and development of the seminiferous tubules. Maung and Follett (1977) studied the effects of ovine and chicken LH on androgen release and c'AMP production **in vitro** by isolated quail Leydig cells. They found that both chicken and ovine LH resulted in increased testosterone production and that c'AMP formation was also stimulated. As with mammals, the dose of LH required to stimulate measurable increases in c'AMP production was greater than that necessary to stimulate testosterone production. This raises the question whether c'AMP is the sole mediator in the action of LH in avian Leydig cells. However, Maung and Follett (1977) have shown that dibutyryl c'AMP stimulates testosterone production and that the LH-stimulated testosterone production is enhanced by the addition of a phosphodiesterase inhibitor, suggesting that,

as in mammals, c'AMP is involved in mediating the response.

Protein synthesis is also involved in the LH-induced testosterone release since the addition of cycloheximide **in vitro** inhibits this response (Maung and Follett, 1977). These results indicate that the mechanism of action of LH in the avian testis is similar to that already summarised for the mammal in Figure 1.5. There is little information about the mechanism of action of FSH in the avian testis.

1.3.4 The Effect of Age on Testicular Sensitivity to Gonadotrophins

A) The Testosterone Response of Leydig Cells during Sexual Maturation

Odell, Swerdloff, Jacobs and Hescox (1973) proposed that during sexual maturation, continued FSH stimulation causes a change in the responsiveness of the testes to LH. They hypophysectomised rats on d21 (sexually immature) or d87 (sexually mature) and injected them daily 5 days later with LH, for 5 days. They found that rats hypophysectomised when sexually mature responded to LH, resulting in an increase in prostate weight. Rats hypophysectomised on d21 and treated with LH showed an increase in testicular weight with no increase in prostate weight until the rats were pretreated with FSH and then given the LH. The results show that FSH had modified the sensitivity of the testis to LH.

This increase in testicular responsiveness to LH during sexual maturation was also seen in the intact rat (Odell, Swerdloff, Bain, Wollesen and Grover, 1974): LH administered intraperitoneally to rats of increasing age between d10 and d62, caused increased levels in serum testosterone 1h after the injection. The increase in testosterone

concentration was least in the d10 animals and greatest in the 42 and 62 day old rats. A similar change in the sensitivity of the testes with age was seen in rabbits: hCG injected into the ear vein caused an increase in plasma testosterone and testicular concentration of testosterone at all ages (d20-d180) (Berger, Chazaud, Jean-Faucher, de Turckheim, Vessiere and Jean, 1976). However, the lowest responses were seen in the immature animals, while an increase in the response occurred between d60 and d180.

Maung and Follett (1978) have also found a marked increase in the magnitude of the LH-induced increases in plasma testosterone in Japanese Quail **in vivo**, 6 to 7 days after the birds had been transferred from short to long photoperiods i.e when the testes are starting to mature. It seems possible therefore that a similar increase in the sensitivity of the testes of Japanese Quail to LH occurs during sexual maturation. It is likely that FSH does not have such a dramatic effect in the quail in modulating LH sensitivity as shown in the rat, since treatment of sexually immature quail with ovine FSH for 10 days had no effect on the rise in testosterone caused by an injection of LH, given on completion of the FSH injections (Follett, 1976). Part of the experimental work in this thesis was devoted to an **in vitro** study on the response of dispersed Leydig cells to hCG stimulation and to see if an increase in the sensitivity of these cells occurred with age, as seen in mammals.

B) On Oestradiol Production by Testicular Cells during Sexual Maturation

Oestradiol secretion by Leydig or Sertoli cells also depends on the state of testicular development. Sertoli cells obtained from immature testes are more active than those from mature rats (Dorrington, Fritz and Armstrong, 1978; van der Molen, Brinkmann, De Jong and Rommerts, 1981) since basal levels of oestradiol secretion are highest at

d20 and decrease by d30. The decrease in the apparent activity of the Sertoli cells with age may have resulted from the decreasing percentage of Sertoli cells in the cell incubates from maturing animals. Thus the low but relatively constant oestradiol production seen during maturation may show that the real activity of the Sertoli cells is increasing. This suggestion is supported by experiments on prenatally irradiated rats which produce relatively large amounts of oestradiol from Sertoli cells at 70 days of age (van der Molen et al, 1981). However, prenatal irradiation may have also modified the normal development of the Sertoli cell.

Leydig cells obtained from normal rats at different ages showed an increase in oestradiol production **in vitro** with increasing age, which cannot be explained by changes in the percentage of Leydig cells within the cell incubates (van der Molen et al, 1981). This suggests that there is an increase in the aromatase activity per Leydig cell with age. However care must be taken in interpreting these results in understanding their relevance to the situation **in vivo**, since the conditions **in vitro** may alter the results obtained. Different species give different responses as already shown in Chapter 1.3.3.(A), where oestrogen production was shown to occur in Sertoli cells in Wistar rats (van der Molen and co-workers) but not in Spague-Dawley rats (Steinberger et al, 1979). Additions to the culture medium can also affect the response, for example, the presence of 1% foetal calf serum has no effect on the aromatase activity in the Leydig cells but results in an increase in oestradiol production from the Sertoli cells (van der Molen et al, 1981).

hCG/LH has been shown to stimulate testicular aromatisation of testosterone after injecting immature rats (Canick, Makris, Gunsalis and Ryan, 1979), or mature rats **in vivo** (Valladares and Payne, 1979b). The increase in the aromatase activity in the immature rat on d15 is found

mainly in the Leydig cells and was 5-7x higher than that found in the seminiferous tubules. In the adult rat the increase in aromatase was only seen in the Leydig cells. This suggests that the Sertoli cells will respond to hCG in the immature rat but not in the mature rat while the increase in aromatase activity in the Leydig cells after hCG occurs at all stages of testicular maturation.

The effect of FSH on oestradiol production also seems to vary with age. Sertoli cells from immature rats (d16-d20) show the greatest stimulation of oestradiol production after FSH has been administered **in vitro** provided that testosterone is present (Dorrington, Fritz and Armstrong, 1978). However, plasma oestradiol is not stimulated by injections of FSH **in vivo** to adult rats but is stimulated by hCG (De Jong, Hey and van der Molen, 1973). In contrast to Dorrington et al (1978), Canick et al (1979) failed to show any stimulation of aromatase activity by FSH in immature rats. Pomerantz (1979) has since shown that testicular oestradiol levels in Spague-Dawley rats can be stimulated by FSH in the 12day old rat and that this capacity is lost by d18. It is not surprising therefore that Canick et al (1979) failed to find an increase in aromatase activity after FSH since their Spague-Dawley rats were 15 days old when used and Pomerantz showed that the response to FSH decreases between d12 and d18. Dorrington, Fritz and Armstrong (1978) using Wistar rats found a response to FSH in Sertoli cells from d16-d20 old rats while the effect was lost in the adult. The age of this decrease in FSH-responsiveness may therefore depend on the strain of rat.

Results now suggest that in the young rat oestradiol production is a function of both Sertoli and Leydig cells and is therefore under FSH and LH control, while as the animal matures the higher percentage of oestradiol production, and therefore aromatase activity lies in the Leydig cells and comes under LH control.

C) Control of Testicular Sensitivity by Luteinizing Hormone

Several hormones have been shown to regulate the concentration of their specific receptor sites on the surface of target cells, resulting in receptor loss and a desensitisation of the cellular response, known as 'down regulation'. Hormones such as insulin (Gavin, Roth, Neville, De Meyts and Buell, 1974), catecholamines acting on β-adrenergic receptors (Kebabian, Zatz, Romero and Axelrod, 1975; Mukherjee and Caron, 1975), thyroptrophin-releasing hormone (Hinkle and Tashjian, 1975), growth hormone (Lesniak and Roth, 1976) and LH acting on Leydig cells (Tsuruhara, Dufau, Cigorraga and Catt, 1977) or on luteal cells (Conti, Harwood, Dufau and Catt, 1977), have been shown to exhibit 'down regulation'. Of particular interest to this thesis is the 'down regulation' caused by LH in Leydig cells.

Purified rat Leydig cells treated with hCG **in vitro** showed dose-related responses in the extent and rate at which LH-receptors were lost, as measured by ^{125}I-hCG binding. This was paralleled by a reduction in the c'AMP response and a decrease in the maximum responsiveness of androgen production (Tsuruhara et al, 1977; Hsueh, Dufau and Catt, 1977). Normal Leydig cell function and binding was only restored 10 days after the hCG was administered. The LH/hCG-receptor complex has been shown to become increasingly resistant to dissociation with time and testicular cells already stimulated by LH or hCG will not bind any additional hormone. It is this lack of dissociation which leads to the apparent loss of receptors (Katikineni, Davies, Huhtaniemi and Catt, 1980).

In vivo, endogenous LH may result in a decrease in the LH-binding capacity of the Leydig cells due to a depletion in the number of available receptors and cause a decrease in

androgen production by these cells: 'down regulation' of the receptor population in the testis was seen after endogenous LH had been raised by injecting LH-RH (Catt, Harwood, Aguilera and Dufau, 1979).

It was hoped that by using an **in vitro** system of semipurified Leydig cells and dispersed testicular cells from quail testis, a study on the possible changes in sensitivity of these cells to hCG not only during sexual maturation, but during the adult life could be undertaken. We hoped to investigate the possibility that 'down-regulation' of any LH/hCG response by LH occurred in these birds, by measuring testosterone production in response to hCG. The possibility of testicular oestradiol production and its site of production from quail testes was also examined, and the effects of hCG on this oestradiol secretion was studied.

1.4 The Egg Cycle

As already discussed the ovary of a sexually mature female bird is composed of a hierachy of follicles, the larger yolky follicles designated F1-Fn according to their decreasing size, see Figure 1.6. The factors involved in the differentiation of the avian ovary and the control of the egg-laying cycle are poorly undertsood. It is not known why the largest follicle is always the next to ovulate nor what determines which follicle will start to take up yolk protein from the blood.

It is, however, well established that in mammals the ovarian steroids, particularly oestrogens, exert a feedback control between the ovary and the hypothalamo-hypophysial system. The question arises whether the same relationship exists in birds, and, if so whether the whole hierarchy of follicles participates in the control, or whether only the largest follicle is responsible for giving the signal necessary for the release of the hormones which induce ovulation. Much work has been done in the hen to study plasma and follicular concentrations of steroids throughout the egg-laying cycle and their inter-relationships with gonadotrophins in order to understand the timing and sequence of events leading to ovulation.

1.4.1 Site of Steroid Synthesis in the Avian Ovary: Dependence on Follicle Size

Preovulatory follicles from the avian ovary contain the steroids progesterone, testosterone, oestrone and oestradiol (Kumagi and Homma, 1974; Shahabi, Norton and Nalbandov, 1975). Later studies revealed that, in the chicken, two major sites of steroidogenesis existed within each follicle, the inner granulosa cell layer and the outer thecal cell

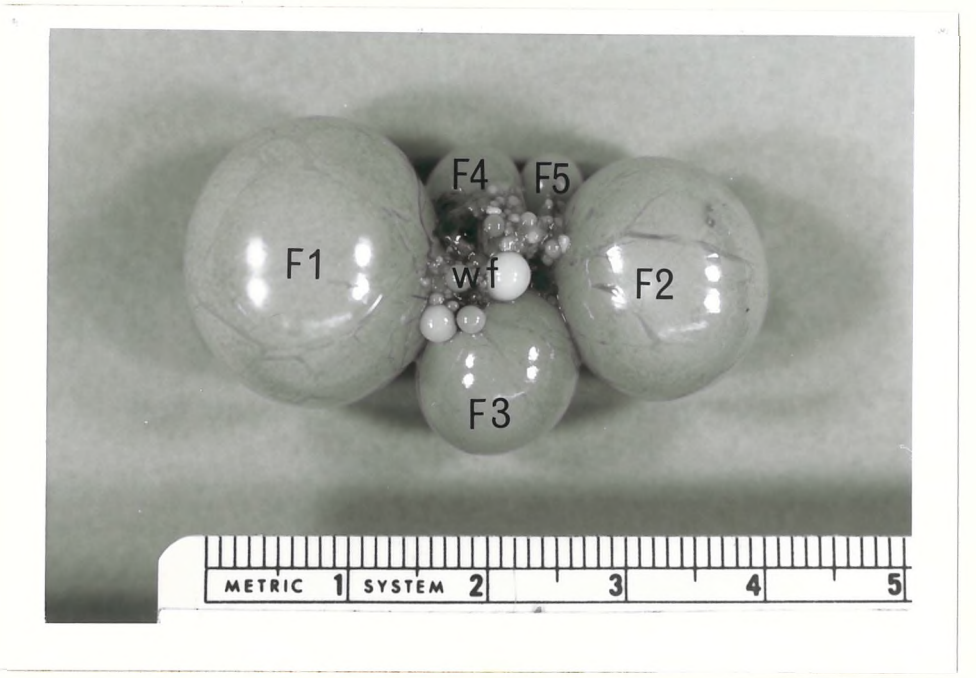


Figure 1.6

The Ovary of a Sexually Mature Female Quail Raised on Long Photoperiods.

A hierachy of follicles exists, with the large yolk-filled follicles designated F1-Fn according to their decreasing size. The F1 follicle being the next in the sequence to ovulate. All non-yolk filled follicles are designated as white follicles (WF). In an immature ovary only small white follicles exist.

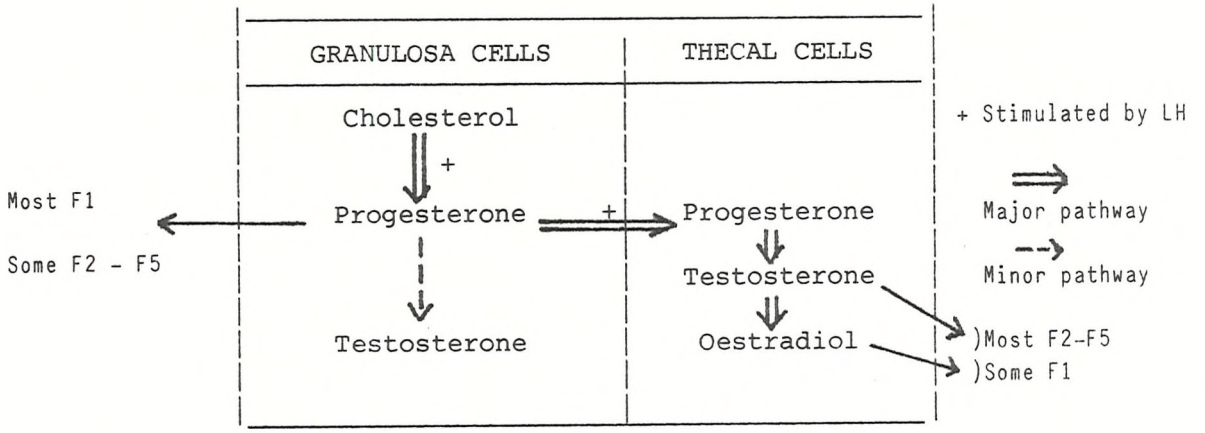
layer. The isolated dispersed granulosa cells produced predominantly progesterone with some testosterone **in vitro**, while the thecal cells produced testosterone and oestradiol in the presence of exogenous progesterone (Huang and Nalbandov, 1979 ; Huang, Kao and Nalbandov, 1979). The production of progesterone by granulosa cells correlates well with histochemical studies on the localisation of the 3β and 17β-hydroxysteroid dehydrogenase (HSD) enzymes. An intense 3β-HSD reaction was found in the granulosa cells of the laying hen (3β-HSD is the enzyme responsible for the conversion of pregnenolone to progesterone) (Chieffi and Botte, 1965; Boucek and Savard, 1970). Some 17β-HSD activity was also seen in the granulosa cells, probably responsible for the small secretion of testosterone from these cells, but intense activity in the thecal layers was seen in the follicles of the molting hen with less in the laying hen (Boucek and Savard, 1970). Dahl (1970) showed that the thecal cells from follicles of laying hens had the morphological characteristics of steroid-producing cells. This is consistent with the later observation that the thecal cells have the capacity to secrete steroids, shown by Nalbandov and co-workers (1979). This led to the 2-cell model hypothesis of avian follicular steroid synthesis shown in Figure 1.7

The amount and type of steroid secreted from avian ovarian follicles varies with the size of the follicle and on the time relative to ovulation at which the follicles were analysed. The secretion by and content of oestrogen (oestradiol-17β and oestrone) were found to be greater for the medium-size follicles (F3 and F4) than for the larger F1 and F2 follicles or the smaller F5 and white follicles (Kumagi and Homma, 1974; Senior and Furr, 1975; Cole and Peddie, 1980). Progesterone secretion and content increased with respect to increasing follicle size, the largest follicle, F1, secreted the greatest amount of progesterone (Shahabi, Norton and Nalbandov, 1975; Cole and Peddie,

Figure 1.7

Two Cell Model Hypothesis of Follicular Steroid Synthesis in the Avian Ovary

Progesterone is synthesised from cholesterol in the granulosa cells where some is metabolised to testosterone. In the largest yolk-filled follicle (F1) most of this progesterone is secreted out of the follicle into the plasma. The remaining progesterone from the F1 follicle and most of the progesterone from the medium sized yolk-filled follicles is secreted from the granulosa cells and taken up by the thecal cells (an LH-stimulated process), where it is converted to testosterone and oestradiol. Testosterone and oestradiol are then secreted out of the follicle into the plasma. As the follicles develop the profile of steroid secretion changes.



(From Huang, Kao, Nalbandov, 1979).

1980), while testosterone was secreted by all the follicles. These results suggested that the steroidogenic potential of the follicular cells changes during follicular maturation, oestrogens were secreted by the medium-sized follicles and as ovulation of the follicle approached, oestrogen secretion from the follicle was 'switched off' and progesterone secreted instead.

1.4.2 Effect of Endogenous and Exogenous Gonadotrophins on Follicular Steroid Secretion.

Luteinizing hormone (LH) significantly increases progesterone secretion from isolated, dispersed granulosa cells **in vitro**. The follicle F1 produced more than the F2 which produced more than F3, (Huang, Kao and Nalbandov, 1979; Scanes and Fagioli, 1980). A similar response was seen in follicles removed from birds at the time of high plasma LH levels (Huang and Nalbandov, 1979) or after plasma LH was raised by an injection of LH (Imai and Nalbandov, 1978; Culbert, Hardie, Wells and Gilbert, 1980) when again the progesterone content of the granulosa cells was increased.

Follicular testosterone production is also stimulated by LH in all sizes of follicles while the effect of LH on oestradiol production remains unclear. Some reports showed that oestradiol secretion was unaltered after LH or hCG injection (Shahabi, Bahr and Nalbandov, 1975; Cole and Peddie, 1980), while other reports showed that avian LH did cause an increase in the oestradiol content in the medium-sized follicles (Imai and Nalbandov, 1978; Huang, Kao and Nalbandov, 1979).

The effect of FSH on follicular steroids has not been studied in such great detail. Follicular levels of progesterone in the three largest chicken follicles are elevated after FSH administration **in vivo** (Imai and

Nalbandov, 1978). Increased progesterone secretion from the granulosa cells also occurred after the addition of FSH **in vitro** (Scanes and Fagioli, 1980). FSH and LH acted synergistically on increasing progesterone synthesis and release from chicken follicles (Imai and Nalbandov, 1978).

Studies on follicular steroid synthesis suggest that while the granulosa cells can synthesise progesterone in the absence of the thecal cells, testosterone and oestradiol production by the thecal cells requires the presence of either the granulosa cells or the addition of the precusor, progesterone. The follicles also respond to LH with an increase in progesterone and testosterone production by the intact follicle. It is possible that the decrease in aromatising activity in follicles of increasing size (F3-F1) coupled with increasing progesterone production by the granulosa cells of the F1 follicle are two of the factors which determine whether the avian follicle is ready to ovulate.

1.4.3 Control of the Preovulatory surge of LH: Role of Reproductive Steroids

The preovulatory release of LH in the hen occurs between 7 and 4h before ovulation and is associated with increased plasma concentrations of progesterone (peaks 4-7h before ovulation), testosterone (peaks 8h before) and oestrogens (oestradiol-17β and oestrone peak 6h before) (Furr, Bonney, England and Cunningham, 1973; Senior and Cunningham, 1974; Laguë, van Tienhoven and Cunningham, 1975; Shodono, Nakamura, Tanabe and Wakabayashi, 1975; Etches and Cunningham, 1977). The role which these steroids play in the induction of the preovulatory surge of LH has been investigated **in vivo** by injecting them into intact or ovariectomised hens. Injections of progesterone, testosterone, deoxycorticosterone or corticosterone but not oestro-

gens stimulates the secretion of LH in laying hens often resulting in premature ovulation (Etches and Cunningham, 1976a; Wilson and Sharp, 1976a), while in the ovariectomised hen, progesterone will induce LH release only after the hen has been 'primed' with injections of oestradiol and progesterone (Wilson and Sharp, 1976b).

The successful elevation of plasma LH and induction of ovulation by injecting these steroids depends on the time in the cycle at which the steroids are injected. LH-RH, progesterone or testosterone injected 25-27h after the terminal ovulation of a clutch all result in an elevation of plasma LH and ovulation while if the injections are given 0-8h after the terminal ovulation, none of the three hormones results in ovulation (Wilson and Sharp, 1976a; Etches and Cunningham, 1976b) despite the fact that progesterone does cause some increase in LH secretion. This correlates with the fact that between 0-8h after the terminal ovulation, there is no mature F1 follicle in the ovary capable of either secreting progesterone or of ovulating in response to endogenous LH (induced by LH-RH injection) or by exogenous LH. In contrast, 25-27h after the terminal ovulation, the F1 follicle has matured to the stage when it can respond to LH.

In chickens, oviposition is normally confined to an eight to nine hour period of each day, with the preovulatory releases of LH that lead to the formation and laying of eggs also restricted to an approximately 8h period per day. The period of LH release has been defined by Fraps as the hypothalamic 'open period' and is the period 'of low thresholds of response to feedback hormones' (Fraps, 1965). The 'closed' period includes the remaining 16h period when the hypothalamus-pituitary axis does not normally respond to feedback hormones in stimulating LH release. The first ovulation of a sequence results from an LH surge occurring near the onset of the 'open' period with successive LH

surges taking place later and later in the 'open' period until the 'open' period is closed, no LH surge seen and the sequence terminated. The start of the 'open' period in chickens is closely related to the onset of dusk (Follett and Davies, 1979 for review). However, attempts to demonstrate a diurnal rhythm in the sensitivity of the hypothalamus to the positive feedback action of progesterone have been unsatisfactory. Injections of progesterone, which produced plasma levels similar to those seen in the spontaneous preovulatory surge of progesterone, resulted in LH release, comparable to the spontaneous preovulatory LH surge, during the proposed period of minimum sensitivity of the hypothalamus (Etches and Cunningham, 1976b).

The lack of evidence for a diurnal rhythm in hypothalamic sensitivity led Williams and Sharp (1978) to study the plasma concentrations of progesterone, androgens and LH throughout the daily cycle of laying hens. They found that there was a small diurnal rise in LH at the onset of the dark period, also seen by Scanes, Chadwick, Sharp and Bolton (1978), which was paralleled by a small increase in progesterone and androgens but this only occurred when there was a mature follicle in the ovary. A large LH surge then followed together with a much larger rise in both progesterone and androgens and ovulation occurred about 8½h later. It seems likely that the diurnal increase in plasma LH levels at night does not induce ovulation directly but acts on the maturing follicle to stimulate progesterone secretion. Thus the timing of the preovulatory release of LH could be due to the diurnal rhythm of basal LH secretion. This complements the proposal of Bastian and Zarrow (1955) that a stimulus sufficient to induce ovulation (such as high LH levels in the plasma) is present for 8-9h every night, irrespective of whether the ovary contains a mature follicle.

In vitro, LH stimulates the F1 follicle to secrete more

progesterone and all the follicles to secrete more testosterone (shown by Nalbandov and co-workers). For the progesterone-induced LH release the hypothalamus must have been 'primed' with oestrogens and progesterone (Williams and Sharp, 1976b). These oestrogens are secreted from the medium-sized follicles and progesterone secreted from the developing follicles. Once plasma progesterone levels have exceeded a threshold level the positive feedback action of progesterone on the hypothalamus would result in a stimulation of LH release and induce the preovulatory surge of LH.

1.4.4 Control of Ovulation in the Japanese Quail

Most studies on the endocrine control of ovulation in birds has been done in chickens and little has been done in the Japanese Quail. Japanese Quail show a different pattern of egg-laying from the chicken (Wilson and Huang, 1961). Oviposition in the chicken occurs earlier in the day than in the quail, with most quail, raised on 14h light per 24h, laying between the 9th and 14th hour of light inclusive (Opel, 1966). The first ovulation in a sequence in chickens occurs around the onset of light while quail do not ovulate the first egg of a sequence until some 8 to 9h after the onset of light (Opel, 1966). It seems that while the onset of night is the signal for laying in the chicken, the onset of day seems to be the signal and the start of the 'open period' in the Japanese Quail. Quail raised on constant light laid uniformly throughout the day (Arrington, Abplanalp and Wilson, 1962).

Recently Doi et al (Doi, Takai, Nakamura and Tanabe, 1980) showed that plasma LH in the quail increased shortly after dawn reaching a peak 4h before the next expected ovulation, coinciding with a peak in plasma progesterone. Plasma oestradiol and testosterone both showed maximal levels 6h before the next expected ovulation while oestra-

diol showed an additional peak some 22h before the next expected ovulation.

The plasma steroid levels in quail correlated with the follicular steroid levels which have been seen in the chicken, with the F1 follicle containing most progesterone and the medium-sized (F3 and F4) containing most oestrogens. The changes in follicular content paralleled the changes seen in plasma steroid levels throughout the cycle. It seems likely that a similar pattern of events occurs in the quail as is seen in the chicken, but that in the quail the events are triggered by the onset of light instead of the onset of dark.

One of the aims of this thesis was to study the histochemical reactions, locating the 3β- and 17β-HSD enzymes within the thecal and granulosa cell layers, in the maturing follicles of Japanese Quail and to see if the changes in the steroidogenic potential of the follicular cells was paralleled by changes in the activities of these two enzymes.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Animals

This research was performed using Japanese Quail (Coturnix coturnix japonica). Fertilised quails' eggs were incubated at 37°C and 60% humidity in a forced draught Westernette incubator (Messrs. Western Incubator Ltd., East Hanningfield, Essex, England). After hatching, quail were either housed in cages maintained at 25°C by an infra-red heater or in an incubator at 25°C for 2 weeks, when they were transferred to cages maintained at 19-20°C. They were raised on turkey starter diet, containing (per Kg) 10g calcium, 8g total phosphorus and 50µg cholecalciferol (2000IU), and water **ad libitum**.

Hatched quail were raised on one of the five lighting systems listed below:-

a) 16L:8D NL - 16 hours light : 8 hours dark per day (Long photoperiods). Lights on 09.00-01.00h GMT (Normal Light, NL).

b) 16L:8D RL - 16 hours light : 8 hours dark per day (Long photoperiods). Lights on 21.00-13.00h GMT (Reverse Light, RL)

c) 8L:16D - 8 hours light : 16 hours dark per day (Short photperiods). Lights on 08.00-16.00h GMT.

d) 24LL - 24 hours light per day. (Continuous light).

e) d70TLD - Hatched onto 8L:16D until somatically mature (i.e raised as group c)) and then transferred when 70 days old to 16L:8D NL.

2.2 Blood Sampling

In experiments where more than one blood sample was taken from each bird, blood samples were collected from the wing vein without the use of anaesthesia. Feathers were plucked from the wing vein area and the vein was cut with a razor blade. Blood was allowed to flow, and was removed using a pasteur pipette into heparinised tubes, until the flow of blood stopped. 300-800µl of blood could be collected in this way. The blood was centrifuged, plasma collected and frozen at -20°C until required. Blood samples taken during the dark period were obtained using a minimal red light source.

2.3 Hormone Treatment

Equimolar amounts of oestradiol-17β, oestradiol-17α, oestrone and oestriol were dissolved in ethyl oleate to yield a 1.8mM solution. Quail were injected intramuscularly into the breast muscle at a dose of 0.18µmoles/100g BW daily for 3 days. The control birds were injected with the ethyl oleate vehicle only.

2.4 Chemicals

The chemicals used in this research and their commercial sources are listed below.

i) Reagents used for preparation of Buffers

Di-sodium hydrogen orthophosphate	British Drug Houses (BDH)
Potassium dihydrogen orthophosphate	BDH
Sodium chloride	BDH
Potassium chloride	BDH
Magnesium sulphate-$7H_2O$	BDH

Calcium chloride	BDH
Sodium bicarbonate	BDH

ii) Reagents used for histology and histochemistry

Chloroform	Koch-Light Labs
Methanol	Koch-Light Labs
Erlich's Haemotoxylin	Hopkin and Williams
Eosin	Hopkin and Williams
DPX mountant	Hopkin and Williams
Nitro Blue Tetrazolium	Sigma Chemical Co
Nicotinamide	BDH
Dimethylformamide	BDH
Nicotinamide Adenine dinucleotide	Sigma Chemical Co
Farrent's medium	Hopkin and Williams
Formalin	BDH
Tissue Tek OCT Compound	Raymond Lamb, London

iii) Antigens

a)	Oestradiol-6-carboxymethyl-oxime-bovine serum albumin (BSA)	Steraloids Inc
b)	Progesterone-11-carboxymethyl-oxime-BSA	Steraloids Inc
c)	Testosterone-11-carboxymethyl-oxime-BSA	Steraloids Inc
d)	Oestrone-6-carboxymethyl-oxime-BSA	Steraloids Inc

iv) Unlabelled Steroids

Oestradiol-17β	Organon Ltd., Morden
Oestradiol-17α	Steraloids Inc
Oestrone	Sigma Chemical Co
Oestriol	Sigma Chemical Co
Testosterone	Sigma Chemical Co
Dihydrotestosterone (DHT)	Sigma Chemical Co
Progesterone	Sigma Chemical Co
17α-hydroxyprogesterone	Sigma Chemical Co
Pregnenolone	Sigma Chemical Co
Dehydroepiandrosterone	Sigma Chemical Co
Epiandrosterone	Sigma Chemical Co
5α-androstan-3α-17β-diol	Sigma Chemical Co
Androsterone	Sigma Chemical Co
Androstenedione	Sigma Chemical Co

v) Radiochemicals

All radiochemicals were obtained from the Radiochemical Centre, Amersham, Buckinghamshire, England.

(1,2,6,7n-3H)	Testosterone	SA 80-100Ci/mmol
(2,4,6,7n-3H)	Oestradiol-17β	SA 80-110Ci/mmol
(1,2,6,7n-3H)	Progesterone	SA 80-110Ci/mmol
(2,4,6,7n-3H)	Oestrone	SA 83 Ci/mmol

vi) Other Reagents and Chemicals

Gelatine	Sigma Chemical Co
Sodium azide	Sigma Chemical Co
Dextran T70	Pharmacia Fine Chemicals
Charcoal Norit A	BDH
D-Glucose	BDH
Collagenase (Type 1)	Sigma Chemical Co
Bovine serum albumen	BDH
Heparin	Boots Co Ltd.
Ethanol	J.Burroughs Ltd
Diethylether (anhydrous puriss)	Koch-Light Labs
Lanthanum chloride (Analar)	BDH
Calcium Carbonate (Analar)	BDH
Ethyl oleate	BDH
Acetone	Koch-Light Labs
Human Chorionic gonadotrophin (hCG) (Batch No.6299 : 3250i.u/mg protein)	Organon Ltd (UK)
Freund's adjuvant	Miles Laboratories Inc.

2.5 Liquid Scintillation Fluids

The liquid scintillation fluids used throughout were tritoscint, for aqueous samples, and Toluene-Butyl PBD, for non-aqueous samples.

i) Tritoscint

Xylene (puriss) 667ml	Koch-Light Labs
Diphenyloxazole (PPO) 4g (scintillation grade)	Int. Enzymes
Dimethyl bis phenyl oxazoly benzene (Scintillation grade) 0.5g	Cambrian Chem.
Synperonic NXP 333ml	I.C.I

ii) Toluene-Butyl PBD

Toluene (sulphur free) 2.5 litres	Koch-Light Labs
Butyl PBD (2-4' tert butyl phenyl-5-4' biphenyl)-1,3,4-oxadiazole 20g	C & G Chemicals

2.6 Preparation of Buffers

i) 50mM Phosphate gelatine buffer, pH 7.4

a) Stock solutions, 50mM disodium hydrogen orthophosphate (Na_2HPO_4) and 50mM potassium dihydrogen orthophosphate (KH_2PO_4), were made up containing 0.01% sodium azide.

b) These were titrated against each other to pH 7.4.

c) 0.1% gelatine was added to the final buffer. (Solubilisation of the gelatine was aided by first adding it to a smaller than required volume of warm buffer, and then making the buffer up to the correct volume once the gelatine had dissolved).

ii) 0.1M Phosphate buffer, pH 7.4

Stock solutions of 0.1M Na_2HPO_4 and 0.1M KH_2PO_4 were made up and titrated against each other to pH 7.4.

iii) Dextran-coated Charcoal Preparation

500mg charcoal and 50mg dextran T70 was added to 200mls of 50mM phosphate gelatine buffer pH 7.4. The solution was mixed using a magnetic stirrer for 1 hour before being stored at 4°C.

iv) Kreb's bicarbonate buffer, pH 7.4

	g/l
Sodium chloride	5.50
Potassium chloride	0.35
Magnesium sulphate $7H_2O$	0.11
Potassium dihydrogen orthophosphate	0.16
Sodium bicarbonate	2.10
*Calcium chloride	0.28

All salts except *Calcium chloride were dissolved in 900mls of distilled water. The calcium chloride was dissolved in 50 mls distilled water before it was added to the remaining salts. The buffer was then made up to 1 litre. The buffer was gassed immediately before use with 95%O_2 : 5% CO_2 for approximately 20 minutes until pH 7.4 was obtained. 0.2% Glucose was then added.

2.7 Histological Procedure

Quail ovary and testis were removed and fixed in Bouin's solution.* They were later dehydrated in graded solutions of alcohol and embedded in paraffin wax. Sections (8µm) were cut on a microtome and mounted on slides. The sections were stained with haematoxylin and eosin. The histol-

* 1:5:15, Glacial Acetic Acid : Formalin sol^n (Formaldehyde) : Saturated Picric Acid, (all chemicals from BDH).

ogy of the follicles of different sizes in the ovary and of testicular development were performed.

2.8 Histochemical Procedure

Tissues were frozen in liquid nitrogen immediately after they had been collected and were fixed to a metal block with Tissue Tek OCT compound. The tissue was then sectioned using a cryostat at 8μm and mounted on slides. The sections were kept at -20°C until stained for either 3β-hydroxysteroid dehydrogenase (3β-HSD) activity or 17β-hydroxysteroid dehydrogenase (17β-HSD) activity.

The method used was that described by Levy, Deane and Rubin (1959). The stain was made up of the following:-

CONSTITUENT	AMOUNT	FINAL MOLARITY
Steroid (substrate)	0.2mg	≈ 0.10mM
Dimethylformamide	0.5ml	-
Nitro Blue Tetrazolium sol^{n} (1mg/ml)	1.0ml	0.16mM
Nicotinamide sol^{n} (1.6mg/ml)	0.7ml	4.00mM
NAD sol^{n} (3mg/ml)	0.8ml	0.54mM
0.1M Phosphate Buffer pH 7.4	4.0ml	0.057M
	7.0ml	

The steroid substrate used depended on the enzyme activity which was to be measured. Testosterone was used as substrate for the 17β-HSD and pregnenolone as substrate for 3β-HSD. In both cases the steroid was first dissolved in the dimethylformamide before the remaining constituents were added to make up the final working solution.

The sections were covered with the working solution and incubated at 37°C for 1 hour. They were then fixed in 50% ethanol/10% formalin and mounted with aqueous mountant, (Farrent's Medium). Control slides were exposed to the

stain in the absence of either steroid. The intensity of the reactions, judged by the density of the formazan granulation deposited in the tissue under microscopic examination, was rated from 0 (negative) to 5 (dark, dense blue).

2.9 Preparation and Incubation of Testicular Cell Suspensions

2.9.1 Leydig cells

Quail Leydig cells were prepared by a method similar to that previously described for rat Leydig cells by Janszen, Cooke, van Driel and van der Molen, (1976). Quail were killed, the testes removed, weighed and decapsulated. After they had been teased apart they were placed in a flask containing 4 mls Kreb's-bicarbonate buffer, pH 7.4 supplemented with 0.2% glucose (KBBG), 1mg/ml bovine serum albumen and 0.25mg/ml collagenase. The flask was incubated at 37°C for 15-20 minutes under an atmosphere of 95% O_2:5% CO_2 in a shaking water bath (140 cycles/min). After incubation, 15mls of 0.9% sodium chloride was added and the flask left to stand at room temperature for 10 mins, allowing the tubule fraction to settle. The supernatent was filtered through a nylon mesh, mixed with an equal volume of KBBG and spun in a bench centrifuge (5 mins at 700g). The supernatent was discarded and the cells washed in KBBG and respun. The supernatent was again discarded and the cells resuspended in 2-3mls KBBG. 50µl of the cell suspension was further diluted and the cells were counted in a haemocytometer in order to determine cell numbers.

Aliquots containing approximately $4x10^3$ cells were incubated in KBBG (final volume 400µl after addition of hCG)in 1.7ml stoppered glass vials. Cells were preincubated for 30mins at 37°C in a shaking water bath (40

cycles/min). At the end of the preincubation period, hCG (final concentration 1-1000I.U./ml) or saline (control) was added and cells incubated under the same conditions for a further 3h. The incubates were gassed with water-saturated 95% O_2:5%CO_2 three times during the incubation:- at the start of preincubation, t=0h, and t=1½h. At the end of the incubation, the vials were centrifuged at 700g for 5 mins at room temperature and the supernatent removed and frozen at -20°C until assayed for testosterone.

The number of cells showing 3β-HSD activity after collagenase treatment was determined by using the histochemical stain previously described (Chapter 2.8). 1ml of the diluted cell suspension was incubated with 1ml of the stain and incubated for 1h at 37°C. The percentage of cells stained blue was then determined by counting these cells in a haemocytometer. The viability of the cells was evaluated by diluting one aliquot with 0.4% Trypan Blue. The percentage of cells which excluded the dye was always greater than 60%.

2.9.2 Dispersed Testis Preparation

A preparation of dispersed testicular cells (i.e. both Leydig and tubule fractions) was made in a similar way as just described for Leydig cells. Whole testes were incubated, after decapsulation, in 4ml KBBG containing 1mg/ml BSA and 1mg/ml collagenase for 15-20 minutes at 37°C under an atmosphere of 95% O_2:5% CO_2 in a shaking water bath (140 cycles/min). Complete dispersion was aided by a pasteur pipette, since mechanical agitation helped to separate the cells. The cells were washed and spun as the Leydig cells and cell number also determined.

Aliquots containing 8-10x10^3 cells were incubated in KBBG (final volume 800µl after addition of hCG) in 1.7ml stoppered glass vials. Preincubation and incubation was

the same as for the Leydig cells (hCG doses 1-100I.U./ml-final concentration). At the end of the incubation the supernatent was removed after centrifugation and frozen until required for assay. 50µl ethanol was added to the cells in order to stop any further reaction. The cells were then resuspended in 500µl KBBG and frozen until aliquots were extracted with 2ml diethyl ether and the extracts assayed for oestradiol and testosterone by RIA.

2.10 Determination of Plasma Calcium

Total plasma calcium concentrations were determined using an Atomic Absorption Spectrophotometer (Perkin Elmer 280), 50µl of plasma was taken and calcium measured in the presence of 0.1% lanthanum chloride. Standards were prepared from Analar calcium carbonate and contained the same concentration of lanthanum chloride.

2.11 Statistical Analysis

Statistical analyses of the data were performed using paired or unpaired Student's t-tests where appropriate, values of $p < 0.05$ were taken to be significant. Regression and correlation coefficients of a graphic plot were calculated using a programmable Hewlett-Packard digital calculator on a program giving regression coefficients (R^2) for a linear, exponential, parabolic or power plot from the data given. The probability level of these plots occurring can then be worked out using t-tests where: -

$$t = \frac{R}{S_r} \quad \text{and} \quad S_r = \frac{1 - R^2}{n - 2}$$

where n = number of points plotted
n-2 = degrees of freedom

2.12 Radioimmunoassay Techniques

2.12.1 Setting up a Radioimmunoassay (RIA)

Antisera were raised in New Zealand white rabbits to one of the following antigens:-

1. 17β-Oestradiol-6-carboxymethyl-oxime-bovine serum albumen
2. Progesterone-11-carboxymethyl-oxime-bovine serum albumen
3. Testosterone-11-carboxymethyl-oxime-bovine serum albumen
4. Oestrone-6-carboxymethyl-oxime-bovine serum albumen

The rabbits were injected intra-dermally with 1.0-2.0mg of the conjugate in a 50:50 mixture of 0.9% saline in complete Freund's adjuvant. They received two supplementary intra-muscular injections 6 and 8 weeks later. The antiserum titre was estimated at 10 to 12 weeks before the animals were killed by exsanguination, under urethane anesthesia, through an aortic cannula. The antisera were stored in 500µl aliquots at -20°C until used.

The assay procedure was identical for all steroids except that the antisera were used at different dilutions. Antiserum titre was found by incubating the tritiated (^{3}H) steroid (≈10,000 dpm/ml) with dilutions of the antisera from 1:10 to 1:100,000 for 3h at 37°C. The free and bound ^{3}H-steroid were separated using dextran coated charcoal, at 4°C, and after counting the radioactivity in the bound fraction, the dilution of the antiserum at which 50% of the label was bound was calculated. These dilutions were: for oestradiol-17β 1:22,000; for progesterone and testosterone 1:8,000 and for oestrone 1:6,000.

2.12.2 Method for Extracting Steroids from Plasma

100µl aliquots of plasma were extracted in 12x100mm glass test tubes with 2ml diethyl ether. 100µl ethanol was added to each plasma sample prior to extraction to denature any binding proteins present. Each sample was vortexed for 1 min, the aqueous phase frozen on cardice (solid carbon dioxide) and the supernatent decanted into 12x75mm glass test tubes and evaporated overnight in the fume cupboard at room temperature.

2.12.3 RIA of Medium Containing Secreted Steroids.

100µl or 150µl aliquots of medium containing secreted steroids were assayed directly from the medium without prior extraction. It was found that assaying direct from the medium yielded similar results as those when extraction was performed. The standard curve of the assay was modified for assays of medium samples. A similar size of aliquot, to that containing the sample, of medium buffer was added to each of the tubes of the standard curve and the assay performed as described in Chapter 2.12.4

2.12.4 Methods used for RIA

Standard curves were constructed for each steroid over the following ranges:

Oestradiol : 2.5×10^{-14} moles to 5×10^{-12} moles per tube.
Progesterone : 5×10^{-14} moles to 5×10^{-12} moles per tube.
Testosterone : 5×10^{-14} moles to 2.5×10^{-11} moles per tube.
Oestrone : 5×10^{-14} moles to 5×10^{-11} moles per tube.

The purity of these stock standards were routinely checked by gas liquid chromatography by Dr. D. Corina, and the steroids were found to give only one retention peak.

The assays were performed as follows:-

Stock antisera was diluted with 50mM phosphate buffer pH 7.4 containing 0.1% gelatine and 0.01% sodium azide to the dilution binding 60-70% of the added ^{3}H-steroid to which the antibody had been raised. The diluted antisera were incubated at 4°C overnight with the homologous ^{3}H-steroid (^{3}H-oestradiol, ^{3}H-progesterone, ^{3}H-testosterone or ^{3}H-oestrone) so that each tube finally contained approximately 22,000dpm.

500µl of the tritrated binding solution were added to each assay tube, the sample mixed and incubated at 37°C for 1½h, vortexed again and left for a further 1½h at 37°C. The samples were then cooled to 4°C. Free and bound steroid were separated using 500µl dextran-coated charcoal. The tubes vortexed, left to stand on ice for 10 min, centrifuged at 4°C at 2,000rpm on an MSE mistral 2L centrifuge. 500µl of the supernatents were pipetted out into scintillation vials, and 500µl distilled water and 10ml tritoscint added. The radioactivity was counted for 4min in a Philip Liquid Scintillation counter with a counting efficiency of 55% and which gave automatic quench correction and printed the results as corrected dpm.

In all assays, pool samples were incubated as well as reagent and assay blanks. This enabled the precision of the assay, intra- and inter-assay variations as well as the sensitivity of the assay to be established.

2.12.5 Analysis of Assay Data

Analysis of the assay data was performed using a Hewlett-Packard digital calculator programmed to perform a logit-transformation of the dpm, and to estimate the amount of steroid in the unknown samples from a calculated line of closest fit to the standard curve. (This programme was

prepared for us by Dr. I. Giles).

Any sample which fell within 9.89% of either end of the line was considered either non-detectable or not accurate since the logit-transformation deviates from linearity beyond these limits.

The coefficient of regression, slope and the intercept value on the Y axis were also calculated. A typical standard curve for one of the steroids is shown in Figure 2.1.

2.12.6 Assessment of Assay Reliability, Reproducibility and Specificity

Minimum sensitivity, i.e. the least detectable amount measured in the assay. This was obtained by finding the least amount of cold steroid needed to show a displacement of the bound ^{3}H steroid from the binding solution and was found using the standard curve data. Oestradiol had a minimum sensitivity of 2.5×10^{-14} moles per tube. Progesterone, testosterone and oestrone all had minimum sensitivities of 5×10^{-14} moles per tube.

The reproducibility of the assay was determined from the pool samples used in each assay and from these results the inter-assay coefficient of variation (CV) of the assay was found using the following equation:

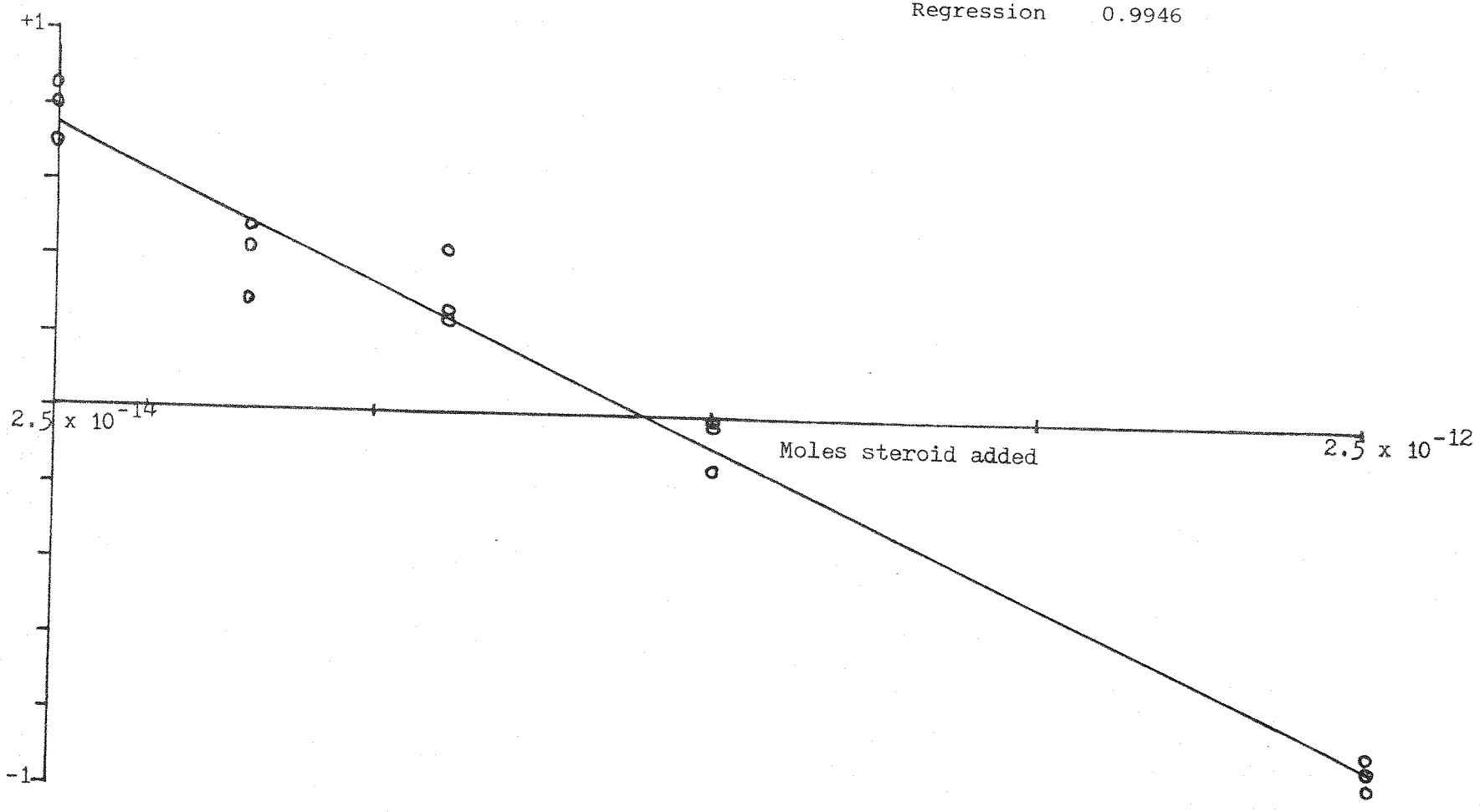
$$CV = \frac{SD}{\bar{X}} \times 100$$

where $\bar{X}$ = the mean value of the steroid pool samples from the different assays and

SD = standard deviation of the mean of the pool samples

Intra-assay variation was also determined in the same way where $\bar{X}$ is the mean value of amount of steroid present in

Figure 2.1: Log-Logit Transformation of a Typical Standard Curve for Oestradiol-17β



Antiserum raised against E_2-6-CMO-BSA at a final dilution of 1:22,000 was incubated with ^{3}H-oestradiol and the radioactivity displaced with cold steroid, over a range between 2.5×10^{-14} moles and 2.5×10^{-12} moles; standards were run in triplicate. The details of the method are in the text.

tubes containing the same amount of added steroid.

The coefficients of variation (CV) of the plasma RIA were:-

	Mean Plasma pool sample pmole/ml	Inter-assay CV	Intra-assay CV
	6.5	17.0%	10.0%
Testosterone	17.8	17.8%	13.0%
	21.4	17.0%	6.0%
	6.19	21.0%	9.0%
Progesterone	15.50	24.0%	5.0%
	39.80	16.0%	9.0%
	0.97	21.0%	8.4%
Oestradiol	2.83	19.1%	6.7%
	7.56	18.4%	4.0%

(n = 9 in each instance)

Inter-assay variation was corrected for if the pool samples were found to be more than 10% away from the average value of the pool samples above. In these cases, all the values obtained were increased or decreased accordingly by the same amount that brought the pool samples back to the average value. Any differences, therefore, in plasma levels reported in this thesis cannot be accounted for by inter-assay variation since this was corrected for, but no correction was made for intra-assay variation. Samples taken from the same bird were all assayed at one time.

The coefficients of variation (CV) of the medium RIA were:-

	Mean Medium pool sample pmole/ml	Inter-assay CV	Intra-assay CV
	1	12%	3%
Testosterone	10	9%	4%
	50	5%	5%
	1	12%	7%
Oestradiol	10	14%	5%
	100	9%	5%

(n = 6 in each instance)

Inter-assay variation was not corrected for in

RIA of medium samples.

Medium and Solvent Blanks were always below the minimum sensitivity of the assays and the reagent blank was taken into account in the programme for the logit-transformation on the Hewlett-Packard digital calculator.

The Recovery of Added Steroids from the plasma was estimated by adding a known amount of steroid and measuring it in the same way as the unknown samples. From the standard curve, the concentration of the steroids were determined and then expressed as the % of the added steroid.

The recovery in the assays were:-

Testosterone - 90.5±2.0%
Oestradiol - 91.5±3.5%
Progesterone - 94.9±2.7%

Shortfall in recovery has not been corrected for since it seemed to be consistent at three different doses (50, 75 and 100µl) of the assayed steroids.

Recoveries during the extraction procedure were checked by adding the homologous ^{3}H-steroid to the plasma prior to ether extraction and counting the radioactivity extracted.

The recoveries were found to be:-

Testosterone - 96.8±0.9%
Oestradiol - 95.4±2.8%
Progesterone - 91.2±2.4%

The recovery of added steroids from the medium assays was:-

Testosterone - 94.5±1.9%
Oestradiol - 95.4±2.8%

The Cross Reactivity of the Antisera were evaluated at

the level at which the added antigen gave a 50% displacement of the tritrated label, 100% being the percentage bound in the absence of any added cold hapten. At this level for the **testosterone antiserum** only dihydrotestosterone caused any significant displacement being 50% of that caused by testosterone. Androstenedione, androstanediols, androsterone, androstenediols, dehydroepiandrosterone, oestradiol-17β, oestrone, progesterone and 11α-hydroxyprogesterone caused less than 1% displacement.

At the 50% level for the **oestradiol-17β antiserum**, oestradiol-17α, oestrone and oestriol caused displacements of 50.0%, 24.9% and 9.1% respectively of that caused by oestradiol-17β. Other steroids tested (testosterone, androstenedione, progesterone and 11α-hydroxyprogesterone), caused less than 1% displacement.

At the 50% level for the **progesterone antiserum**, 11α-hydroxyprogesterone and 17α-hydroxyprogesterone gave 95% and 25% cross-reactivity respectively of that caused by progesterone. All the androgens and oestrogens tested caused less than 1% displacement.

At the 50% level for the **oestrone antiserum**, oestradiol-17β and oestriol gave 21.9% and 18.8% cross-reactivity respectively of that caused by oestrone. Androstanediol gave 22%, dihydrotestosterone - 9.4%, dehydroepiandrosterone - 9.4%, progesterone - 6.3% and testosterone - 3.1% cross-reactivities; while androstenedione and androsterone showed less than 1% displacement.

CHAPTER 3. STUDIES ON GENERAL DEVELOPMENT AND PLASMA STEROID LEVELS DURING MATURATION OF JAPANESE QUAIL MAINTAINED ON DIFFERENT PHOTOPERIODS

3.1 Introduction

The onset of puberty and sexual maturation involves many changes in the hormonal and physical state of the species. None more so than in the female bird who must prepare her body for the high calcium requirement needed for egg-shell calcification in the laying state as well as for the high rates of lipoprotein and protein synthesis required for egg production.

Development of the ovary and testis also occurs and increased secretion of gonadal hormones ensues. The development of a functional oviduct is dependent on these ovarian hormones. Some work has been done in birds (chickens and quail) to measure the levels of plasma steroids during development and it was hoped that the work reported in this thesis would complement these results.

Steroid hormones, in particular the oestrogens, have been shown to be involved in Vitamin D_3 metabolism. It was proposed (Sedrani, 1979) that the changes in the metabolism of Vitamin D_3 in the female, just prior to the onset of lay, could be dependent on plasma steroid levels at this time. I therefore decided to look at the levels of three of the plasma steroids (testosterone, oestradiol-17β and progesterone) in quail that would either become mature (raised on long photoperiods), or stay immature (raised on short photoperiods) and then later mature when they were transferred to long photoperiods. The steroid levels found were correlated with the sexual maturity of the bird and particular attention was paid to any differences in steroid

levels between male and female quail just prior to the onset of lay. The possible relationship which these levels have in the regulation of Vitamin D_3 metabolism is discussed.

3.2 Methods

350 quail were raised from hatching on one of four lighting regimes: 16L:8D NL, 8L:16D, 24LL or d70TLD (see Materials and Methods, Chapter 2.1 for details). Quail were killed by decapitation in either their first or second hour of light and trunk blood collected. Plasma was stored at -20°C until assayed for progesterone, testosterone and oestradiol by specific RIAs (as described in Materials and Methods, Chapter 2.12). Testis, ovary and body weight were recorded at autopsy. The testes were fixed in Bouin's solution and histological sections prepared for staining with Haematoxylin and Eosin (H and E) (see Chapter 2.7 for details). Yolk-filled follicles from mature ovaries were either frozen and sectioned immediately for histochemical studies or they were fixed in Bouin's solution for later sectioning and staining with H and E (results of this staining are described in Chapter 5.3).

A second group of quail were raised on d70TLD but on transfer to long photoperiods of 16L:8D NL were caged out separately, each cage containing a male and female quail. These birds were then bled by the wing vein (see Chapter 2.2), in their 2nd (female) or 3rd (male) hour of light, two or three times a week throughout the next 70 days. Plasma was collected and stored at -20°C until all the samples from one bird could be assayed together. Progesterone and oestradiol RIAs were performed for female plasma and testosterone and oestradiol RIAs for male plasma. Egg-laying records were kept for each female.

3.3 Results

Onset of egg-laying in quail kept under 24LL or 16L:8D NL occurred between 42 and 49 days after hatching. Egg-laying was prevented completely in birds kept on 8L:16D but in quail raised on d70TLD, onset of egg-laying began between the 22nd and 29th long day (92 and 99 days after hatching).

Female quail raised on d70TLD and then caged out with a male at the time they were transferred to long photoperiods, came into lay between the 30th and 44th long day.

3.3.1 Body Weights

There is a linear increase in male and female body weights between 11 and 35 days (11 and 35d) in quail raised under each lighting system (Figure 3.1). Significant increases in body weight were seen between the ages 11 and 18d ($p<0.001$), 18 and 21d ($p<0.001$), 21 and 29d ($p<0.001$), and 29 and 35d ($p<0.001$) for both male and female quail in all the groups studied. No significant difference was found in the body weights of male and female birds at these ages. After 35d the increase in body weight was not significant within any one seven day period.

Quail raised on either 16L:8D NL or 24LL showed no significant difference in the body weights recorded at the same age on the two lighting regimes. Male quail on these two lighting systems showed no further significant increase in body weight with age after 35d (within one 7 day period), but body weights increased slowly to reach their highest values at 70d on 16L:8D (94.7±4.1g, n=5) and at 49d on 24LL (106.0±2.4g, n=5). Female quail on both lighting systems showed a significant increase in body weight between 35d and 49d, $p<0.01$, (16L:8D NL - from 90.0±4.2g, n=9 to

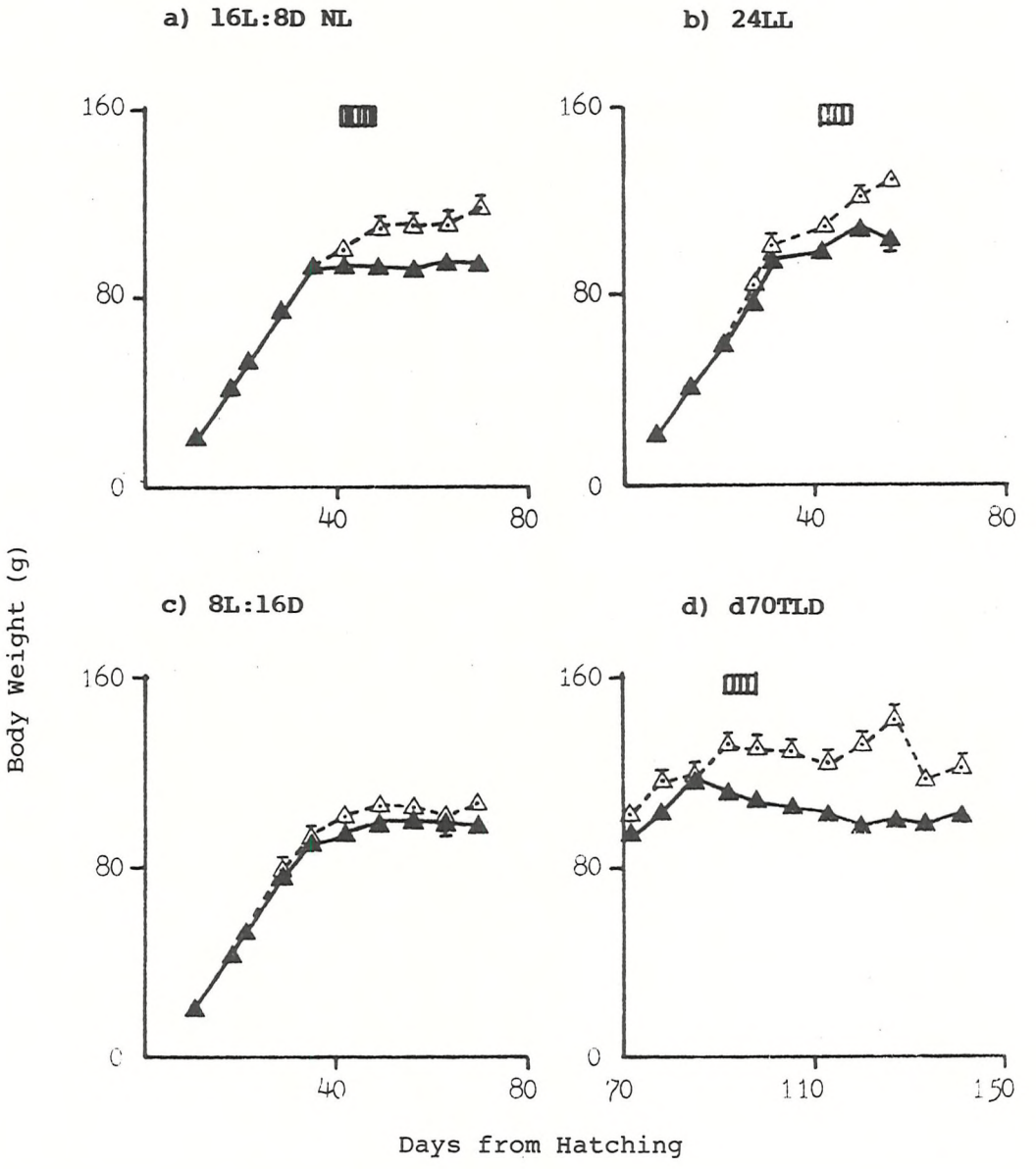


Figure 3.1 - Changes in Body Weight for Male (▲——▲) and Female (△--△) Quail with Age

Quail were raised on a) 16L:8D NL, b) 24LL, c) 8L:16D and d) d70TLD. (The first 70d of the latter group was identical to that of group c)). The period when onset of lay occurred is shown by the hatched area. Birds raised on 8L:16D did not come into lay during the period studied. Results are shown as mean ± S.E.M with the number in each group between 5-10 birds, except for birds aged 11d or 18d raised on a), b) or c) which could not be sexed, when n=20-30.

108.7±4.3g, n=13; and 24LL - from 101.5±2.5g, n=5 to 121.4±5.0g, n=4). Body weights of female quail (16L:8D NL and 24LL) were significantly higher than males of the same age at 42d ($p<0.02$) under 24LL and at 49d ($p<0.01$) under 16L:8D NL, and remained significantly higher for the remainder of the period studied.

Quail raised on 8L:16D showed no further significant increases in body weight after 35d in either sex (Figure 3.1 c)). There was no significant difference in the body weights of female and male quail until 70d when female body weights were higher, (female - 105.7±1.6g, n=6; male - 96.7±2.7g, n=6: $p<0.02$). These results are similar to the quail raised on d70TLD up to 70d. After they had been transferred to 16L:8D NL, the d70TLD female quail showed a significant increase in body weight ($p<0.001$) between the 2nd long day (72d - 102.0±2.4g, n=10) and the 22nd long day (92d - 131.4±3.1g, n=11), with no further significant increase with age thereafter,(Figure 3.1d). d70TLD male quail showed a significant increase in body weight ($p<0.05$) between the 2nd long day (72d - 94.2±1.6g, n=5) and the 35th long day (105d - 104.5±2.8g, n=11). Male quail body weights remained unchanged with continuous exposure to 16L:8D.

Body weights of female quail, being significantly higher ($p<0.02$) than the males on the day of transfer to 16L:8D NL, were no longer significiantly higher by the 2nd long day and remained not significantly different until the 22nd long day. Body weights at this time were significantly higher ($p<0.01$) in the female (131.4±3.1g, n=11) than the male (112.4±6.1g, n=5).

There was no significant difference in body weights recorded for male quail raised on 8L:16D and 16L:8D NL, 24LL or d70TLD. No significant difference was seen in body weights recorded for female quail raised on 8L:16D and

16L:8D NL although female body weights tended to be higher in quail raised on 16L:8D NL after 49d. Body weights of female quail raised on 24LL were significantly higher ($p<0.01$) at 49d, and remained higher compared to body weights of females of the same age, but raised on 8L:16D. Females' body weight on the 28th long day (d70TLD group) were not significantly different to those recorded for female birds of 49d on either 16L:8D NL or 24LL, i.e. once laying had been established then body weights for all three photostimulatory groups were the same. Laying quail had higher body weights than non-laying quail.

3.3.2 Gonadal Weights

Gonadal development occurred under all four lighting regimes, although growth under the 8L:16D was very small, remaining in the mg range, and so as a result these birds never reached sexual maturity. The results are shown in Figure 3.2. Regression analysis showed parabolic growth patterns of testis and ovary ($p<0.001$) in quail raised on 24LL, 16L:8D NL and d70TLD after transfer to long days. There are no significant differences in testicular or ovarian weights in birds of the same age and sex raised on 16L:8D NL compared with those raised on 24LL.

Significant increases in testicular weights were seen between 21d (0.03 ± 0.01g, n=7) and 35d (0.55 ± 0.11g, n=8) ($p<0.001$) and between 35d and 42d (1.78 ± 0.19g, n=5) ($p<0.001$) in quail raised on 16L:8D NL (Figure 3.2 a)). Quail raised on 24LL showed significant increases in testicular weight between 21d (0.03 ± 0.01g, n=6) and 27d (0.13 ± 0.03g, n=6) ($p<0.02$) and between 27d and 42d (1.97 ± 0.38g, n=6) ($p<0.001$), (Figure 3.2 b)).

Ovarian weight of quail raised on either 16L:8D NL or 24LL remained low until 35-42d, with a small but significant

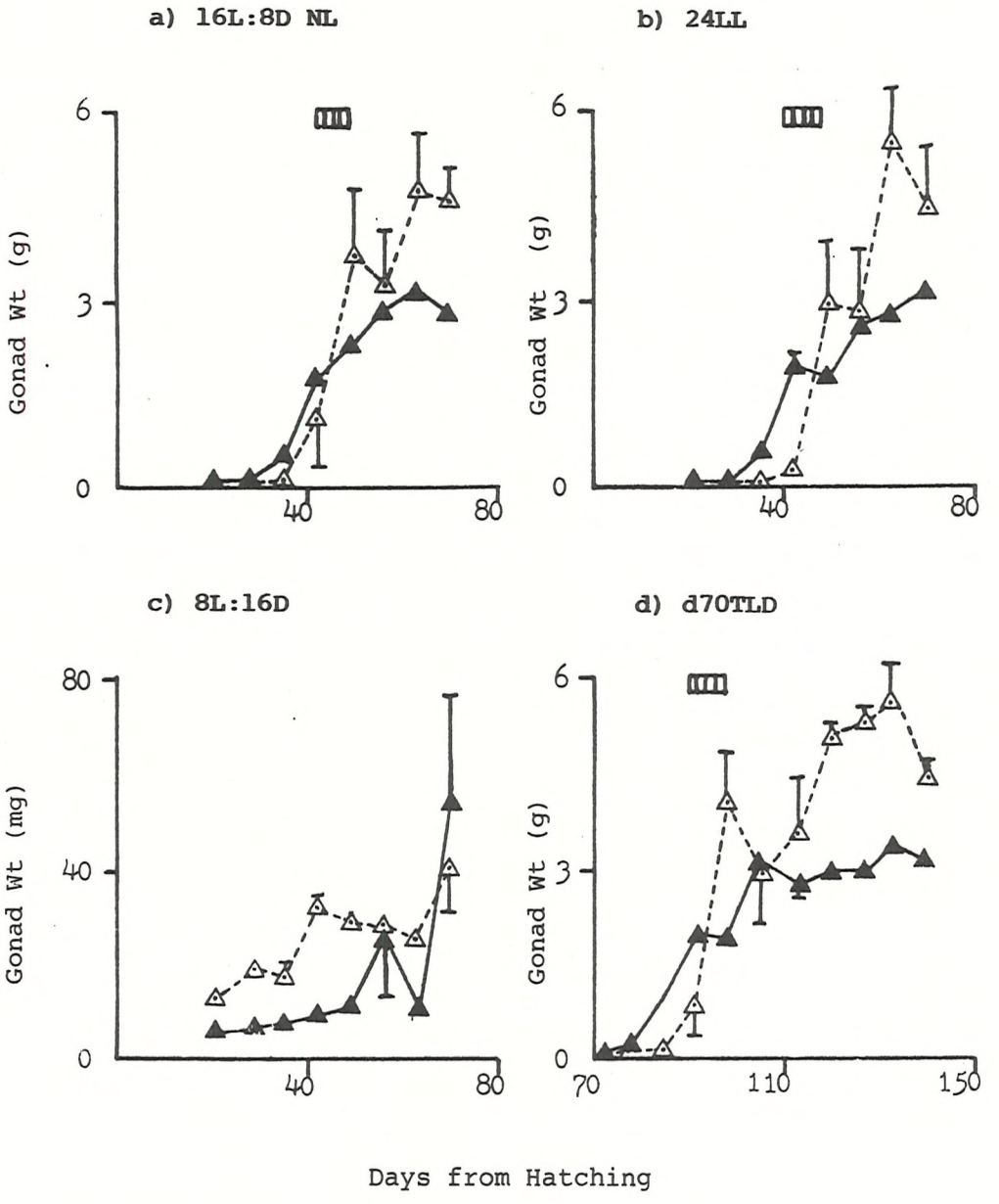


Figure 3.2 - Changes in Gonadal Weight for Male (▲——▲) and Female (△---△) Quail with Age

Quail were raised on a) 16L:8D NL, b) 24LL, c) 8L:16D and d) d70TLD. (The first 70d of the latter group was the same as that of group c)). The period when onset of lay occurred is shown by the hatched area. (N.B. Results for group c) are expressed in mg and these birds did not come into lay during the period studied). Results are shown as mean ± S.E.M with n=5-10.

increase in weight between 21d (0.02±0.01g, n=5) and 35d (0.07±0.01g, n=6) ($p<0.01$) on 16L:8D NL and between 21d (0.03±0.01g, n=6) and 42d (1.97±0.38g, n=6) ($p<0.01$) on 24LL (Figure 3.2 a) and b)). A sudden rise in ovarian weight occurred around the onset of lay, corresponding to the development of large yolky follicles within the ovary. Significant increases in ovarian weight were seen during the onset of lay, between 35 and 49d (3.77±1.03g, n=6) ($p<0.01$) on 16L:8D NL and between 42 and 63d (2.77±0.14g, n=19) ($p<0.01$) on 24LL.

Figure 3.2 c) shows the gonadal development of quail raised on 8L:16D expressed in milligrams (mg). Ovarian weights showed a small significant increase from 21d (12.5±1.3mg, n=5) to 42d (32.0±3.4mg, n=5) ($p<0.001$). Ovarian weights were found to fluctuate after 42d and no further significant changes in weight with age was seen. Ovarian weights in quail raised under 8L:16D were significantly lower at 35d (16.9±3.5mg, n=5) than those seen at 35d in quail raised under either 16L:8D NL (70±10mg, n=6) ($p<0.01$) or 24LL (590±140mg, n=22) ($p<0.01$).

Testicular weights in quail raised on 8L:16D showed a small significant increase between 21d and 42d (from 5.2±1.0mg, n=6 to 8.6±0.7mg, n=5) ($p<0.05$). Testicular weight then fluctuated but showed no further significant increase with age although by 70d testicular weight had increased to 55.1±21.2mg, (n=5). Testicular weights in quail raised under 8L:16D were always significantly lower than those in quail of the same age raised under 16L:8D NL or under 24LL.

Quail raised on d70TLD showed the same small degree of gonadal development as the quail hatched onto 8L:16D up to 70d at which time they were transferred to 16L:8D NL. Ovarian weights then showed significant increases between the 2nd long day and the 15th long day (from 0.03±0.01g, n=5

to 0.13±0.03g, n=5) ($p<0.02$) and a further increase by the 28th long day (to 4.13±0.77g, n=4) ($p<0.001$). Ovarian weights showed no further significant changes with age after the 28th long day, which is after the onset of lay in these birds. Ovarian weights recorded on the 28th long day (98d) were significiantly higher ($p<0.001$) than those seen at 28d in quail raised on either 16L:8D NL or 24LL, but were similar to those seen in quail of 49d. The weights of ovaries recorded in these groups were not significiantly different once egg-laying had been established, but the length of exposure to long photoperiods necessary to cause egg-laying was shorter for the d70TLD quail (22-28d) than for either the 16L:8D NL quail or for the 24LL quail (42-49d).

Testicular weights in quail raised on d70TLD showed significant increases between the 2nd and 8th long day (from 0.03±0.01g, n=5 to 0.25±0.02g, n=8) ($p<0.001$), the 8th and 15th long day (to 0.91±0.21g, n=5) ($p<0.01$), the 15th and 22nd long day (to 1.91±0.21g, n=5) ($p<0.01$) and the 22nd and 35th long day (to 3.13±0.24g, n=4) ($p<0.01$). Testicular weights did not change significantly after the 35th long day through the rest of the study. Testicular weights on the 22nd long day (92d) were significantly higher ($p<0.001$) than those seen at 21d in quail raised on either 16L:8D NL or 24LL, but were similar to those seen in quail of 42d raised on these lighting systems.

3.3.3 Morphological Development of the Gonads : A Study using Light Microscopy

Sections of testes from quail raised on either 24LL or 16L:8D NL showed a similar pattern of development and therefore only the sections of testes from quail with increasing age on 16L:8D NL have been shown (Figure 3.3). With the fixative and staining techniques used, a detailed

Figure 3.3 - Testicular Development during Maturation in Quail Raised on 16L:8D NL : A Study Using Light Microscopy

A. Cross section of the testis from a 21d quail. The seminiferous tubules (st) are small, with no conspicuous lumen present. The interstitial spaces (i) are filled with Leydig cells and blood vessels.

B. Cross section of the testis from a 27d quail. The seminiferous tubules have become larger and the lumen is developing. The interstitial spaces are filled with Leydig cells and blood vessels.

C Cross section of the testis from a 35d quail. The seminiferous tubule wall has become thicker, presumably as a result of spermatogensis occurring, and a well defined lumen (lu) can be seen. The interstitial space is filled with Leydig cells (lc) and blood vessels.

D. Cross section of the testis from a 42d quail. Spermatogenesis is complete as the presence of mature sperm (sp) in the tubule lumen indicates. The interstitial space appears to contain less Leydig cells and blood vessels as the tubules increase in size. The larger tubules cause the interstitial cells to become more dispersed throughout the testis.

E. Cross section of the testis from a 49d quail. The testicular development is as for the 42d quail, (Section D)

The sections were prepared from testes fixed in Bouin's solution, and stained with haematoxylin and eosin as described in Chapter 2.7. All photographs are x50 magnification.

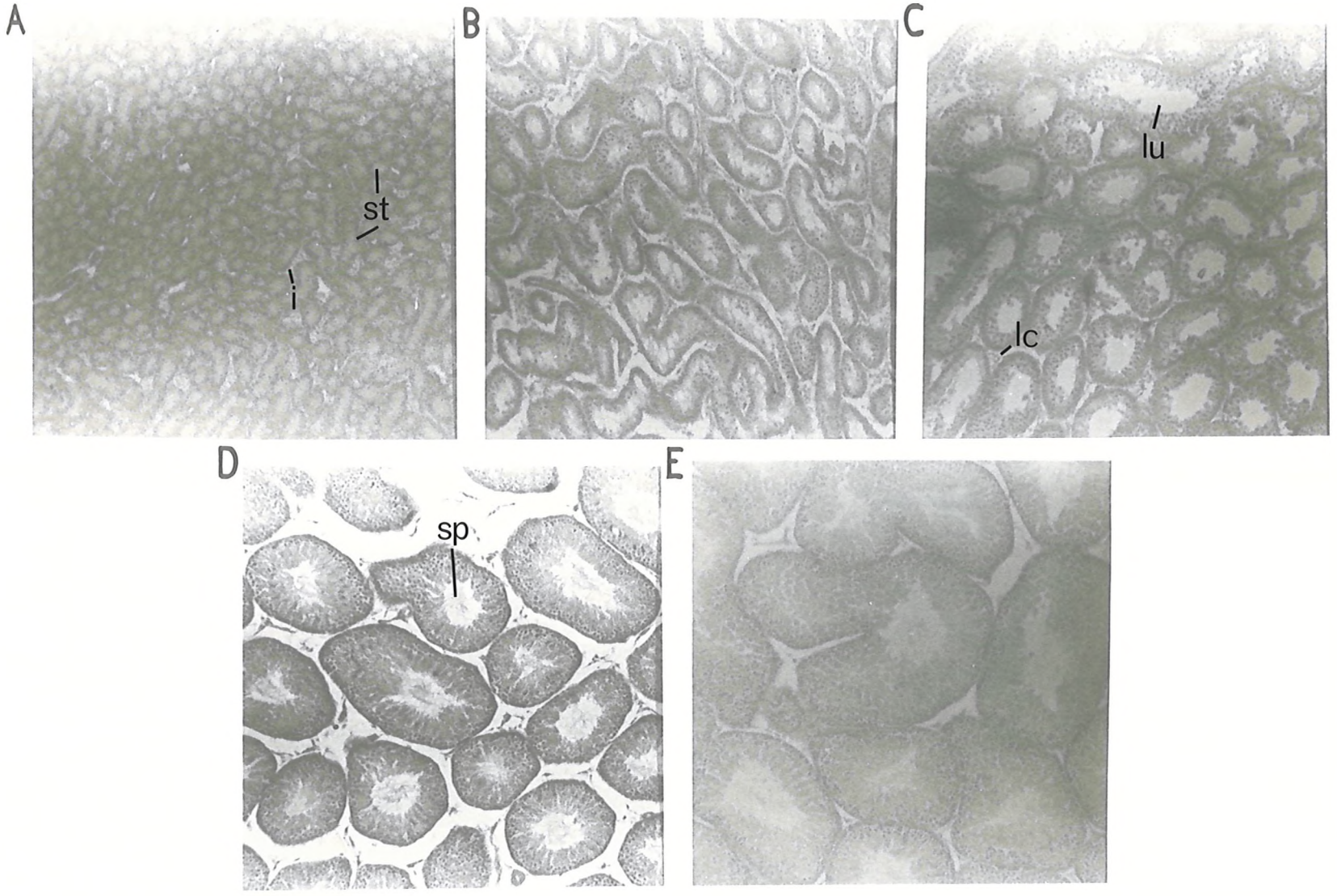

A
st
i
B
C
lu
lc
D
sp
E

Figure 3.4 - Testicular Development during Maturation in Quail Raised on d70TLD after Increasing Exposure to Long Photoperiods : A Study using Light Microscopy

A. Cross section of the testis from a quail after 70 days on 8L:16D. There was only a slight increase in seminiferous tubule (st) size from hatching to 70 days which corresponded to a small increase in the testicular weight. i = the interstitial space.

B. Cross section of the testis from a quail after 8 days photostimulation by 16L:8D. Seminiferous tubules have increased slightly in diameter. The interstitial space is filled with Leydig cells (lc) and blood vessels.

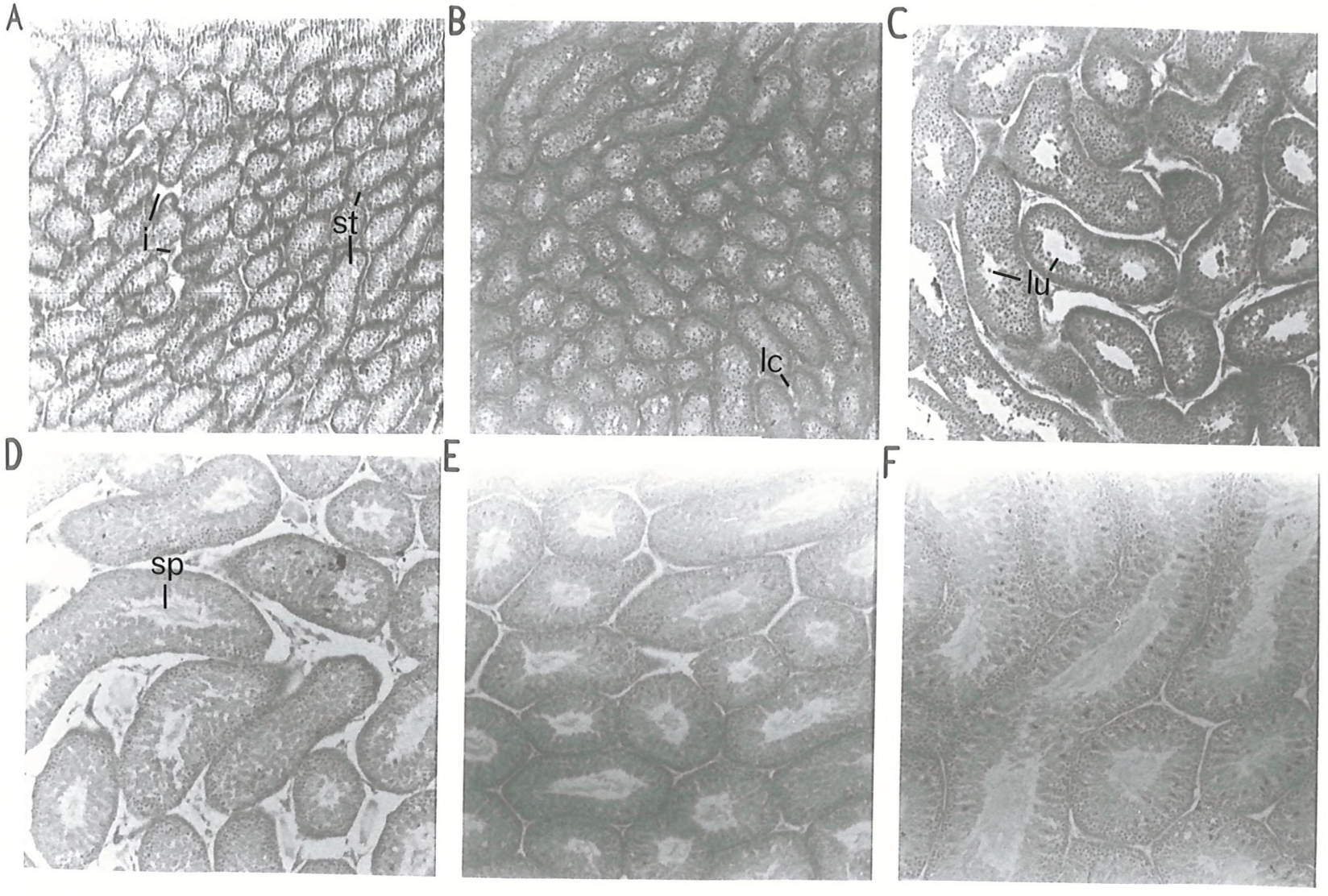
C. Cross section of the testis from a quail after 15 days photostimulation by 16L:8D. The tubular lumen (lu) is now apparent while the interstitial space contains the Leydig cells.

D. Cross section of the testis from a quail after 22 days photostimulated by 16L:8D. Mature sperm (sp) are present in the tubule lumen. The interstitial tissue is becoming less conspicuous as the tubule size increases.

E. Cross section of the testis from a quail after 29 days photostimulation by 16L:8D. No further development has occurred compared with that for Section D.

F. Cross section of the testis from a quail after 56 days photostimulation by 16L:8D. Testicular development is as for Sections D and E.

The sections were prepared from testes fixed in Bouin's solution, and stained with haematoxylin and eosin as described in Chapter 2.7. All photographs are x50 magnification.


A
i
st
B
lc
C
lu
D
sp
E
F

examination of the morphology of spermatogenesis was not possible. By 35d (Figure 3.3 C), the first sperm were seen within the lumen of the tubules in 2 out of 5 quail, while by 42d (Figure 3.3 D), sperm were present, in the tubule lumen, in all quail.

Quail raised on 8L:16D showed no testicular development and no spermatogenesis. A typical testicular section after 70d exposure to 8L:16D is shown in Figure 3.4 A. Quail raised on d70TLD had similarly underdeveloped testes at 70d as shown in Figure 3.4 A. The presence of sperm, by visual assessment, within the lumen of the seminiferous tubules of quail raised on d70TLD was not detected until the 22nd long day, when they were seen in all quail (Figure 3.4 D). By the 15th long day, although no sperm were present, the lumen of the seminiferous tubules had increased in size (Figure 3.4 C).

In all quail raised on long photoperiods testicular development occurred. Sperm were seen within the tubule lumen at 35-42d in testes from quail raised on 16L:8D NL or 24LL and around the 22nd long day in testes from quail in the d70TLD group. This is in contrast to mammals where the sperm cycle lasts about 70d. In all these quail the presence of sperm was preceded by an increase in the diameter of the tubule as can be seen in Figure 3.3 C and Figure 3.4 C. The interstitial tissue became less conspicuous with increasing age, although Leydig cell number increased. Leydig cell number, although not accurately determined, was found to increase, since in the isolation of Leydig cells for the experiment in Chapter 6, more cells could be isolated from the testes of mature birds than immature ones.

Development of the ovary is marked by the sudden transition of the ovary from containing small white foll-

icles of less than 2mm in diameter to containing the larger yellow yolk-filled follicles (up to 18mm in diameter). These yolk-filled follicles show a hierarchical arrangement, as discussed in the initial introduction (Chapter 1.1). These yellow yolk-filled follicles did not occur in quail raised on 8L:16D, but first appeared in quail raised on 16L:8D NL or 24LL around 42-45d and around the 22nd to 29th long day in quail raised on d70TLD. The histologically stained sections of quail ovaries and follicles are shown later in Chapter 5.

3.3.4 Plasma Progesterone, Oestradiol and Testosterone Levels in Female Quail

i) Plasma Progesterone

Plasma progesterone levels in female quail raised under the four different lighting systems are shown in Figure 3.5a.i and 3.5b.i and Figure 3.6a.i and 3.6b.i.

Progesterone levels remained low in the quail raised on **8L:16D** (Figure 3.6a.i), while levels rose across the period of onset of lay on all the other photoperiods. In quail raised on **24LL** a significant increase in progesterone levels was seen, prior to the onset of lay, between 35d and 42d (from 2.32±0.38 pmole/ml,n=6 to 9.46±2.39 pmole/ml,n=6) ($p<0.02$). Although no significant increases were seen, plasma progesterone also rose in quail raised on **16L:8D NL** across the time of onset of lay, (35d - 5.66±0.75 pmole/ml,-n=7, to 56d - 8.14±1.57 pmole/ml,n=6), (Figure 3.5a.i and 3.5b.i).

Progesterone levels in quail raised on d70TLD showed significant increases between the 2nd and 22nd long day (2.87±0.64 pmole/ml,n=5 rising to 4.89±0.47 pmole/ml,n=5) and between the 22nd long day and the 28th long day (to

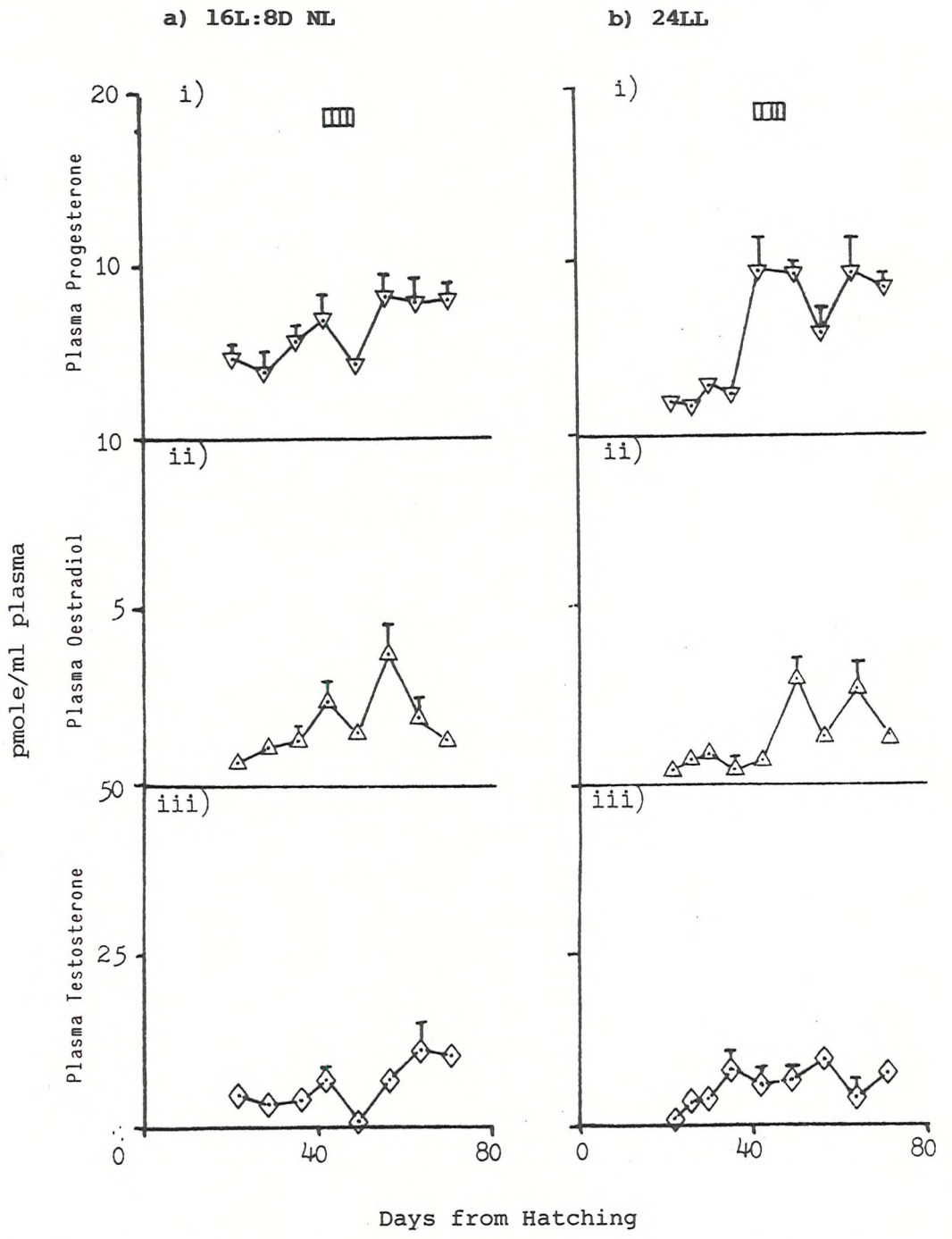


Figure 3.5 - Changes with Age in Mean Plasma Steroid Levels in FEMALE Quail.

(i) Progesterone
(ii) Oestradiol-17β
(iii) Testosterone

Quail were raised on a) **16L:8D NL** and b) **24LL**. Hatched area indicates onset of lay. The steroids were measured using specific RIAs (see Chapter 2.12 for details). Results show mean ± S.E.M, n=5-8 at each age.

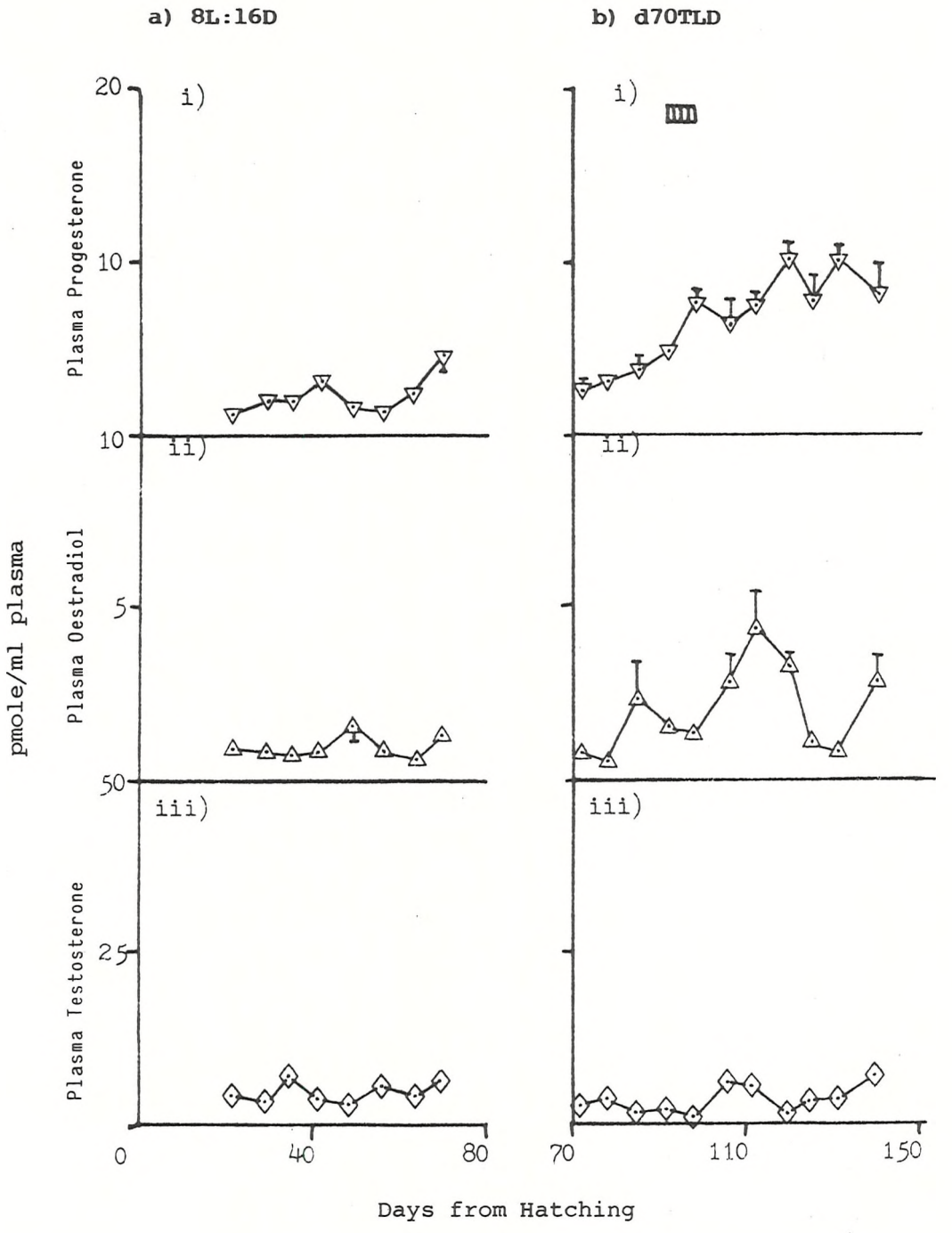


Figure 3.6 - Changes with Age in Mean Plasma Steroid Levels in FEMALE Quail.

(i) Progesterone
(ii) Oestradiol-17β
(iii) Testosterone

Quail were raised on a) **8L:16D** and b) **d70TLD**. Hatched area indicates onset of lay. The steroids were measured using specific RIAs (see Chapter 2.12 for details). Results show mean ± S.E.M, n=5-8 at each age.

7.90±0.77 pmole/ml,n=6), (Figure 3.6b.ii).

There was no significant difference in the mean progesterone levels, once laying had been established, in quail raised under either 16L:8D NL, 24LL or d70TLD, with progesterone levels around 8.5 pmole/ml, while levels seen on 8L:16D rarely exceeded 3.5 pmole/ml throughout the period studied. Progesterone levels were significantly lower in quail raised on 8L:16D at 49d compared to levels seen around the onset of lay in quail on the other lighting regimes at 50d on 24LL ($p<0.001$), 49d on 16L:8D NL ($p<0.001$) and on the 28th long day on d70TLD ($p<0.001$).

ii) Plasma Oestradiol

Oestradiol levels in female quail raised on **8L:16D** showed no significant changes with age and levels rarely rose above 1 pmole/ml, (Figure 3.6a.ii).

Oestradiol levels in female quail raised on **16L:8D NL** showed a significant increase between 21d and 42d (from 0.56±0.04 pmole/ml,n=5 to 2.48±0.60 pmole/ml,n=6) ($p< 0.02$), (Figure 3.5a.ii). After 42d, i.e. once egg-laying was established, plasma oestradiol levels fluctuated showing maximal levels at 56d (3.60±0.94 pmole/ml,n=6) followed by a non-significant decrease in oestradiol levels (to 1.32±0.14 pmole/ml,n=6) at 70d.

Oestradiol levels in female quail raised on **24LL** showed the first significant increase between 42d and 50d when levels rose from 0.65±0.19 pmole/ml,(n=6) at 42d to 2.93±0.54 pmole/ml, (n=8) at 50d ($p<0.02$) (Figure 3.5b.ii). Plasma oestradiol then fluctuated, as seen in the quail raised on 16L:8D NL, with levels remaining above 1 pmole/ml.

Plasma oestradiol levels in quail raised on **d70TLD** remained low until they were transferred to long photoperiods. A significant rise in plasma oestradiol occurred during maturation rising from the 2nd long day (0.86±0.29

pmole/ml,n=5) to the 42nd long day (4.47 ±1.06 pmole/ml,n=6) ($p<0.02$). However, the rise in plasma oestradiol seen between the 2nd and 28th long day was not a statistically significant increase, but was followed by a significant rise between the 28th and 42nd long days (from 1.37±0.16 pmole/ml,n=8 to 4.47±1.06 pmole/ml,n=6) ($p<0.01$).

Quail raised on 16L:8D NL and d70TLD showed a slight rise before the onset of lay, while quail raised on 24LL only showed increasing oestradiol levels around the onset of lay. Mean plasma oestradiol levels were similar once laying had been established in quail raised on 16L:8D NL, 24LL and d70TLD. Levels were variable but remained above 1 pmole/ml during the egg laying period. Plasma oestradiol levels in laying birds were significantly higher than the plasma oestradiol levels found in the unstimulated birds of a similar age, but raised on 8L:16D.

iii) Plasma Testosterone

Plasma testosterone levels in female quail showed no relationship to the light regime on which the birds were raised (Graphs iii in Figure 3.5 and Figure 3.6).

Plasma testosterone levels in quail raised on **16L:8D NL** and **24LL** showed no significant changes with age with levels varying around 7.5 pmole/ml. Quail raised on **8L:16D** or **d70TLD** also showed only small changes with age with a small decrease at the time of transferral to long days (from 6.27 ±0.51 pmole/ml,n=5 on the day of transferral to 2.63 ±1.28 pmole/ml,n=5 by the 2nd long day) ($p< 0.05$). Plasma testosterone levels rarely exceeded 10 pmole/ml in females raised under any of the lighting systems.

3.3.5 Plasma Testosterone, Oestradiol and Progesterone in Male Quail

i) Plasma Testosterone

Plasma testosterone in male quail raised on **16L:8D NL** showed significant increases between 21d and 35d (from 3.12±0.85 pmole/ml,n=7 to 8.11±1.68 pmole/ml,n=7) ($p<0.05$) and between 35d and 49d (to 26.76±6.65 pmole/ml,n=10) ($p<0.05$) (Figure 3.7a.i). Plasma testosterone then fell to 10.69±2.40 pmole/ml,(n=7) by 56d and remained around 12 pmole/ml for the rest of the period studied.

Plasma testosterone in male quail raised on **24LL** also increased with age, although plasma levels never exceeded 15 pmole/ml. Across the period studied, 21d to 70d, plasma testosterone showed a significant increase (from 2.82±0.42 pmole/ml,n=7 to 8.95±1.24 pmole/ml,n=6) ($p<0.001$) with the greater part of this increase occuring between 21d and 49d (7.23±1.07 pmole/ml,n=21) ($p<0.05$), (Figure 3.7b.i). Plasma testosterone, in both these groups of quail, increased during the time of testicular maturation.

Male quail raised on the non-stimulatory photoperiod, **8L:16D** showed no rise in plasma testosterone during the period studied (Figure 3.8a.i), correlating with the lack of testicular development in this group. Testosterone levels became significantly lower in quail raised on 8L:16D at 49d (3.84±1.25 pmole/ml,n=5) compared to those found at 49d in quail raised on 16L:8D NL (26.76±6.65 pmole/ml,n=10) ($p<0.05$). Plasma testosterone levels at 42 and 49d in quail raised on 24LL were higher than those seen at the same age in quail raised on 8L:16D but at no age were the levels significantly different on the two lighting systems.

Plasma testosterone levels in male quail raised on **d70TLD** showed no significant increases throughout the time on short photoperiods. When the birds had been transferred

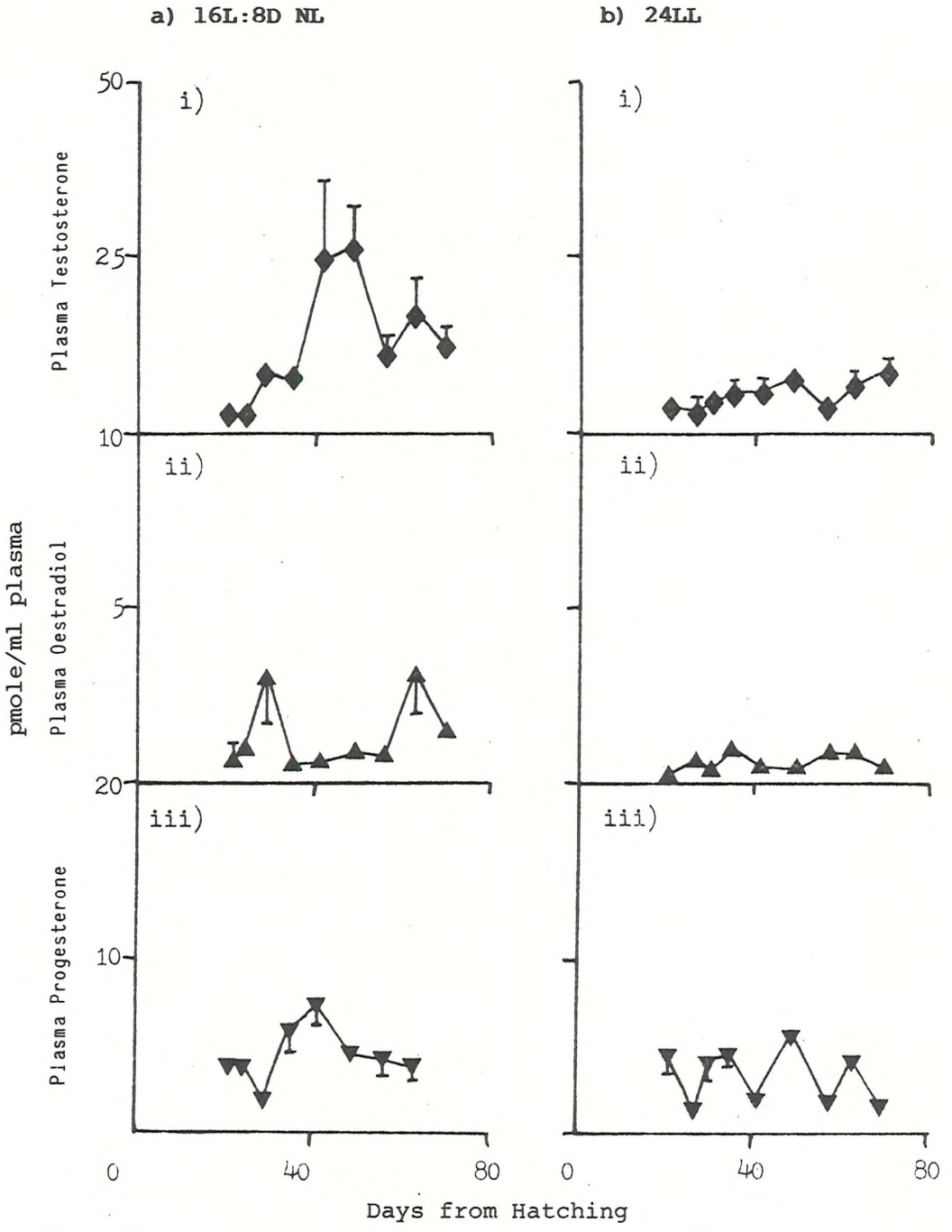


Figure 3.7 - Changes with Age in Mean Plasma Steroid Levels in MALE Quail.

(i) Testosterone
(ii) Oestradiol-17β
(iii) Progesterone

Quail were raised on a) **16L:8D NL** and b) **24LL**. The steroids were measured using specific RIAs (see Chapter 2.12 for details). Results show mean ± S.E.M, n=5-20 at each age.

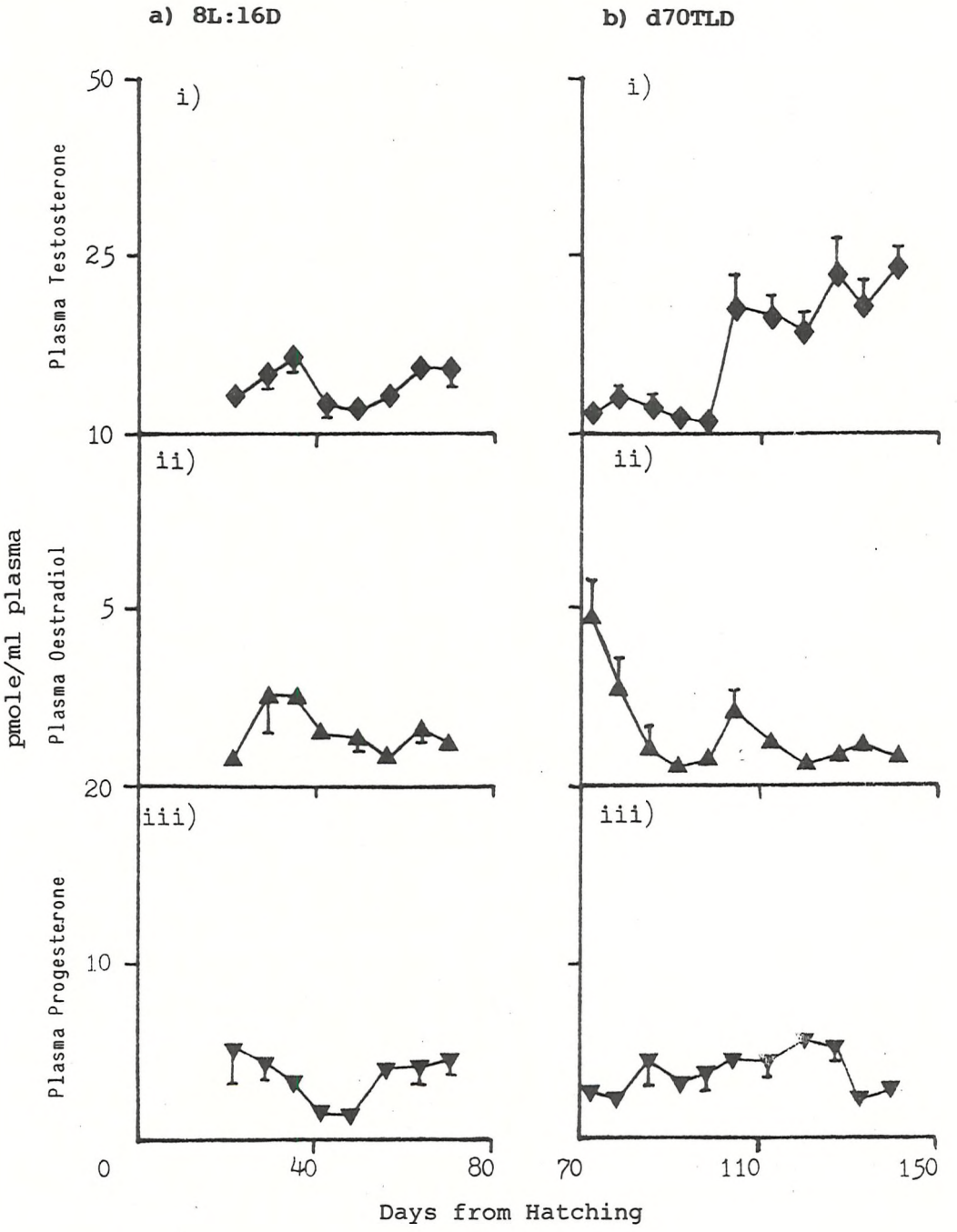


Figure 3.8 - Changes with Age in Mean Plasma Steroid Levels in MALE Quail.

(i) Testosterone
(ii) Oestradiol-17β
(iii) Progesterone

Quail were raised on a) **8L:16D** and b) **d70TLD**. No testicular development occurred in quail raised on 8L:16D. The steroids were measured using specific RIAs (see Chapter 2.12 for details). Results show mean ± S.E.M, n=5-20 at each age.

to long photoperiods, testosterone levels fell from 70d to 72d (the 2nd long day) from 9.97±3.15 pmole/ml,(n=5) to 2.59±0.40 pmole/ml,(n=5) ($p<0.05$) (Figure 3.8a.i and 3.8b.i). Plasma testosterone then remained low until between the 28th long day and the 34th long day when a rapid increase was seen (from 0.97±0.61 pmole/ml,n=6 to 17.60±5.60 pmole/ml,-n=6) ($p<0.02$). Testosterone levels then remained high for the rest of the period studied, around 19 pmole/ml.

Plasma testosterone levels in quail raised on d70TLD and 16L:8D NL were similar in birds whose testes weight exceeded 2g, while levels found on 24LL were lower than those seen in quail raised on d70TLD or 16L:8D NL. Testosterone levels in quail on 8L:16D were always lower than those seen in mature adult quail on 16L:8D NL or d70TLD.

ii) Plasma Oestradiol

Plasma oestradiol levels showed no change with age in quail maintained on **16L:8D NL** (Figure 3.7a.ii). A small increase in oestradiol levels was seen in quail raised on **24LL** between 21d and 35d (from 0.28±0.07 pmole/ml,n=7 to 1.06±0.11 pmole/ml,n=22) ($p<0.001$) (Figure 3.7b.ii). Oestradiol levels in quail raised on 24LL were not significantly different, at any age, from those seen in quail raised on 16L:8D NL.

Oestradiol levels in male quail raised on **8L:16D** were similar to those in birds raised on 16L:8D NL and on 24LL at all ages. A significant increase in plasma oestradiol was seen between 21d and 35d (from 0.79±0.12 pmole/ml,n=5 to 2.51±0.66 pmole/ml,n=5) ($p<0.05$) which was followed by a drop in oestradiol levels (Figure 3.8a.ii).

Plasma oestradiol levels in quail raised on **d70TLD** remained low while the birds were on short photoperiods.

Once the birds had been transferred to long photoperiods, oestradiol levels showed a transient increase from the day on which they were transferred (70d) to the 2nd long day (72d) (from 1.22±0.34 pmole/ml,n=5 to 4.72±1.12 pmole/ml,-n=5) ($p<0.02$). The oestradiol concentration then fell back to low levels by the 15th long day (to 1.12±0.59 pmole/ml,-n=6) ($p<0.02$) (Figure 3.8b.ii). Plasma oestradiol remained low until the 34th long day when levels rose transiently to 2.02±0.78 pmole/ml, (n=6). Oestradiol levels then remained low for the rest of the period studied, at around 0.9 pmole/ml.

Plasma oestradiol levels in male quail were similar at all ages and did not depend on the lighting regime the quail were raised on, except for quail raised on d70TLD between the day they were transferred and the 15th long day. During this time oestradiol levels showed a transient rise to levels not seen in male quail at any other time or under any of the other lighting systems.

iii) Plasma Progesterone

Plasma progesterone levels in male quail raised on any of the four photoperiods were around 4 pmole/ml at all ages, (Figure 3.7 and Figure 3.8a and 3.8b.iii). There was no difference in the levels recorded between quail of the same age raised under different lighting systems. Plasma progesterone was found to fluctuate throughout the period studied on all lighting systems with small and sometimes significant changes observed.

3.3.6 Comparisons of Plasma Steroid Levels Between Male and Female Quail Raised on the same Photoperiod.

i) 24LL

Plasma **progesterone** levels were significantly higher

($p<0.02$) by 42d in female quail raised on 24LL compared to those seen in male quail at the same age (♀:- 9.46±2.39 pmole/ml,n=6; ♂:- 2.22±0.44 pmole/ml,n=6). Progesterone levels in female quail remained higher than those in males for the rest of the period studied.

Plasma **oestradiol** levels were higher ($p<0.001$) by 49d in female quail raised on 24LL compared to plasma oestradiol levels in male quail of the same age (♀:- 2.93±0.54 pmole/ml,n=8; ♂:- 0.42±0.05 pmole/ml,n=21). Oestradiol levels, like progesterone, remained higher in the laying females compared with those seen in the male quail, for the rest of the period studied.

There was no significant difference between males and females, in plasma **testosterone** levels at any age in quail raised on 24LL.

ii) 16L:8D NL

Plasma **progesterone** levels in female quail although higher in the laying female compared with those seen in the male, were never significantly higher during the laying period.

Plasma **oestradiol** levels in female quail raised on 16L:8D NL were significantly higher ($p<0.05$) at 42d than oestradiol levels in males, observed at the same age (♀:- 2.48±0.60 pmole/ml,n=6; ♂:- 0.64±0.21 pmole/ml,n=5). Oestradiol levels were always higher in the laying female compared to those of the male quail of the same age.

Plasma **testosterone** levels in female quail were significantly lower ($p<0.02$) at 49d than those seen in male quail (♀:- 0.90±0.65 pmole/ml,n=5; ♂:- 26.76±6.65 pmole/ml,n=10). Testosterone levels in the laying females were always

lower than levels seen in adult male quail.

iii) 8L:16D

There was no significant difference in plasma **progesterone** or plasma **testosterone** levels found in female quail compared to those seen in male quail at any age under this photoperiod. **Oestradiol** levels were only significantly different at 35d, when oestradiol levels were higher in male quail compared with the female (♂:- 2.51±0.66 pmole/ml, n=5; ♀:- 0.70±0.10 pmole/ml,n=5) ($p<0.05$).

iv) d70TLD

While on short photoperiods (0 to 70d), the comparison between steroid levels in males and females was similar to that described in the preceding section, (iii). Once transferred plasma **progesterone** levels in female quail became significantly higher ($p<0.05$) by the 22nd long day compared with those seen in the males of the same age (♀:- 4.89±0.47 pmole/ml,n=5; ♂:- 3.28±0.44 pmole/ml,n=5). Progesterone levels remained higher in the laying female than those seen in the male.

Plasma **oestradiol** levels in the male quail were significantly higher by the 2nd long day compared with those seen in females (♂:- 4.72±1.12 pmole/ml,n=5; ♀:- 0.86±0.29 pmole/ml,n=5) ($p<0.02$), but by the 15th long day oestradiol levels in male and female quail were not significantly different. Plasma oestradiol levels in female quail became higher than those seen in male quail by the 28th long day (♀:- 1.37±0.16 pmole/ml,n=8; ♂:- 0.69±0.14 pmole/ml,n=6) ($p<0.01$), remaining higher in the laying female compared with those of the male.

Plasma **testosterone** levels in female quail, although lower than those in male quail by the 34th long day, did not become significantly lower until the 42nd long day (♀:- 5.99±0.62 pmole/ml,n=5; ♂:- 17.04±5.60 pmole/ml,n=6) ($p<0.01$). Plasma testosterone levels in laying females remained lower than those seen in males of comparable ages.

3.3.7 Plasma Steroid Levels in Quail Bled Sequentially Through Maturation

Changes in plasma **progesterone** levels in eight individual female quail through sexual maturation are shown in Figure 3.9. In all eight females, progesterone levels had started to increase prior to the onset of lay. Onset of lay in these quail was between the 30th and 42nd long day. There was considerable individual variation, between quail of the same age, in the plasma progesterone levels recorded. This did not appear to be dependent on the fact that the quail did not all come into lay on the same day.

Changes in plasma **oestradiol** levels, in the same plasma samples in which progesterone had been measured are shown in Figure 3.10. Oestradiol levels in five of the eight quail (Q25, Q29, Q31, Q33 and Q35) did show an increase (exceeding 1.5 pmole/ml) prior to the onset of lay. These increased levels were seen at least a week before the onset of lay. Q21, Q23, and Q27 did not show such a marked increase in oestradiol levels prior to the onset of lay, although oestradiol levels had increased by the time laying had begun in these birds, reaching oestradiol levels of at least 1 pmole/ml. All eight females did show increases in oestradiol prior to their onset of lay.

Changes in the plasma **testosterone** levels through

Figure 3.9

Plasma Progesterone Levels in Eight Individual Female Quail throughout Sexual Maturation.

Quail were raised on d70TLD and once 70d, each female was caged out with a male. Blood was collected from the wing vein, during their 2nd hour of light, two or three times a week after transfer to the long photoperiods (16L:8D NL). Plasma was stored at -25°C until assayed for progesterone by RIA. Egg-laying records were kept for each female by checking for the presence of an egg in the cage at 09.00h every day. The arrow indicates the day on which each female laid her first egg.

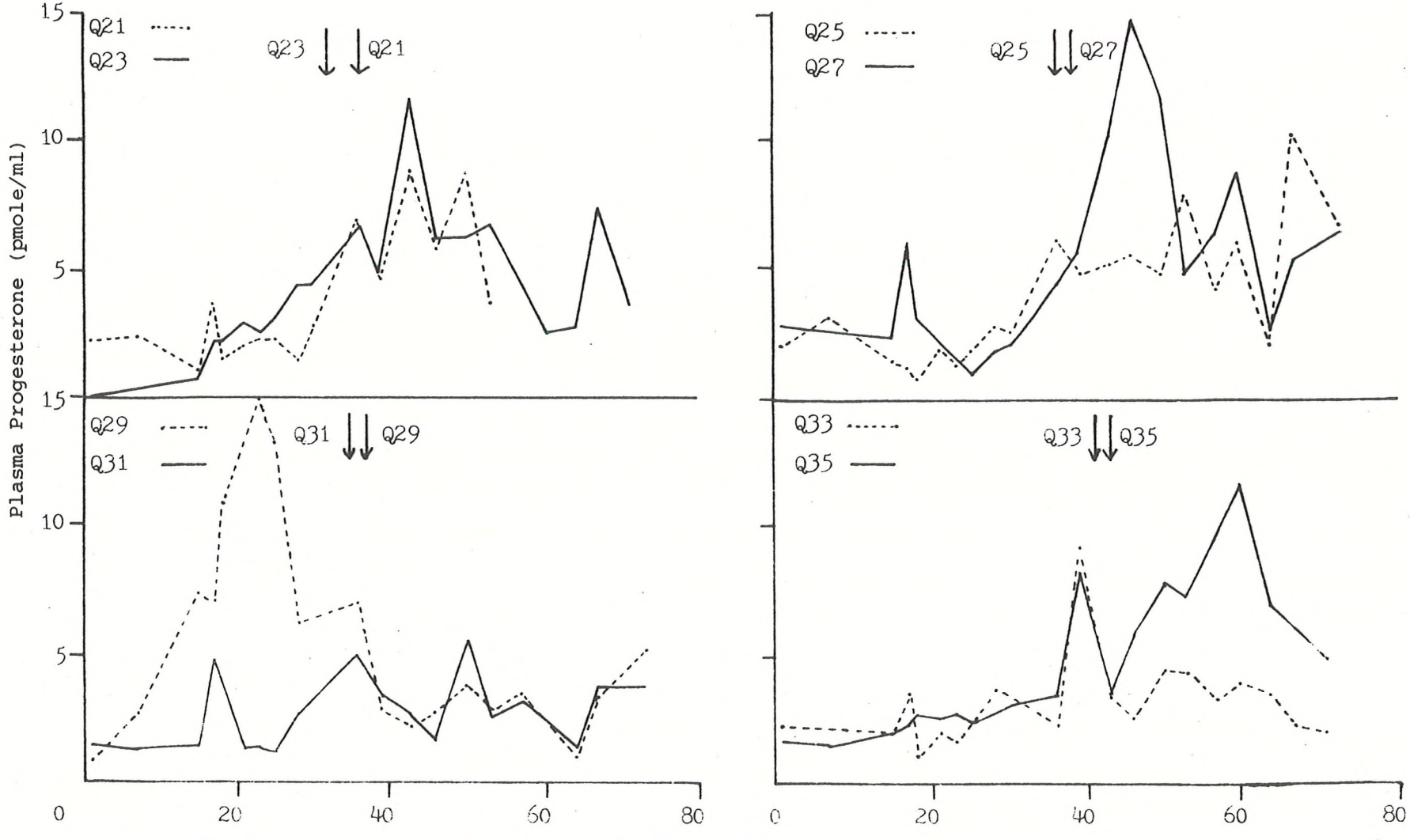


Figure 3.9

Days since transfer to 16L:8D NL from 8L:16D

Figure 3.10

Plasma Oestradiol Levels in Eight Individual Female Quail throughout Sexual Maturation.

These eight quail were the same quail as those used for the determination of plasma progesterone levels. Quail were therefore raised and bled as described in the legend for Figure 3.9. Plasma was stored at -25°C until assayed for oestradiol by RIA. Egg-laying records were recorded as before. The arrow indicates the day on which each female laid her first egg.

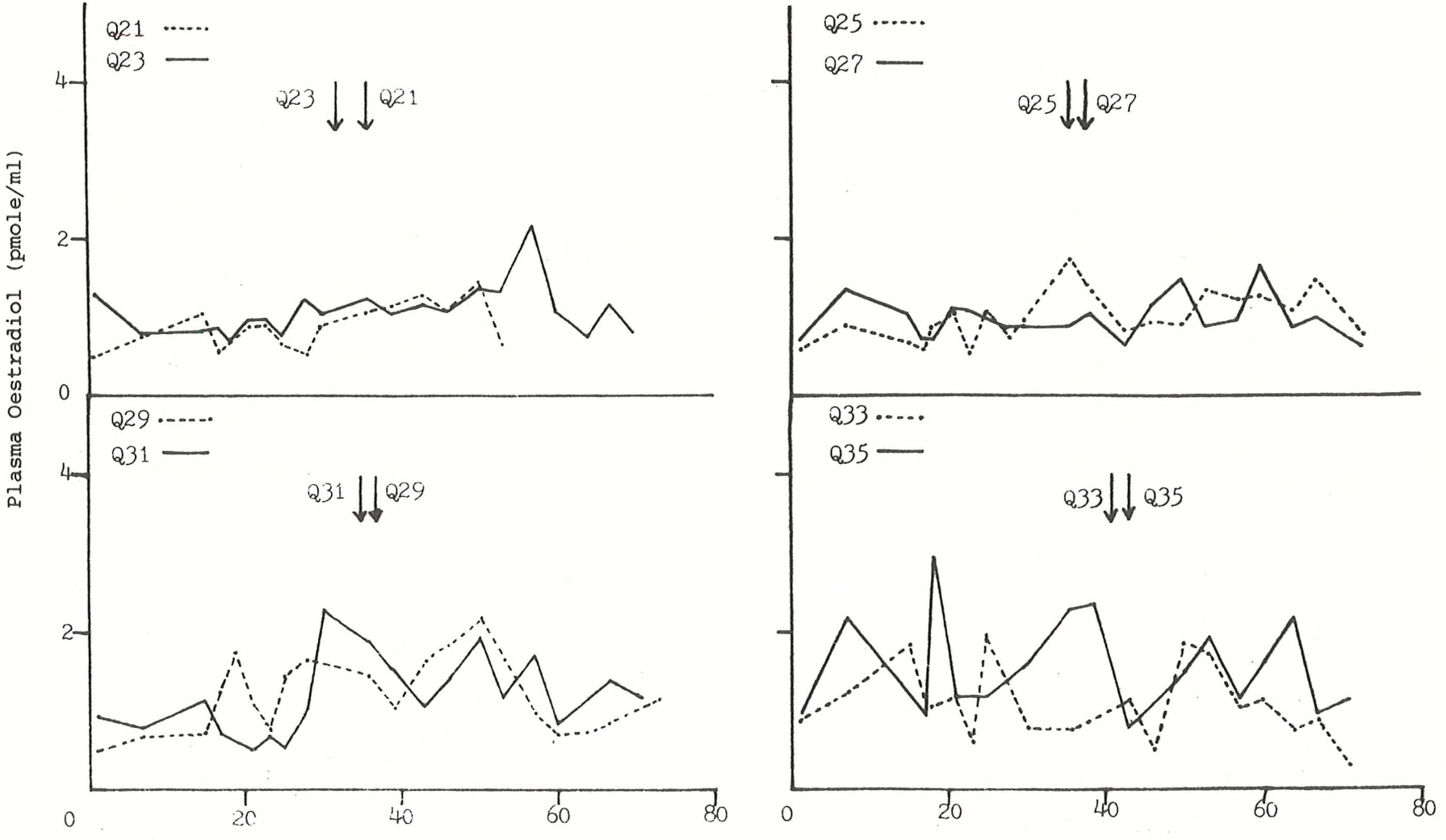

Plasma Oestradiol (pmole/ml)
Q21
Q23
Q23 Q21
Q25
Q27
Q25 Q27
Q29
Q31
Q31 Q29
Q33
Q35
Q33 Q35
0
2
4
0
20
40
60
80
Days since transfer to 16L:8D NL from 8L:16D

Figure 3.10

sexual maturation in six individual male quail are shown in Figure 3.11. Plasma testosterone levels in all six quail increased during sexual maturation, with testosterone levels starting to increase by the 15th long day in all birds. Testosterone levels seemed to show a peak between the 35th and 50th long day in all six quail and then decreased to lower levels, around the 60th long day. Testosterone levels then rose again in all 6 quail. Individual variations were seen between testosterone levels found in quail of the same age.

Figure 3.12 shows the changes in plasma **oestradiol** levels in five individual male quail during sexual maturation. All quail showed levels greater than 1 pmole/ml at some time within the first 10 long days. By the 30th long day, oestradiol levels had fallen in 4 out of the 5 quail below 1 pmole/ml with levels remaining low for the rest of the period studied. Q28 continued to have oestradiol levels greater than 1 pmole/ml up to the 50th long day, although levels had started to fall by the 36th long day.

3.4 Discussion

The results presented here have shown the profiles of plasma levels of oestradiol, progesterone amd testosterone in Japanese Quail, during sexual maturation and development under several different lighting systems.

Quail raised on short photoperiods did not become sexually mature, shown by the small growth of both ovaries and testes, compared with that seen in quail raised on long photoperiods. The histological sections of the testes from quail raised on short photoperiods also failed to show any signs of sexual development. Female body weights were lower in sexually immature compared to sexually mature

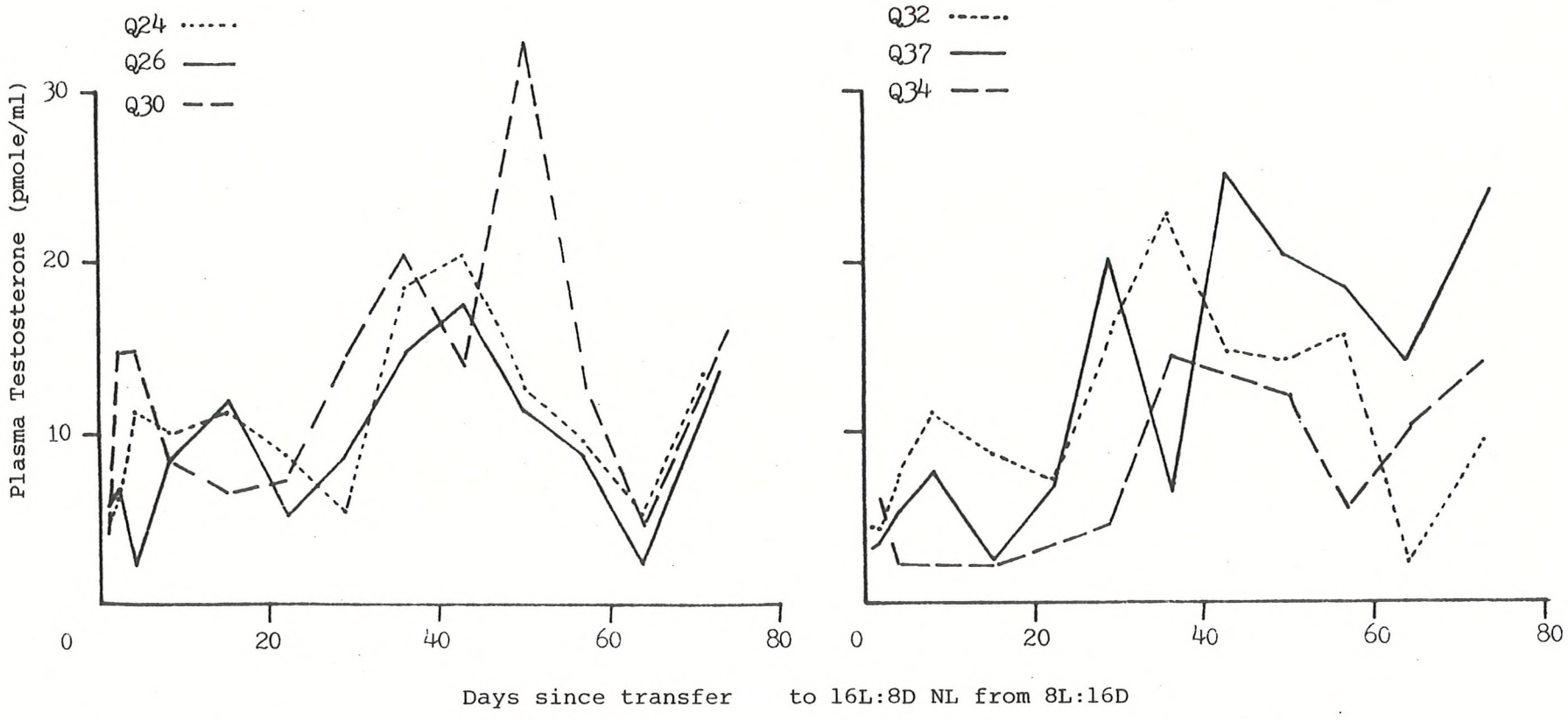


Figure 3.11 - Plasma Testosterone Levels in 6 Individual Male Quail throughout Sexual Maturation.

Quail were raised on d70TLD and once 70d, caged out with a female. Blood was collected from the wing vein, during their 3rd hour of light, about once a week, after transfer to the long photoperiods (16L:8D NL). Plasma was stored at -25°C until assayed for testosterone by RIA.

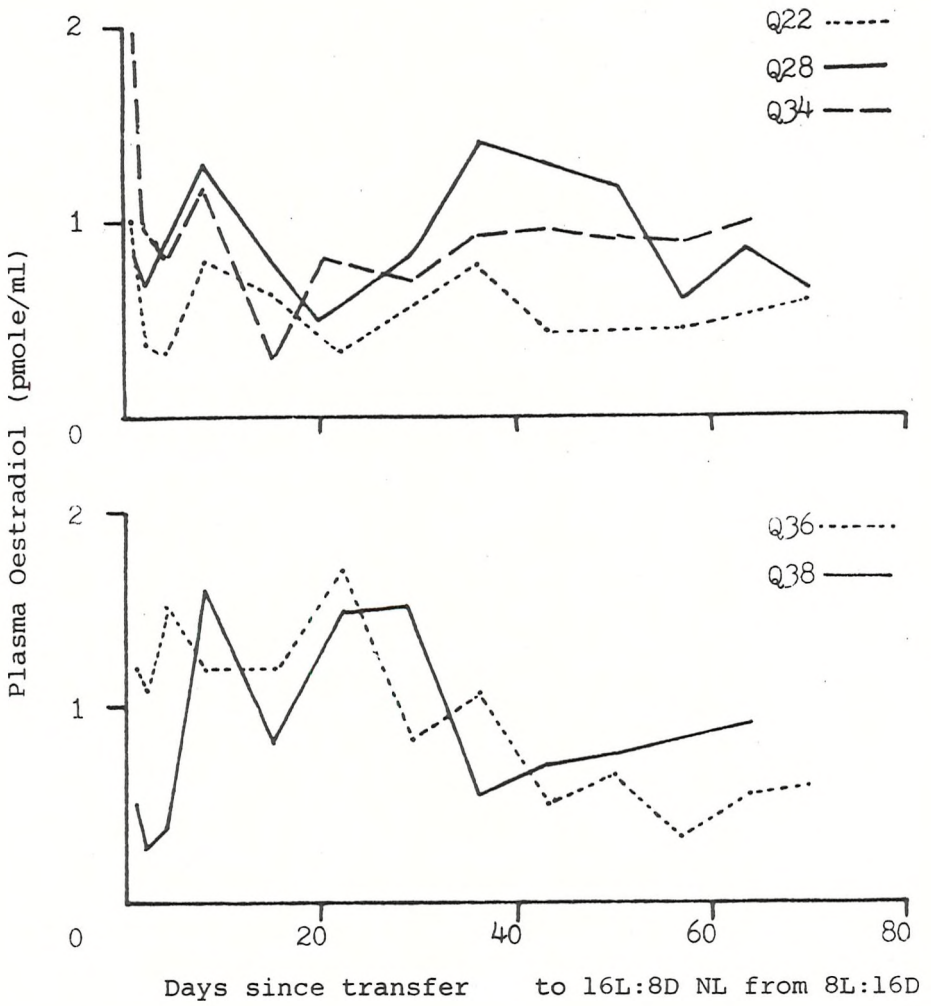


Figure 3.12 - Plasma Oestradiol Levels in Five Individual Male Quail throughout Sexual Maturation

Quail were raised on d70TLD and once 70d, caged out with a female. Blood was collected from the wing vein, during their 3rd hour of light, about once a week, after transfer to long photoperiods (16L:8D NL). Plasma was stored at -25°C until assayed for oestradiol by RIA.

quail, but were comparable to those of sexually immature and those of sexually mature male quail. The extra weight contributed by the ovary, oviduct and developing eggs of a mature female accounted for the difference in body weight between sexually mature and sexually immature female quail. This lack of sexual development in quail raised on short photoperiods, has been reported previously by Follett and Maung (1978). Large standard errors are found in ovarian weights around the onset of lay, since not all quail come into lay at exactly the same time. Some ovaries still consist of small white follicles, instead of the larger yolk-filled ones. Large errors found after the onset of lay were due to those birds which had already released a follicle into the oviduct, prior to being killed, having lower ovarian weights than those which had not yet released the F1 follicle.

Plasma oestradiol and progesterone levels in female quail were dependent on the sexual maturity of the bird. Immature female quail had lower plasma progesterone and oestradiol levels than their sexually mature counterparts. Plasma progesterone and oestradiol levels in laying quail were also higher than those found in adult males. This agrees with measurements of progesterone levels in the domestic fowl, where levels were higher in laying birds (3.13±0.37 ng/ml) than in either non-laying hens (0.35±0.02 ng/ml) or in cockerels (0.44±0.06 ng/ml) (Furr, 1973). Similarly, plasma oestradiol levels in laying hens (48-54 pg/ml) have been found to be higher than those in either immature females or in adult males (6-8 pg/ml) (Schrocknadel, Bator and Frick, 1973). Plasma oestradiol and progesterone levels in male quail showed no dependence on the state of sexual maturity of the bird and, as already stated, were always lower than in laying females.

In female chickens, plasma progesterone was found to increase up to the 15th week of age when levels showed a

decrease before and after maturation (21 weeks) (Muray, Pethes and Pëczely, 1980). In female quail, plasma progesterone increased with age and was always higher in photostimulated quail than in non-photostimulated birds, especially prior to the onset of lay. There was not such a marked increase in progesterone levels with sexual maturation in photostimulated immature quail, compared with those seen in the chicken, although levels did tend to decrease after the onset of lay. Thus plasma progesterone levels in quail during maturation show similar trends to those seen in chickens. It is likely that progesterone is the hormone involved in positive feedback mechanisms to the hypothalamic-pituitary axis in the quail as proposed for the chicken, and is important in initiating the LH surge.

The elevation of plasma progesterone just prior to the onset of lay is also important for the development of the fully functional oviduct, since progesterone is required for ovalbumen and avidin synthesis by the oviduct.

Plasma oestradiol levels in photostimulated quail were elevated prior to the onset of lay. This was seen more clearly in female quail bled sequentially throughout maturation where, in all but one bird, oestradiol levels increased at least 7 days prior to their first egg being laid. This was followed by a slight decrease in oestradiol levels after maturation. Similar changes in plasma oestradiol levels have been reported in both chickens (Senior, 1974; Peterson and Webster, 1974) and in turkeys (Wineland and Wentworth, 1975).

The elevation of plasma oestradiol levels prior to egg-laying is important, since in birds, oestrogens play a role in calcium and phosphorous metabolism prior to egg-laying in the formation of medullary bone for egg-shell calcification, as well as in the morphological and functional 'preparation' of the oviduct for the egg-laying state.

It is also possible that oestrogens are involved in regulating hypothalamic function in laying quail, since in chickens oestrogens have been shown to facilitate the mechanism of progesterone stimulated preovulatory LH release and in maintaining low basal LH levels by decreasing the pituitary responsiveness to LH-RH (Wilson and Cunningham, 1981).

Plasma testosterone levels in male quail were higher in sexually mature male quail, compared to either immature or laying quail, except when birds were raised under 24LL, when levels apparently remained low despite testicular development. The low levels of testosterone in these birds may have resulted from a lack of diurnal changes in plasma testosterone. These quail were raised on continuous light, which makes the timing of diurnal events either impossible or uncoordinated. It is likely that these quail show a low but continuous secretion of testosterone throughout the day, as opposed to a period of high plasma testosterone and a period of low plasma testosterone during the day, as seen in quail raised on 16L:8D NL or 16L:8D RL (reported in Chapter 4.3). Further studies are required to see if there is a lack of diurnal changes in quail raised on 24LL, in order to understand fully why these low testosterone levels exist. Schrocksnadel, Bator and Frick (1971), using a competitive protein binding method, also found that plasma testosterone levels in mature cocks were higher than those found in immature cocks or laying hens.

Follett and Maung (1978) showed that plasma testosterone levels in male quail started to rise by the 4th long day after transfer from 8L:16D to 20L:4D. Transfer to shorter stimulatory photoperiods resulted in a longer delay in the significant rise in testosterone:- 7th long day - 14L:10D; 9th long day - 13L:11D and 22nd long day - 12L:12D.

In the quail raised on d70TLD mean plasma levels of testosterone showed only a small increase a few days after

transfer, with a large signficant increase occuring after the 30th long day. However, in quail bled individually throughout maturation, plasma testosterone levels were elevated in most quail by the 8th long day.

The rise in plasma testosterone levels is preceded by elevated levels of LH and FSH, since plasma levels of these hormones have been found to increase as early as the 2nd long day (quail transferred from 8L:16D to 20L:4D) (Gledhill and Follett, 1976). Plasma testosterone levels in male quail reflect the state of sexual maturity of the bird and the level of testicular development. Immature birds have the lowest plasma levels. As testicular weight increases under the control of FSH, levels of plasma testosterone also increase, due to increasing LH levels stimulating testosterone release from the Leydig cells.

3.4.1 The Relationship of Plasma Sex Steroids and Vitamin D_3 Metabolism during Sexual Maturation in the Japanese Quail

The ability of injections of oestrogens **in vivo**, in particular oestradiol benzoate, to stimulate the **in vitro** production of 1,25DHCC (the active metabolite of Vitamin D_3) and to inhibit the production of 24,25DHCC has been well documented (Baksi and Kenny, 1977; Pike et al, 1978; Sedrani, 1979). Kenny (1976) also reported that the **in vitro** metabolism of Vitamin D_3 depended on the physiological state of the bird at the time of killing. When the calcium requirement was high, as in the egg-laying state, 1,25DHCC was the major product, while in the non-egg laying state, the main metabolite was 24,25DHCC. Sedrani (1979) showed that the metabolism of Vitamin D_3 depended on the maturity of the quail. In quail raised on constant light, production of 1,25DHCC was found to increase with age in the females and decrease in the males. The change in Vitamin

D_3 metabolism occurred prior to the onset of lay at about 35d, reaching peak levels at the onset of lay (45d) and was paralleled by increased plasma calcium concentrations and the rapid growth of the ovarian follicles.

In the results from quail reported here (raised on 24LL), the increase in growth of the ovarian follicles coincided with the time of increasing plasma oestradiol levels. The timing of this growth phase was different from that of Sedrani's, with the rapid increase in growth of the ovary not occurring until the quail were about 40d. This different time scale must be borne in mind when considering the role of the reproductive steroids in Vitamin D_3 metabolism. As one can see from the individually bled quail, each quail does not come into lay at exactly the same time. Therefore, it is difficult to know in advance exactly when each quail will come, or would have come into lay. This could be even more apparent in quail raised on 24LL, since if the onset of light triggers the ovulation sequence in the quail, these birds must have some other trigger for ovulation, since they have no dark period. This would result in a lack of synchronisation in, (a) the time of onset of lay and (b) time of daily lay, between quail raised on the same lighting system. Indeed, studies on egg-laying in quail raised on continuous light showed that eggs were laid randomly throughout the day and were not restricted to an 8h period towards the end of the light period, as found in quail raised on 14L:10D (Arrington, Abplanalp and Wilson, 1962).

Injections of testosterone propionate have been shown to inhibit the 1α-hydroxylase enzyme in quail. In male quail the activity of the 1α-hydroxylase enzyme is completely suppresed by sexual maturity (Sedrani, 1979). He suggested that this decrease in 1α-hydroxylase enzyme activity may reflect a gradual increase in the level of testosterone secreted by the developing testis. In quail

raised on 16L:8D NL plasma testosterone levels did increase with sexual maturation and levels were higher in the males than in the females. However, in quail raised on 24LL, plasma testosterone levels were similar in both sexes with only a slight increase seen in the males as they matured. This increase may account for the inhibition of the 1α-hydroxylase enzyme activity seen in sexually maturing male quail raised under constant light.

Pike et al (1978) found that injections of oestrogens and androgens together potentiated the stimulation of 1,25DHCC production **in vitro** seen after a single oestrogen injection. Sedrani (1979) failed to confirm this and found that the combined injections led to a reduced response, unless parathyroid extract was injected as well when an even greater stimulation of the 1α-hydroxylase was seen. The presence of testosterone in the plasma of female quail could therefore enhance the oestrogen stimulation of the 1α-hydroxylase enzyme, especially if levels of parathyroid hormone were elevated, just prior to the onset of lay when plasma oestradiol levels are rising. The possible mechanisms of oestrogen action on Vitamin D_3 metabolism is discussed later in Chapter 7.4.

The results presented here show that plasma levels of oestradiol, testosterone and progesterone are dependent on the sexual maturity of the quail. Changes in the levels of these hormones just prior to the onset of lay and during sexual development in the male, could contribute towards the differences in Vitamin D_3 metabolism seen between the sexes. In particular, the absence of high levels of oestradiol in the male compared to those of the female could be one cause of the lower 1α-hydroxylase enzyme activity found in maturing male quail. It is possible that other factors may also lead to the sex differences in Vitamin D_3 metabolism seen. Other steroid hormones may be involved, for instance, oestrone, oestriol and oestradiol-17α; levels of

which may also be elevated prior to the onset of lay, and progesterone, which is high prior to the onset of lay. It maybe that specific combinations of these hormones are required for a physiological effect.

Another factor that must be taken into account is the ratio of free to bound steroid (steroid bound to plasma proteins). In all plasma measurements reported here the total steroid levels were measured. It may be that it is only the 'free' steroid which is effective in regulating Vitamin D_3 metabolism. Equally, high binding might increase the steroid effect, by prolonging any diurnal changes and pulses of steroids, as clearance of the steroid is reduced.

Preliminary studies done within this department (Nagvekar, unpublished results) have shown that the ratio of free : bound steroid does alter with age. The proportion of plasma protein-bound oestradiol and progesterone decreases with the onset of laying and then increases again, while the proportion of plasma protein-bound testosterone increases with age and then decreases. There was, however, little difference between the sexes.

The results presented in this thesis have also produced a profile of plasma steroid levels in birds which become somatically mature while remaining sexually immature (quail raised on short photoperiods, 8L:16D) where plasma levels remain low. This provides a good model for future studies on the regulation of Vitamin D_3 metabolism by exogenous steroid hormones in somatically mature but sexually immature quail.

CHAPTER 4 - DIURNAL CHANGES IN GONADAL STEROID HORMONES IN SEXUALLY MATURE MALE AND FEMALE QUAIL AND THE RELATIONSHIP BETWEEN THESE CHANGES IN THE FEMALE AND THE EGG-LAYING CYCLE

4.1 Introduction

Many species show changing hormone levels during a 24h period, the pattern being dependent on the time of day at which the samples are collected. Diurnal changes in gonadotrophic hormones have been shown to occur in the laying hen: at the onset of the dark period, plasma LH levels increase (Williams and Sharp, 1978). They have postulated that this may stimulate progesterone secretion from a mature ovarian follicle. This progesterone will then feedback onto the hypothalamus-pituitary axis to initiate the LH surge and subsequently result in ovulation. Williams and Sharp (1978) showed that the increase in LH at the onset of dark was a light-related phenomenon and not an event related to ovulation per se, since it still occurred on days when there was no ovulation. Hens lay during their first hours of light, while quail lay towards the end of their light period (Opel, 1966). The difference in the time of lay must also be reflected in a different sequence and diurnal rhythm of hormone secretion. The preovulatory release of LH in hens is associated with increased plasma progesterone levels (Furr, Bonney, England and Cunningham, 1973) with the rises in progesterone and LH occurring about 4-7h before ovulation. Opel (1966) showed that the timing of oviposition in quail is closely related to the time of ovulation, with ovulation occuring 0.5 to 1h after oviposition.

Male and female quail were used in this experiment to determine whether any diurnal changes in plasma hormone levels occurred and in the females, to relate the diurnal

changes in plasma hormones with the presence or absence of an ovulation on the day on which the birds were bled.

4.2 Methods

Male and female quail were raised from hatching either on 16L:8D NL or 16L:8D RL. Once egg-laying was established the birds were housed in cages containing one male and one female quail. Egg-laying records were kept by recording the presence or absence of an egg in the cage at 09.00h every day. Quail raised on 16L:8D NL were bled from the wing vein either in their 2nd or 5th hour of light (females), or in their 3rd, 5th or 7th hour of light (males). Quail raised on 16L:8D RL were bled in their 13th hour of light and 4th hour of dark (females), or in their 14th hour of light and 5th hour of dark (males). Plasma for these quail was stored at -25°C until assayed by RIA for progesterone and oestradiol (females) and testosterone and oestradiol (males). Only 2 steroids per plasma sample were measured since often insufficient blood was collected to ensure that other steroids could be measured in all samples.

The mean plasma levels of the hormones at the different times was found for male quail and the significance of the changes throughout the day assessed using unpaired Student's t-tests. Plasma hormone levels for females were grouped into those taken on the day an ovulation occurred and those taken when there was no ovulation on the day of bleeding. Statistical analysis was performed using paired and unpaired Student's t-tests.

4.3 Results

The egg-laying records obtained from the quail used in this experiment were unable to show the exact time of

oviposition since eggs were only checked for at one time of day. A pilot study on the time of oviposition was performed on another group of quail raised on 16L:8D and the results are summarised in Figure 4.1. In this group of quail, the presence of an egg in the cage was looked for at half-hourly intervals throughout the day. None of the quail had laid until after the 8th hour of light, while all the quail which laid an egg that day had laid by the end of their light period. The time of oviposition for each bird did not vary over the 4-day period in which the survey was carried out. The relative time of oviposition in these quail is in close agreement with Opel (1966), who found that his quail, kept on 14L:10D, also laid towards the end of their light period. However, later studies showed that some quail, which were bled in their 4th hour of dark, were found to lay as late as this on some days, since eggs were found in the cage during the time of bleeding.

Since the time of oviposition is closely related to the time of ovulation (Opel, 1966), ovulation in the Japanese quail must also occur during the last hours of light or first hours of dark. An ovulation which, therefore, occurred on the day of bleeding in quail raised on 16L:8D NL or 16L:8D RL would result in an egg being laid approximately 25h later. The timing of ovulation to oviposition in the Japanese Quail has been shown to be around 25h (Woodward and Mathur, 1964). This meant that in quail raised on 16L:8D NL an ovulation would occur too late in the day (20.00-23.00h) for an egg to be found the next morning at 09.00h. The criteria for an ovulation on Day 1 (the day the quail was bled) was that an egg was found in the cage at 09.00h, two days after this (Day 3). For quail raised on 16L:8D RL ovulation could occur any time between 08.00-11.00h and therefore, an egg could be found on Day 2 (the day after bleeding) at 09.00h, or not on Day 2 but on Day 3 depending on the exact time of ovulation. The criteria for an ovulation on the day of bleeding in this group was that

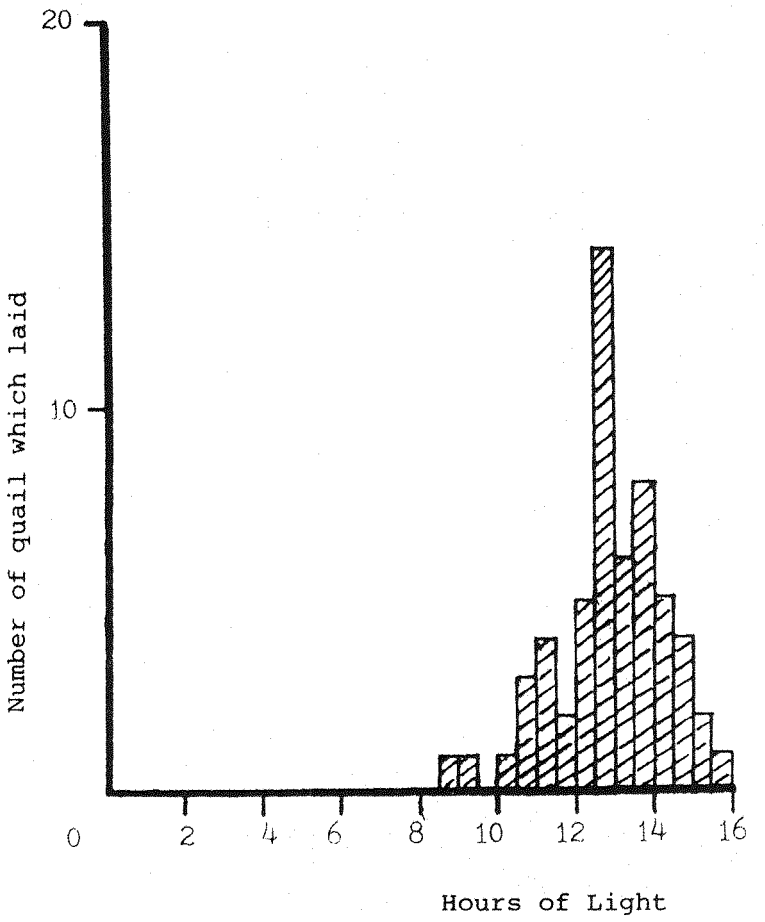


Figure 4.1 - Histogram Showing the Number of Quail to Lay at Different Times During the 16h Period of Light

The presence of eggs within the cage was checked at the end of each half hour over 4 consecutive days, using 15 female quail. No quail laid prior to the 8th hour of light and all quail that laid that day had laid by the last hour of light.

an egg had to be found in the cage both on Day 2 and Day 3. An ovulation was presumed not to have occurred on Day 1, when no eggs were found in the cage on Day 3 for quail raised on 16L:8D NL and no eggs were found in the cage on either Day 2 or Day 3 for quail raised on 16L:8D RL.

Plasma **progesterone** levels and **oestradiol** levels in female quail do show changes throughout the day, (Figure 4.2). In quail which ovulated on the day of bleeding plasma progesterone levels were at their lowest in the 2nd hour of light (8.10±0.69 pmole/ml,n=22). Progesterone levels then rose and by the 13th hour of light had risen significantly to 12.5±1.21 pmole/ml,n=22 ($p<0.01$). Progesterone levels then fell significantly to 8.70±0.62 pmole/ml-,n=19 ($p<0.01$) by the 4th hour of dark. Quail which failed to ovulate on the day of bleeding showed a small non-significant increase between the 4th hour of dark and the 5th hour of light. Levels then fell. The plasma progesterone levels found at the 13th hour of light in non-ovulating quail (7.90±0.93 pmole/ml,n=19) were significantly lower than that seen in the 13th hour of light in quail which had ovulated ($p<0.01$). There was no other significant difference at the times studied in plasma progesterone levels between a female which had ovulated and one which had not.

Plasma oestradiol levels showed a significant fall between the 4th hour of dark and the 2nd hour of light (from 1.97±0.16 pmole/ml,n=19 to 1.04±0.08 pmole/ml,n=18)($p<0.01$) in quail which had ovulated. The fall in oestradiol levels still occurred in quail which had not ovulated, but this was not a significant decrease. Oestradiol levels in quail which had ovulated then significantly increased to 1.62±0.37 pmole/ml, (n=5) ($p<0.05$) by the 5th hour of light. Oestradiol levels continued to increase until reaching maximal levels at the 4th hour of dark. A similar pattern of changes in oestradiol levels in quail which had not

Figure 4.2

Mean Plasma Progesterone and Plasma oestradiol Levels in Laying Female Quail throughout the Day

(i) Progesterone **(ii) Oestradiol**

A. Shows the levels in quail which ovulated on the day of bleeding, while - closed symbols (● and ▼).

B. shows the levels in quail bled on a day when no ovulation took place, (see text for criteria for presence/absence of an ovulation). - open symbols (○ and ▽).

Quail were raised on either 16L:8D NL or 16L:8D RL, and caged out with a male once egg-laying was established. Birds were bled by the wing vein either in their 2nd, 5th or 13th hour of light, or in their 4th hour of dark. Steroid levels were determined by RIA. Statistical analysis was performed using unpaired Student's t-tests: *$p<0.05$, **$p<0.01$, ***$p<0.001$. Results show the mean ± S.E.M. for at least 5 observations per group.

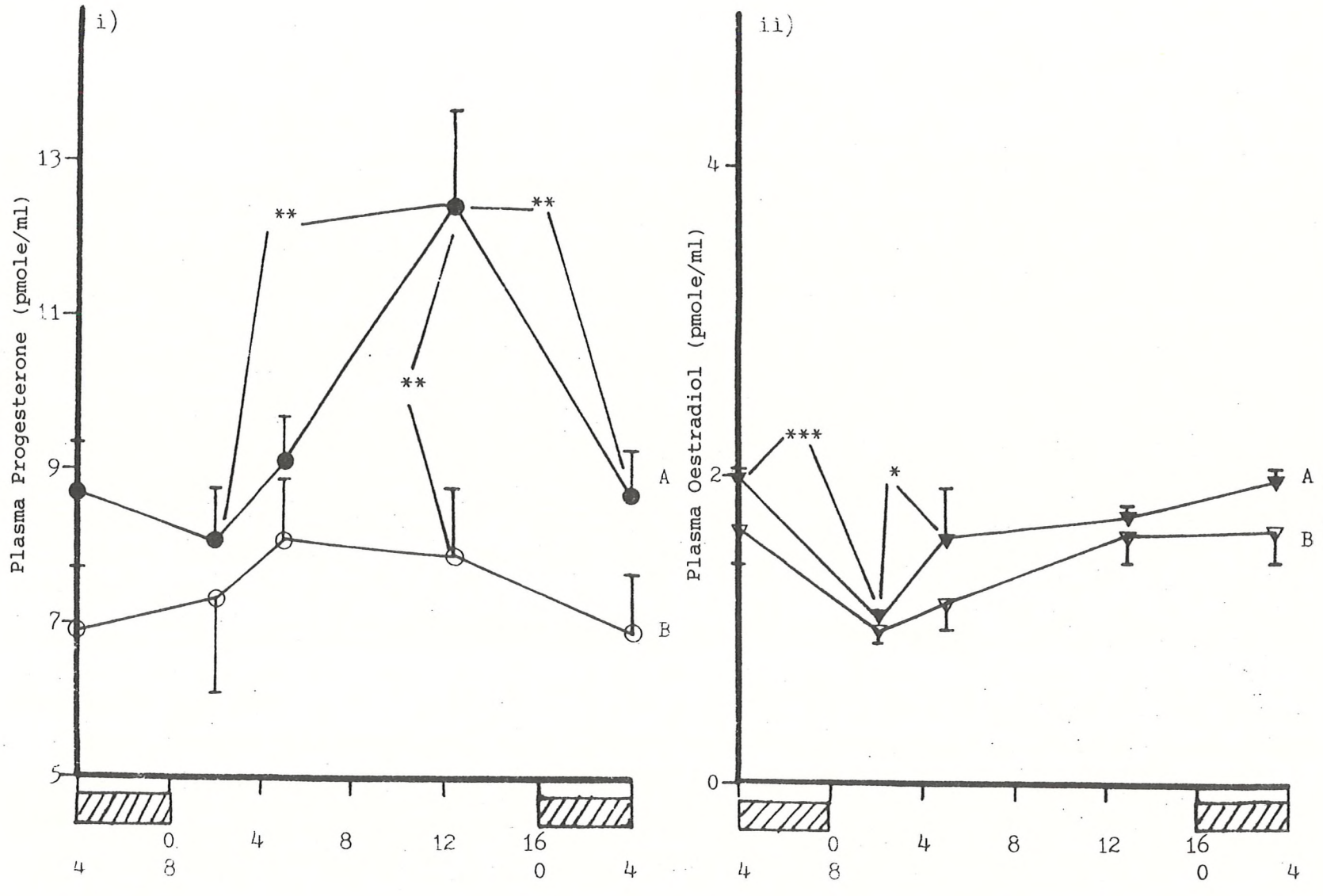

i)
ii)
Plasma Progesterone (pmole/ml)
Plasma Oestradiol (pmole/ml)
13
11
9
7
5
4
2
0
**
**
**
***
*
A
B
0
4
8
12
16
0
4
4
8
Hours of Light (unhatched) or Hours of Dark (hatched)

Figure 4.2

ovulated to those seen in quail which had ovulated was observed and no significant difference was seen between the two groups at any time of day.

Plasma levels from quail which were bled in their 13th hour of light and 4th hour of dark were also subjected to paired Student's t-tests, since these birds were often bled at both these times on the same day. These statistics confirmed those of the unpaired t-tests, since in quail ovulating on the day of bleeding progesterone was significantly higher at the 13th hour of light than at the 4th hour of dark ($p<0.01$, $n=14$), while oestradiol levels were significantly higher in the 4th hour of dark compared to the 13th hour of light ($p<0.01$, $n=13$). In the non-ovulating quail, levels of progesterone or oestradiol were not significantly different between the two times of bleeding.

Plasma **testosterone** and **oestradiol** levels in sexually mature male quail also show diurnal variations as shown in Figure 4.3. Plasma testosterone levels were maximal at the 7th hour of light (14.99±1.14 pmole/ml,n=46). Mean plasma testosterone levels were significantly higher at this time compared to those seen at the 5th hour of light (8.63±0.92 pmole/ml,n=19) ($p<0.001$), or those seen at the 14th hour of light (9.23±0.87 pmole/ml,n=25) ($p<0.001$). Plasma oestradiol levels were also highest in the 7th hour of light (1.06±0.19 pmole/ml,n=23). A significant decrease in oestradiol levels was seen between the last hours of light (14th hour of light) and the 5th hour of dark (from 0.79±0.05 pmole/ml,n=24 to 0.49±0.10 pmole/ml,n=4)($p<0.05$).

Both sexually mature male and female quail show diurnal changes in the steroids measured. Levels of progesterone in female quail were dependent on the presence or absence of an ovulation on the day of bleeding with higher levels recorded on the day when an ovulation occurred, particularly at the 13th hour of light. Oestradiol levels in females

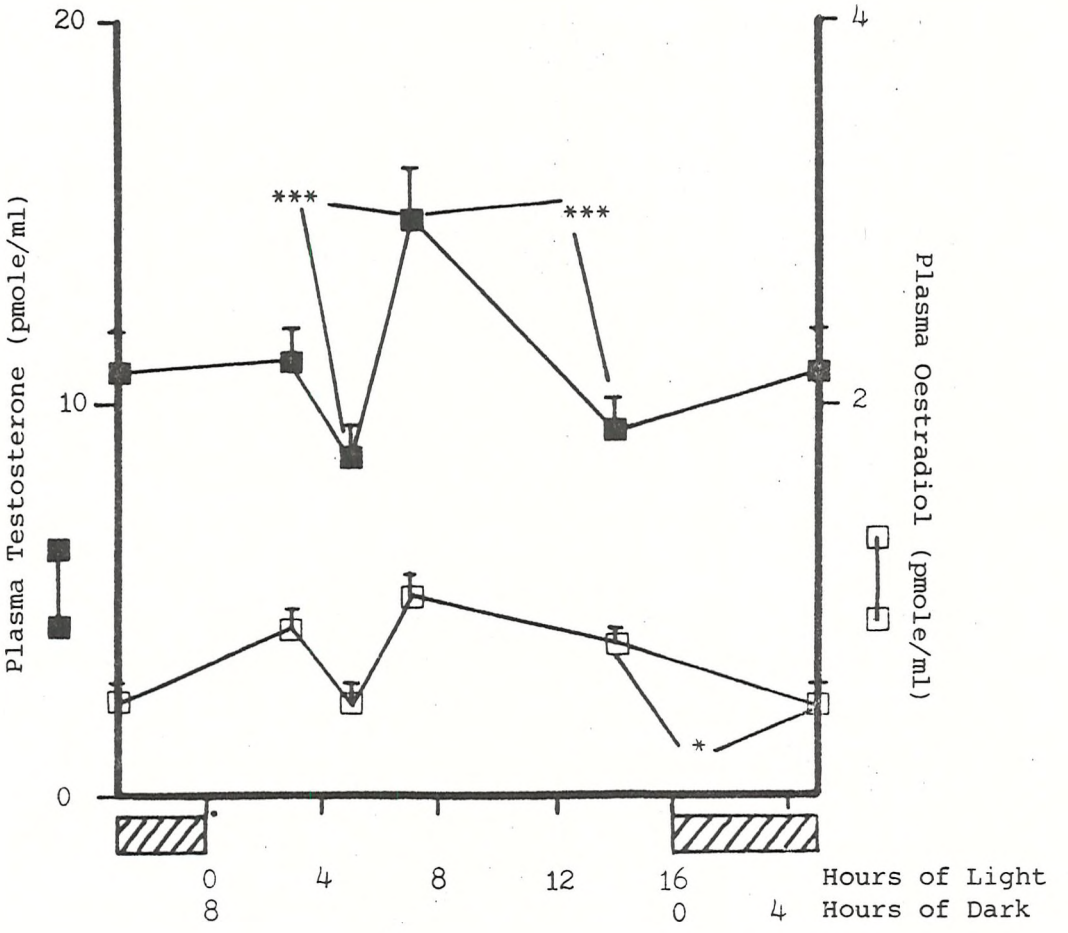


Figure 4.3 - Mean Plasma Testosterone (■—■) and Plasma Oestradiol (□—□) Levels in Sexually Mature Male Quail throughout the day

Quail were raised on either 16L:8D NL or 16L:8D RL, caged out with a female. They were bled by the wing vein in either their 3rd, 5th, 7th, 14th hour of light, or 5th hour of dark. Steroid hormones were assayed using RIA. Results show mean±S.E.M for at least 4 observations per group. Statistical analysis was performed using unpaired Student's t-tests: *** $p<0.001$, * $p<0.05$.

which had ovulated were again higher than those which had not, with lowest levels seen just after the onset of light (2nd hour of light). Plasma testosterone and oestradiol in the male quail showed maximal levels in the 7th hour of light.

4.4 Discussion

Diurnal changes do exist in both the mature male and mature female quail. Whether these changes also occur in the immature developing quail remains to be seen. However, the presence of the diurnal changes seen in these birds shows that care must be taken in the time of sampling from any bird. In the previous chapter quail were bled in their 2nd hour of light, which in retrospect may have been the wrong time of day to bleed these quail. They were bled at a time when oestradiol levels are lowest in the female, although not in the male, and before progesterone in the female, and testosterone in the male reach their maximum daily levels (in the 13th and 7th hour of light respectively). Gulati, Nakamura and Tanabe (1981) also found that testosterone in male quail, raised under 14L:10D, peaked in the 8th hour of light and again in their last hour of dark. Testosterone was always higher in the males than in the females. Unfortunately, in the quail used for this thesis only 2 steroids per sample could be assayed, since insufficient blood was obtained to allow any more to be determined.

If diurnal changes exist in the sexually maturing quail, levels of gonadal steroids may show more marked differences between the sexes at different times of day. However, bleeding mature females later on in their light period would have led to larger standard errors of the mean plasma progesterone levels, since quail ovulating have higher plasma progesterone than non-ovulating quail.

Levels of progesterone and oestradiol reported here from female quail ovulating on the day of bleeding, are similar to those found by Doi et al (1980) and by Gulati et al (1981). Progesterone levels showed a peak around 4h before the expected time of ovulation. The timing of the progesterone rise agrees with the findings of Furr et al (1973) in chickens and of Marshaly, Birrenkott, El-Bergearmi and Wentworth (1976) in turkeys. Progesterone levels did not peak in quail which failed to ovulate on the day of bleeding, comparable with the results of Marshaly et al (1976) in non-ovulating turkeys, although in non-laying quail the levels did rise slightly during the hours of light. Gulati et al (1981) also found that there was no peak of progesterone in male quail or in non-ovulating quail at any time of day.

Plasma oestradiol levels in females showed minimum levels just after the onset of light with levels increasing during the day. The highest levels were found during the 4th hour of dark. Oestradiol levels were always higher in ovulating or non-ovulating quail, compared with males. Doi et al (1978) found that there were two peaks of oestradiol, one at 20-22h and again 6h before ovulation. This was not seen in the ovulating quail used here, possibly because sampling times were infrequent. There were no differences in oestradiol levels between ovulating and non-ovulating quail.

Since, in hens and quail, LH has been shown to peak 4-8h before the next ovulation (Furr et al, 1973; Doi et al, 1980; Gulati et al, 1981), and progesterone peaks at a similar time, but only when an ovulation occurs, the present results are consistent with the hypothesis that progesterone triggers the LH surge. This increase in plasma progesterone would seem to occur only if there is a mature follicle capable of ovulating, since no progesterone peak is seen in quail which did not ovulate. The mature F1 follicle has

been shown to secrete predominantly progesterone (Shahabi et al, 1975; Cole and Peddie, 1980). It is likely that the small LH increase seen around the onset of light in quail (Doi et al, 1980) causes the mature follicle to secrete progesterone. Therefore, as seen, progesterone starts to rise soon after the onset of light in quail about to ovulate. This progesterone one can hypothesise, will feedback onto the hypothalamic-pituitary axis causing an LH surge and ovulation will result. This feedback regulation of LH is also dependent on the presence of oestrogens, since removal of the oestrogen secreting medium-sized follicles (Kumagi and Homma, 1974) in quails or treatment with the anti-oestrogen tamoxifen in chickens (Wilson and Cunningham, 1981) delays ovulation.

The results presented from female quail confirm the fact that progesterone levels are significantly elevated just prior to ovulation with no such rise seen in the non-ovulating bird. It is likely, therefore, that progesterone secreted by the mature F1 follicle, is the hormone involved in the control of the LH surge in quail as has been proposed for chickens.

CHAPTER 5 - A STUDY OF OVARIAN STEROIDOGENESIS USING HISTOCHEMICAL METHODS

5.1 Introduction

Ovarian steroidogenesis has been shown to occur in the granulosa and thecal cell layers of the pre-ovulatory follicles as reported in the main introduction of this thesis (Chapter 1.5.1). The granulosa layer synthesises and secretes most of the progesterone with some testosterone, while the thecal layer synthesises and secretes testosterone and oestradiol. It has also been shown that the size of each yolk-filled follicle is related to the major steroid secreted by that follicle, with the largest follicle (F1) secreting progesterone (Huang and Nalbandov, 1979) while the medium sized follicles (F2, F3, F4) metabolise progesterone to testosterone and oestradiol (Kumagi and Homma, 1974; Senior and Furr, 1975). This differential secretion of steroids from follicles of varying size suggests that the localisation and relative activities of enzymes involved in their synthesis may also depend on follicular size.

Some of these enzymes can be localised using histochemical techniques. Cells in which progesterone synthesis occurs should contain the enzyme 3β-hydroxysteroid dehydrogenase (3β-HSD), the enzyme which catalyses the conversion of pregnenolone to progesterone (see Figure 5.1a). Cells synthesising testosterone and oestrogens should contain the enzyme 17β-hydroxysteroid dehydrogenase (17β-HSD), the enzyme which catalyses the interconversion of androstenedione and testosterone (see Figure 5.1b).

By using specific steroids as substrates for these two dehydrogenase enzymes and in the presence of the oxidized co-factor NAD, NADH will be produced. The NADH produced

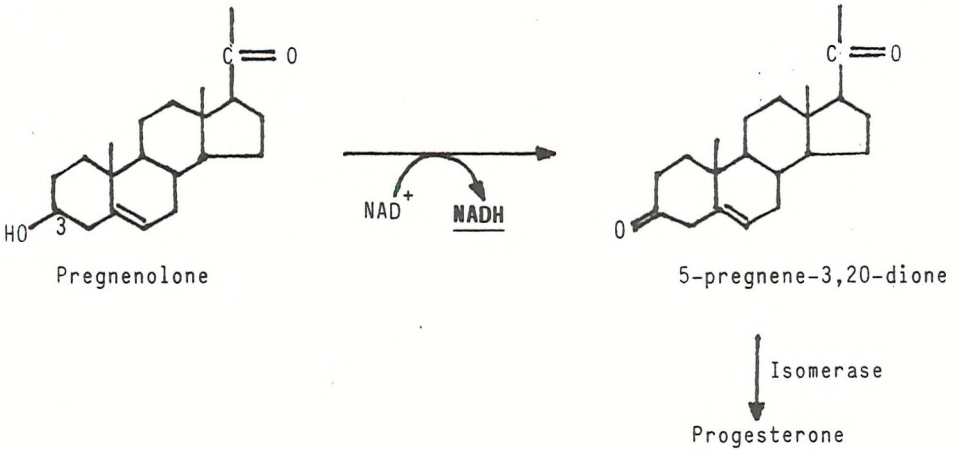
Figure 5.1

a) Production of progesterone from pregnenolone requires NAD^+ and the enzyme 3β-HSD in order to convert the hydroxyl group at position 3 into a ketone. An isomerase reaction then converts the 5-pregnene-3,20-dione into progesterone.

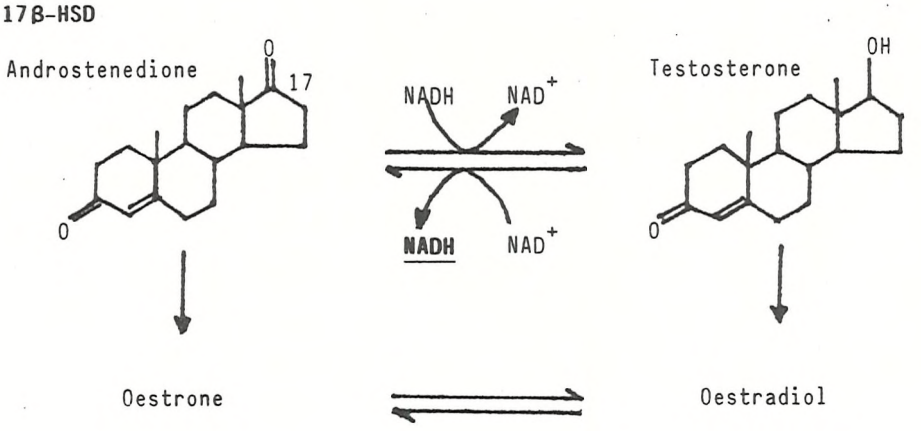
b) Production of testosterone from androstenedione by 17β-HSD converts the ketone at position 17 into an hydroxyl group. Oestrogens are synthesised from both testosterone and androstenedione.

It is the production of **NADH** from these reactions which can then be reoxidised to NAD^+ by reduction of the dye, nitro BT, resulting in a deposition of dark blue formazan granules in cells containing these enzymes.

a) 3β-HSD



b) 17β-HSD



can then be reoxidised to NAD by reacting with a hydrogen acceptor such as the dye nitro blue tetrazolium (nitro BT) which in its reduced form, will precipitate out as blue formazan granules. Most histochemical studies in avian ovarian tissue have only studied the localisation and activity of these enzymes during ovarian maturation, with little reference to any differences pertaining to follicles of varying size.

In the present study, the yolk-filled follicles from laying female quail were removed. Once sectioned, the presence or absence of the 3β-HSD and 17β-HSD within the cell layers was examined using pregnenolone and testosterone as substrates for the two enzymes. It was hoped that the differential pattern of steroid secretion seen in follicles of different diameters would be paralleled by changes in the localisation and relative activities of these two enzymes, as seen by changes in localisation and intensity of the formazan granules deposited within the cell layers. Studies on the profile of steroids secreted by these follicles **in vitro** have been reported elsewhere (Cole and Peddie, 1980; Onagbessan and Peddie, (in preparation)).

5.2 Methods

Female quail raised from hatching on 16L:8D NL were killed once laying had been established. The yolk-filled follicles were removed and their diameter measured. Follicles were grouped according to their size into five groups:- greater than 15mm, 12-15mm, 9-12mm, 6-9mm and less than 6mm. These corresponded approximately to the numbering of the follicles F1, F2, F3, F4 and F5, although some ovaries contained more than five yolk-filled follicles and would often have two or more follicles in the middle-sized ranges.

The follicles were frozen and then sectioned at 8μm using a cryostat and stained for the presence of 3β-HSD and 17β-HSD as described in Materials and Methods (Chapter 2.8). Control sections were incubated with the stain in the absence of any substrate. A comparable set of follicles were fixed in Bouin's fixative to be sectioned at 8μm and stained later with Erlich's haematoxylin and eosin (H and E) (see Materials and Methods, Chapter 2.7).

The localisation and relative activity of the two enzymes was judged by the site and density of the formazan granules which were deposited and graded on a scale of 0 (no granules) to 5 (intense dark blue granules). The sites of histochemical staining were compared with the morphology of follicles seen in the sections stained with H and E.

5.3 Results

Figure 5.2A shows a section through the ovary from a laying female quail stained histologically with H and E. In the centre an atretic follicle (a) can be seen surrounded by blood vessels and other developing follicles. The thecal cells lie on the perimeter of the follicle (t) with the granulosa cells (g) inside (Figure 5.2B and C). As the follicle develops, yolk is deposited into the central lumen (lu) and the follicles become yellow in colour as opposed to white.

Typical sections through an F3 and F1 follicle wall are shown in Figures 5.2B and 5.2C respectively. While the granulosa cell layer is at least two cells thick in the F3 follicle, the thickness of the granulosa cell layer of the larger follicle is reduced to a single layer of cells. The lumen size also increases with increasing follicular size as more yolk proteins are deposited in droplet form into the

Figure 5.2

Histological Study of the Ovary from a Laying Japanese Quail

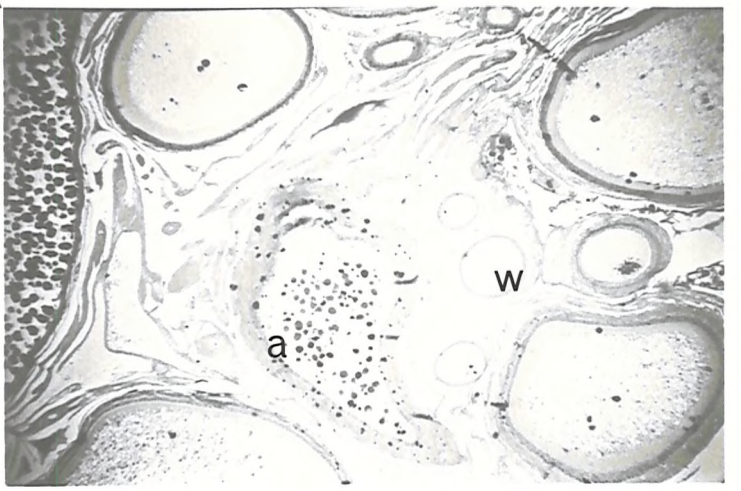
A. Cross section of an ovary of a laying Quail raised on 16L:8D NL. The ovary contained 5 yolk-filled follicles (not shown) as well as many smaller white follicles (w) and some atretic follicles (a). Magnification x22.

B. Cross section of the follicle wall of a typical F3 follicle. The outer thecal layer (t) has become disrupted in the staining process. Several layers of granulosa cells (g) exist. The lumen (lu) is becoming filled with deposited yolk proteins. Magnification x500.

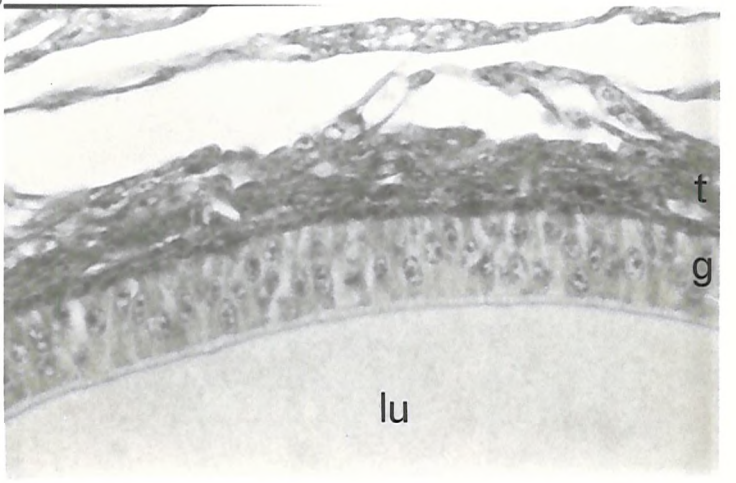
C. Cross section of the follicle wall of an F1 follicle. The outer thecal layers (t) have been disrupted in the staining process. The granulosa cell layer is now only one cell thick. The yolk droplets can also be seen in the lumen (lu). Magnification x400.

The sections were prepared from tissue fixed in Bouin's solution and stained with haematoxylin and eosin as described in Material and Methods, (Chapter 2.7).

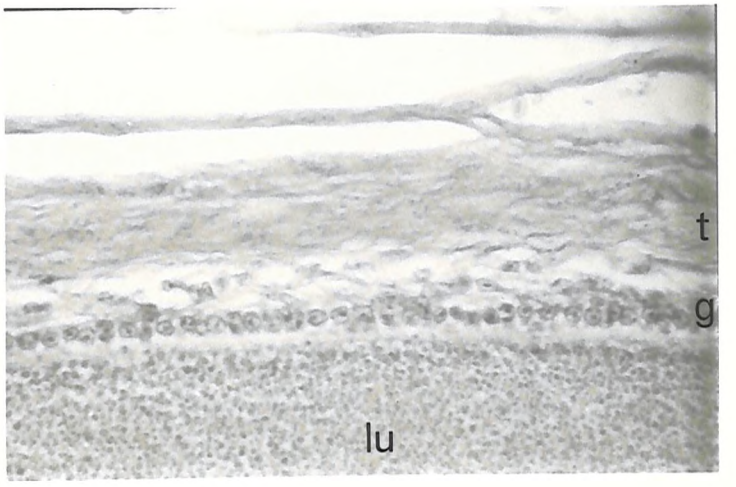
A



B



C



lumen. In the immature ovary and in all quail raised on 8L:16D only small white follicles, together with some atretic follicles, were present in the ovary. There was no deposition of yolk into the follicles in the immature ovary.

Control histochemical sections showed some deposition of formazan granules, but when these granules were seen, they were located both within and without the cells. The intensity of staining was never as great as the intensity which appeared when either pregnenolone or testosterone were used as substrate.

Figure 5.3 shows the relative activities of the 3β-HSD (when pregnenolone was substrate) in a series of follicles of different sizes, taken from a single bird. In all of the follicles examined dense blue granulation was seen in the granulosa cell layer (g). The intensity of staining was lowest in the small and medium sized follicles (intensities 2-4) while in the largest follicle (Figure 5.3A), the maximum intensity was seen (intensity 5). Some granulation was also seen in the thecal cell layer (t) of the smaller follicles (intensities 2-3; Figure 5.3D and E) and in the larger follicles (intensity 1; Figure 5.3A, B and C).

With testosterone as substrate the site and activity of the 17β-HSD was observed and the results are pictured in Figure 5.4. Granulation was found in the thecal cells of the 3 medium-sized follicles (intensity 3, Figure 5.4B, C and D), size range 6-15mm, but in the largest size group (greater than 15mm), the deposition of formazan granules was lost (intensity 0, Figure 5.4A). Some granulation was also seen within the granulosa cell layer, but the intensity was never above 1, (Figure 5.4A, B and D).

A summary of the results is shown in Table 5.1 and is the average result from at least five follicles within each size range. This shows that the 3β-HSD is present mainly

Figure 5.3 - Histochemical Localisation of the Enzyme 3β-hydroxysteroid Dehydrogenase, using Pregnenolone as Substrate, with the Five Yolk-filled Follicles of a Laying Quail

A. Cross section of the wall of an F1 follicle (>15mm diameter). Intense staining (scale 5) is seen in the granulosa cell layer (g), while little staining is seen in the thecal cell layers (t) (scale 1).

B. Cross section of the wall of an F2 follicle (12-15mm diameter). Staining is again seen in the granulosa cell layer (scale 5).

C. Cross section of the wall of an F3 follicle (9-12mm diameter). Staining is in the granulosa cell layer (scale 5).

D. Cross section of the wall of an F4 follicle (6-9mm diameter). Staining is in the granulosa cell layer (scale 4) and in the thecal layer (scale 3).

E. Cross section of the wall of an F5 follicle (<6mm diameter). Staining is in the granulosa cell layer (scale 4) and in the thecal layer (scale 1).

All sections magnified x500.

The intensity was estimated from microscopic examination of the tissue sections, which had been histochemically stained using pregnenolone as substrate (as described in Materials and Methods, Chapter 2.8). The intensity was graded as follows:-

0 = negative
1 = light blue
2 = medium light blue
3 = medium blue
4 = medium dark blue
5 = intense dark blue.

The follicles were removed from laying quail raised on 16L:8D NL.

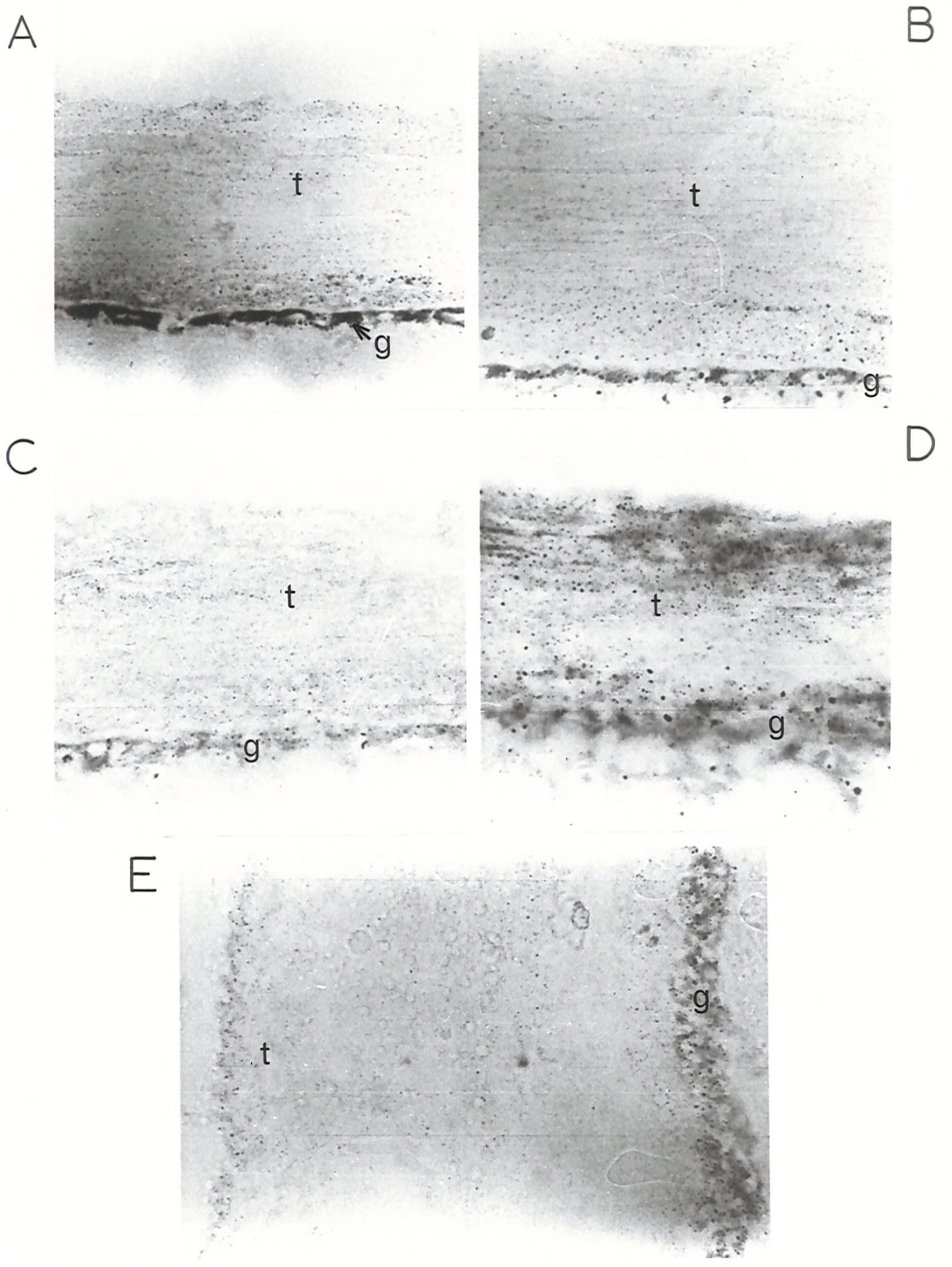

A
t
g
B
t
g
C
t
g
D
t
g
E
t
g

Figure 5.4

Histochemical localisation of the Enzyme 17β-hydroxy-steroid Dehydrogenase, using Testosterone as Substrate, within the Five Yolk-filled Follicles of a Laying Quail

A. Cross section of the wall of an F1 follicle (>15mm diameter). There was no staining in either the thecal (t) or granulosa cell layers (g). Magnification x500.

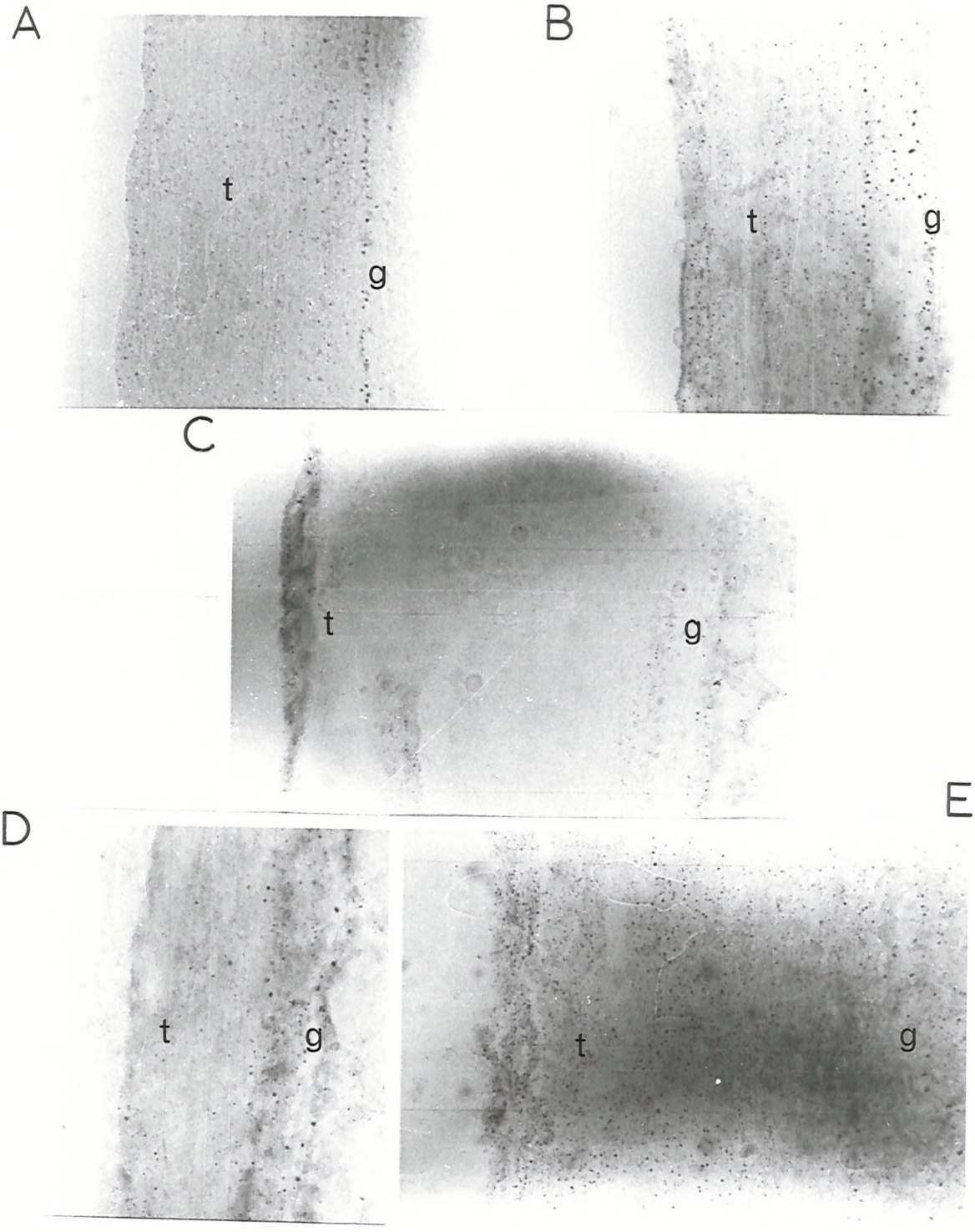
B. Cross section of the wall of an F2 follicle (12-15mm diameter). Staining was seen only in the thecal cell layers (scale 3). Magnification x500.

C. Cross section of the wall of an F3 follicle (9-12mm diameter). Staining was seen only in the thecal cell layers (scale 3). Magnification x404.

D. Cross section of the wall of an F4 follicle (6-9mm diameter). Staining was seen in the thecal cell layers (scale 3) with some in the granulosa cell layers (scale 1). Magnification x500.

E. Cross section of the wall of an F5 follicle (<6mm diameter). Staining was seen only in the thecal cell layers with a reduced intensity (scale 1). Magnification x500.

The intensity was estimated from microscopic examination of the tissue sections and relative intensity scaled as described in the legend for Figure 5.3. The follicles were removed from laying quail raised on 16L:8D NL.


A
t
g
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t
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C
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D
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E
t
g

Table 5.1

		Follicle size (mm diameter)				
		<6	6-9	9-12	12-15	>15
Thecal cells	3βHSD	1	2	2	1	1
	17βHSD	1	3	3	3	0
Granulosa cells	3βHSD	4	4	5	5	5
	17βHSD	0	1	0	1	0

Table 5.1 - Histochemically Demonstratable 3β-HSD and 17β-HSD Activity within the Thecal and Granulosa Cell Layers in Cryostat Sections of Yolk-filled Follicles of Different Sizes

The intensity recorded in each size group is an average interpretation of five follicles that fell within that group. These follicles were removed from five laying Japanese Quail raised on 16L:8D NL. The intensity was estimated from microscopic examination of the tissue sections which had been histochemically stained, using either pregnenolone or testosterone as substrates (as described in Materials and Methods, Chapter 2.8). The intensity was graded as follows:-

0 = negative
1 = light blue
2 = medium light blue
3 = medium blue
4 = medium dark blue
5 = intense dark blue.

in the granulosa cell layer with some activity also in the thecal cell layer. The 17β-HSD is present in the thecal cell layers and not the granulosa cells of the medium sized follicles but is absent from the thecal cell layers of the largest group of follicles.

5.4 Discussion

The histochemical localisation of the 3β-HSD and 17β-HSD reported within this thesis has shown that the localisation of the enzymes is dependent on the size of the follicle and the cell type within the follicle. The method used can only present qualitative results accurately, since to measure quantitative results an assay system would have to be set up for these enzymes. The sections were exposed to a uniform amount of stain and therefore a visual interpretation of the relative intensity could be assessed. While the sites of these enzymes can be identified using this histochemical technique, care has to be taken in interpreting the relative intensities of, and thus the relative activities of, these two HSD enzymes.

The cross sections of quail ovaries stained with H and E have shown that the thickness of the granulosa cell layer decreases as the follicle size increases. This could be due to the fact that the larger follicles contain more yolk and results in the granulosa cell layer having to cover a larger surface area with the same number of cells. The decreasing thickness of the follicle wall would aid the transport or diffusion of yolk proteins from plasma to the lumen of the follicle and aid the rupturing of the follicle wall at the time of ovulation to exude the ovum and yolk.

In ovaries from quail raised on 8L:16D only small white follicles are seen together with some atretic follicles. This observation was also made by Saylor, Dowd and Wolfson

(1970), who found that quail raised on 8L:16D had a larger number of atretic follicles than did ovaries from quail raised on long photoperiods (16L:8D).

The presence of the 3β-HSD activity within the granulosa cell layer and the presence of the 17β-HSD activity within the thecal cells of quails' follicles parallels the postulated sites of steroidogenesis in chicken follicles. Huang, Kao and Nalbandov (1979), using **in vitro** incubations of different follicular components of hens' follicles, found that the granulosa cells produced most progesterone, while the thecal cells, provided that progesterone was added, produced testosterone and oestradiol. Therefore, if a similar pattern occurs in the quail as in the hen, one would anticipate that the 3β-HSD enzyme would be localised within the granulosa cells, since it is involved in the synthesis of progesterone. One would also predict that the 17β-HSD enzyme would be located within the thecal cells.

It was observed that progesterone had to be added to the thecal cells **in vitro** for them to produce testosterone (Huang, Kao and Nalbandov, 1979). However, some 3β-HSD activity was found within the thecal cells, but the activity was always low. One can therefore suggest that the amount of progesterone produced would also be small and that little testosterone would be formed by thecal cells incubated alone, unless exogenous or endogenous progesterone was supplied to them.

The presence of the 3β-HSD enzyme within the granulosa cells of Japanese quail has also been shown histochemically by Sayler, Dowd and Wolfson (1970). They followed the changes in 3β-HSD activity in the ovaries of developing quail raised on different photoperiods, by visual assessment of the intensity of formazan deposits in tissue sections using either epiandrosterone (EA), dehydroepiandrosterone (DHEA) or pregnenolone as substrates. Formazan deposits

were found in thecal, granulosa, interstitial and medullary layers. However, little difference was seen in the intensity found in the thecal, interstitial and medullary cells of birds raised on the stimulatory photoperiod (16L:8D) to that seen on the non-stimulatory one (8L:16D). The formazan deposit in the granulosa layer of birds raised on 16L:8D became denser with age, whereas the granulosa cells of birds on 8L:16D remained consistently non-reactive.

The follicles used in this thesis were all from mature laying quail and therefore the dense formazan deposits seen within the granulosa cells when pregnenolone was used as substrate agrees with the results of Sayler et al (1970).

The localisation of the 3β-HSD and 17β-HSD enzymes in the quails' follicles correlates with those seen in chickens' follicles by Chieffi and Botte (1965) and Boucek and Savard (1970). They also found that the granulosa cells showed some 17β-HSD activity which was only seen in a few of the quails' follicles studied. The difference is perhaps due to the different species of bird, but the 17β-HSD activity within the granulosa cells of chickens could explain the small amounts of testosterone produced **in vitro** by incubations of isolated chickens' granulosa cells.

Boucek and Savard (1970) looking at sections of maturing follicles found low levels of 17β-HSD activity within the thecal cells of laying hens but a high degree of activity was seen in moulting hens. They correlated this high 17β-HSD activity in the theca to the large production of androstenedione seen when the ovarian slices from moulting hens were incubated with ^{14}C-labelled acetate. Testosterone and oestradiol were also labelled while only trace amounts of labelled progesterone were found, presumably as a result of any progesterone formed being rapidly metabolised. In the laying hen, the incorporation of the labelled acetate was equally distributed between progesterone, testosterone, androstenedione and oestradiol. In the

quail, the largest follicles, which secrete the majority of the progesterone, had little or no 17β-HSD activity within the thecal cells, while the smaller follicles, which secrete predominantly testosterone and oestradiol, had high activity. It is possible that the follicles Boucek and Savard were studying in the laying hens were only those represented here in the largest size group; this would explain why they found little 17β-HSD activity within their thecal cells. Follicles secreting larger amounts of testosterone and oestradiol, such as those of the non-laying hen and the smaller follicles of the quail, do show high 17β-HSD activity within the thecal cell layer.

This dependence of follicle size on the relative activities of the two enzymes, 3β-HSD and 17β-HSD, correlates with the steroids secreted by follicles of different sizes. Oestrogen content (oestradiol and oestrone) of quail follicles was found, by RIA of the homogenised follicle extract, to be high in the medium-sized follicles (6-15mm diameter), while the content within the remaining follicles was small (Kumagi and Homma, 1974; Doi, Takai, Nakamura and Tanabe, 1980). Senior and Furr (1975) found low oestradiol levels in the largest follicles of chickens with higher levels in the smaller follicles. Incubations **in vitro** have also shown that oestradiol production is greatest in the medium-sized follicles of the chicken and Japanese quail. Huang, Kao and Nalbandov (1979) incubated thecal cells, isolated from the three largest follicles of hens, with exogenous testosterone and found that the F2 and F3 follicles secreted the greatest amounts of oestradiol per 10^6 cells. Cole and Peddie (1980) found that oestradiol secretion **in vitro** was greatest by the medium-sized follicles of Japanese quail. These follicles were incubated as complete follicles and the total secretion of oestradiol into the medium after a 4h incubation measured. 17β-HSD is required for the interconversion of androstenedione and testosterone, a necessary step in oestradiol production.

The high intensity of granulation seen, when using testosterone as substrate, within the medium-sized quail follicles correlates with the **in vivo** and **in vitro** production of oestradiol from this size range of quail follicles.

The secretion and follicular content of progesterone are also dependent on follicular size. Shahabi, Norton and Nalbandov (1975) found that the largest chicken follicles (F1) contained most progesterone, while Cole and Peddie (1980) by incubating whole quail follicles **in vitro**, showed that progesterone secretion into the medium was dependent on follicle size: increasing progesterone secretion was seen with increasing follicular size. Since progesterone production is necessary for oestradiol and testosterone synthesis, the presence of 3β-HSD within follicles secreting oestradiol and testosterone is also required. Histochemical localisation of the 3β-HSD enzyme showed its presence within the granulosa cells of all sizes of follicles. The intensity of granulation increased from 4 to 5 as the follicle size increased beyond 9mm in diameter. The presence of 3β-HSD activity within the granulosa cells from the largest follicles together with the abrupt disappearance of any 17β-HSD activity coincides with the largest follicles no longer secreting oestradiol but secreting predominantly progesterone. This also explains the findings of Shahabi et al (1975) and of Cole and Peddie (1980) that the largest follicle contains the most progesterone **in vivo** and produces most progesterone **in vitro**.

Nakamura, Tanabe and Hirano (1979) have shown that differences in enzyme activities also exist within the two largest follicles of chickens. By incubating homogenates of the F1 and F2 follicles with ^{14}C-labelled pregnenolone and progesterone, they found that the percentage conversion of pregnenolone to progesterone was always higher in the largest follicle. The percentage conversion of progesterone to 17α-hydroxy-progesterone (and other products) was

always lower in the largest follicle (F1) compared to F2. They also found that relatively higher combined activities of the Δ^5-3β-HSD and $\Delta^5\,\Delta^4$-isomerase, and a significantly lower activity of the 17α-hydroxylase, occurred within the largest follicle (F1) compared to the second largest follicle (F2).

The histochemical localisation of the 3β-HSD enzyme in the granulosa cells and the 17β-HSD enzyme in the thecal cells from quail follicles parallels the postulated sites of steroidogenesis in chicken follicles. The relative activities of these two enzymes, as shown by the intensity of formazan granulation, within follicles of varying size correlates with the observed changes in the ability of the follicles to secrete steroids. The reduction in 17β-HSD activity within the thecal cells, from F2 to F1 follicles, coincides with the maturation of the follicle from secreting oestradiol to secreting progesterone. It is this ability to secrete progesterone in preference to oestradiol, which has resulted from changes in the enzyme activities within the follicle, which possibly determines whether the follicle is ready to ovulate and ensures ovulation of one follicle only.

CHAPTER 6 - TESTOSTERONE AND OESTRADIOL SECRETION AND PRODUCTION BY SEMI-PURIFIED LEYDIG CELLS AND BY DISPERSED TESTICULAR CELLS FROM IMMATURE JAPANESE QUAIL

6.1 Introduction

Plasma testosterone levels in male quail increase rapidly during sexual maturation on long photoperiods (16L:8D NL) between 28 and 45 days of age, as shown in Chapter 1.3 of this thesis. However, when birds are transferred from short photoperiods (8L:16D) to long photoperiods (16L:8D), there is a transient increase in plasma oestradiol levels from 1.22±0.34 pmole/ml (n=5) on day 0, to 4.72±1.12 pmole/ml (n=5) on day 2, decreasing to 2.74±0.82 pmole/ml (n=8) by day 8, while plasma testosterone levels in these birds only start to rise after day 28. Within 20h of transferring quail from short to long photoperiods, there is an increase in plasma LH (Follett, Davies and Gledhill, 1977).

I set out to study the response of testicular Leydig cells to gonadotrophin during sexual maturation, and to determine whether the transient rise in plasma oestradiol could be of testicular origin.

6.2 Methods

Quail raised on 16L:8D NL were killed between 09.00 and 10.00h at various ages between 24d and 120d. The testes were removed and Leydig cell suspensions prepared and incubated in the presence or absence of hCG as described in Chapter 2.9.1. Aliquots of the medium after the 3h incubation were assayed for testosterone and oestradiol by RIA.

A second group of quail raised on d70TLD were killed between 09.00 and 10.00h after 0, 2, 9, 21, 37, 43 and 70 days exposure to the long photoperiods. Cell suspensions from dispersed testis (Leydig and tubule fractions) were prepared for the above ages and incubated with hCG as previously described in Chapter 2.9.2. Both cellular content and medium content (secreted steroid) of testosterone and oestradiol after the 3h incubation were determined by RIA. Further Leydig cell suspensions were prepared from this group of birds after 9 and 21 days exposure to long photoperiods, and incubated in the presence or absence of hCG. The medium was then assayed for testosterone and oestradiol by RIA.

Testes of maturing quail raised under each photoperiod were fixed in Bouin's solution and later sectioned and stained with Haematoxylin and Eosin (see Materials and Methods, Chatper 2.7). These sections were examined under a light microscope. The percentage of 3β-hydroxysteroid dehydrogenase positive cells was determined histochemically as described in Materials and Methods, Chapter 2.9.1.

6.3 Results

6.3.1 Incubations of Semi-purified Leydig Cells

The histological examination of testicular sections from quail of increasing age has been reported previously in this thesis (Chapter 3.3.3; Figures 3.3 and 3.4). While differential cell counts were not performed, it was possible to see that Leydig cell numbers increased with age. The number of semi-purified Leydig cells obtained per pair of testes after collagenase treatment was found to increase with age. While five or more pairs of quail testes were

required to obtain sufficient Leydig cells for Leydig cell incubations from young quail aged 24 to 30d old, only one or two pairs of testes were required to obtain sufficient numbers of cells from the older testes (days 35-120). The percentage of 3β-HSD positive cells within the semi-purified Leydig cell incubations was consistent with age. 48.5±5.2% (n=10) of the semi-purified Leydig cells showed 3β-HSD activity.

As with all **in vitro** incubations care must be taken in extrapolating results found **in vitro** to those obtained **in vivo** situations. However, **in vitro** incubations give an idea of the responsiveness of different cell types to external stimuli. In this case, it was the actions which exogenous hCG had on the endogenous production and secretion of testosterone and oestradiol from testicular fractions, that was examined.

Testosterone secretion by Leydig cells obtained from quail 24d, 29d, 35d, 43d, 51d and 120d after hatching onto 16L:8D NL and their response to increasing doses of hCG is shown in Figure 6.1.

At 24d, there was a significant increase ($p<0.01$) in testosterone secretion in response to 1 I.U/ml hCG (from 2.30±0.30 pmole/10^4 cells/3h, n=5 to 4.23±0.49 pmole/10^4 cells/3h, n=5). At 29d there was no significant increase in testosterone with 1 I.U/ml hCG, but a significant increase ($p<0.001$) was seen with the addition of 10 I.U/ml hCG (from 2.08±0.37 pmole/10^4 cells/3h, n=5 to 5.08±0.44 pmole/10^4 cells/3h, n=5). This shift in the dose-response curve to the right was also seen at 35d when a significant increase ($p<0.001$) was only seen when 100 I.U/ml hCG was added (from 2.62±0.82 pmole/10^4 cells/3h, n=5 to 18.11±2.72 pmole/10^4 cells/3h, n=5). By 120d, the Leydig cells do respond to 100 I.U/ml hCG, but it is only significant at $p<0.02$.

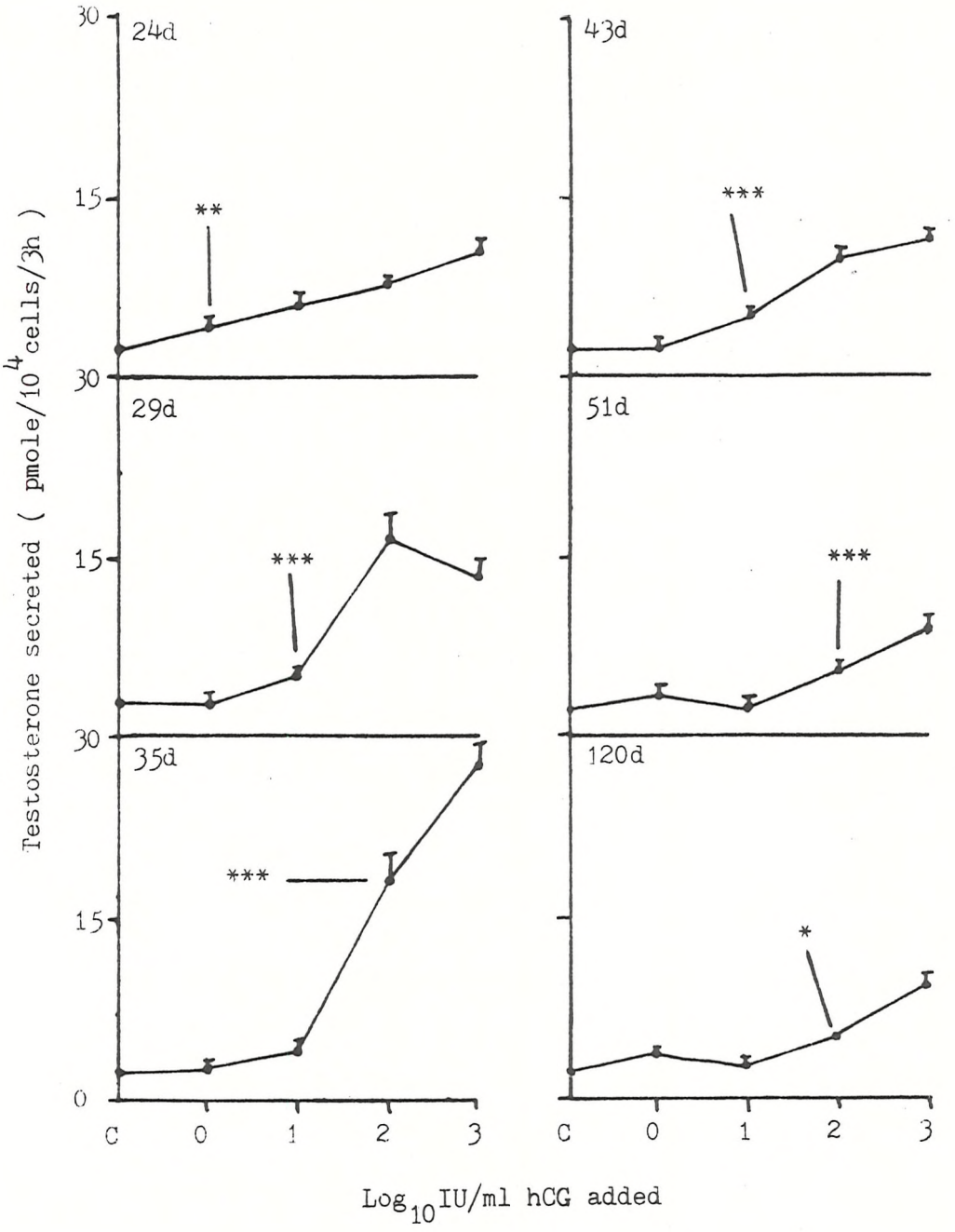

Figure 6.1 - Testosterone Secretion from Semi-purified Leydig Cells in Response to Increasing Doses of hCG

Quail were raised from hatching on 16L:8D NL and killed at 24d, 29d, 35d, 43d, 51d or 120d. Isolated Leydig cells were prepared using collagenase to disperse the cells. Testosterone secretion was measured in Krebs-bicarbonate Buffer pH7.4 by specific RIA (see Chapter 2.12.3 for details). Results are expressed as mean ± S.E.M. for 5 observations. C indicates the incubation with no added hCG. The point at which the response was significantly greater than that with no added hCG is shown; ***$p<0.001$, **$p<0.01$ and *$p<0.02$.

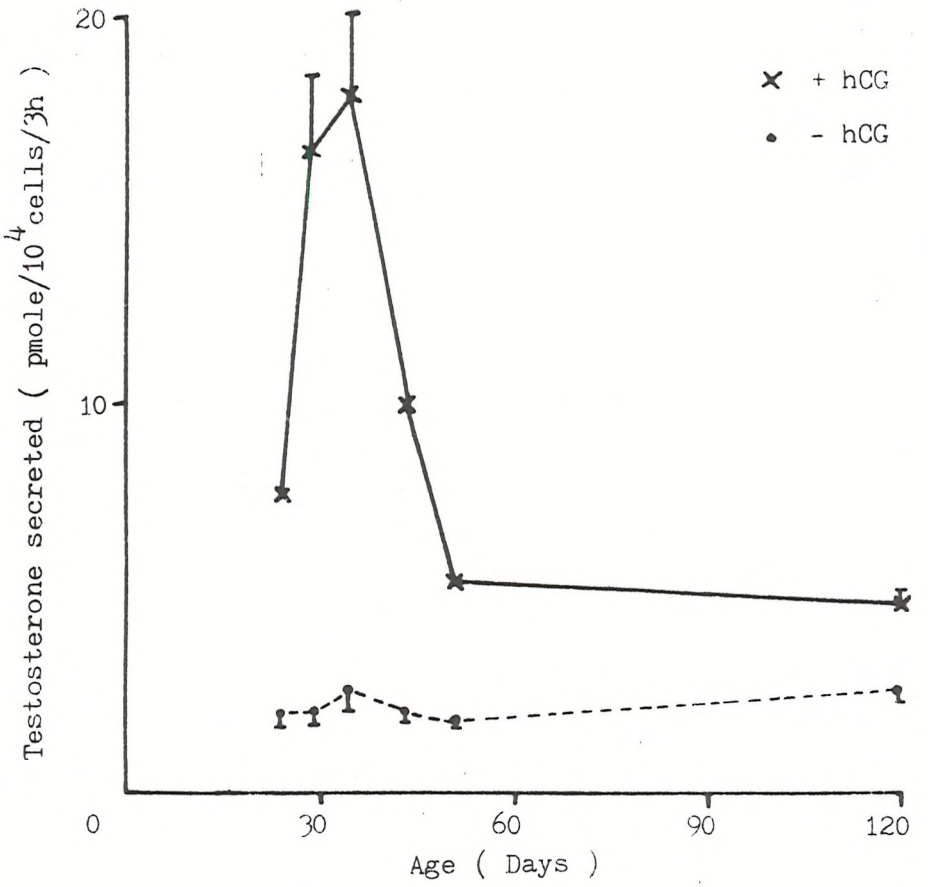

Figure 6.2 - Effect of Age on the Ability of Semi-purified Leydig Cells to Secrete Testosterone in the Absence (●----●) or Presence of 100 I.U/ml hCG (×——×)

Isolated Leydig cells were prepared, and testosterone measured as described for Figure 6.1. Results are expressed as mean ± S.E.M. for 5 observations.

Figure 6.2 shows that the response of these Leydig cells to a particular dose of hCG is dependent on the age of the bird. There is no significant difference in the amount of testosterone secreted in the absence of hCG with age (around 2 pmole/10^4 cells/3h). In the presence of 100 I.U/ml hCG there is a significant increase in the amount of testosterone secreted at 29d (to 16.63±2.73 pmole/10^4 cells/3h, n=5: $p<0.02$) and 35d ($p<0.01$) compared with the amount secreted at 24d (7.69±0.24 pmole/10^4 cells/3h, n=5). This was followed by a significant decrease to 43d (to 10.00±0.38 pmole/10^4 cells/3h, n=5 :$p<0.02$) and a further decrease to 51d (to 5.42±0.26 pmole/10^4 cells/3h, n=5: $p<0.001$). Similar effects of age are seen after the addition of either 1000 I.U/ml hCG or 10 I.U/ml hCG.

There was no detectable accumulation of **oestradiol** in the medium in the absence or presence of hCG at any age by these Leydig cell incubates.

Leydig cell incubates were also prepared from the second group of birds raised on d70TLD and killed on the 9th and 21st long day after transfer. Again no **oestradiol** was found in the medium in the absence or presence of hCG at either age. The two dose response curves for **testosterone** are shown in Figure 6.3. The greater response was found in the Leydig cell incubates from the younger birds after the 9th long day, being significantly greater at this age compared to after the 21st long day ($p<0.001$) in the presence of 10, 31.6 or 100 I.U/ml hCG. The response after addition of 10 I.U/ml hCG was significantly greater at both ages ($p<0.001$) compared with the control levels.

6.3.2 Incubations of Dispersed Testicular Cells

Suspensions of dispersed testicular cells from birds raised on d70TLD, after transfer to long photoperiods,

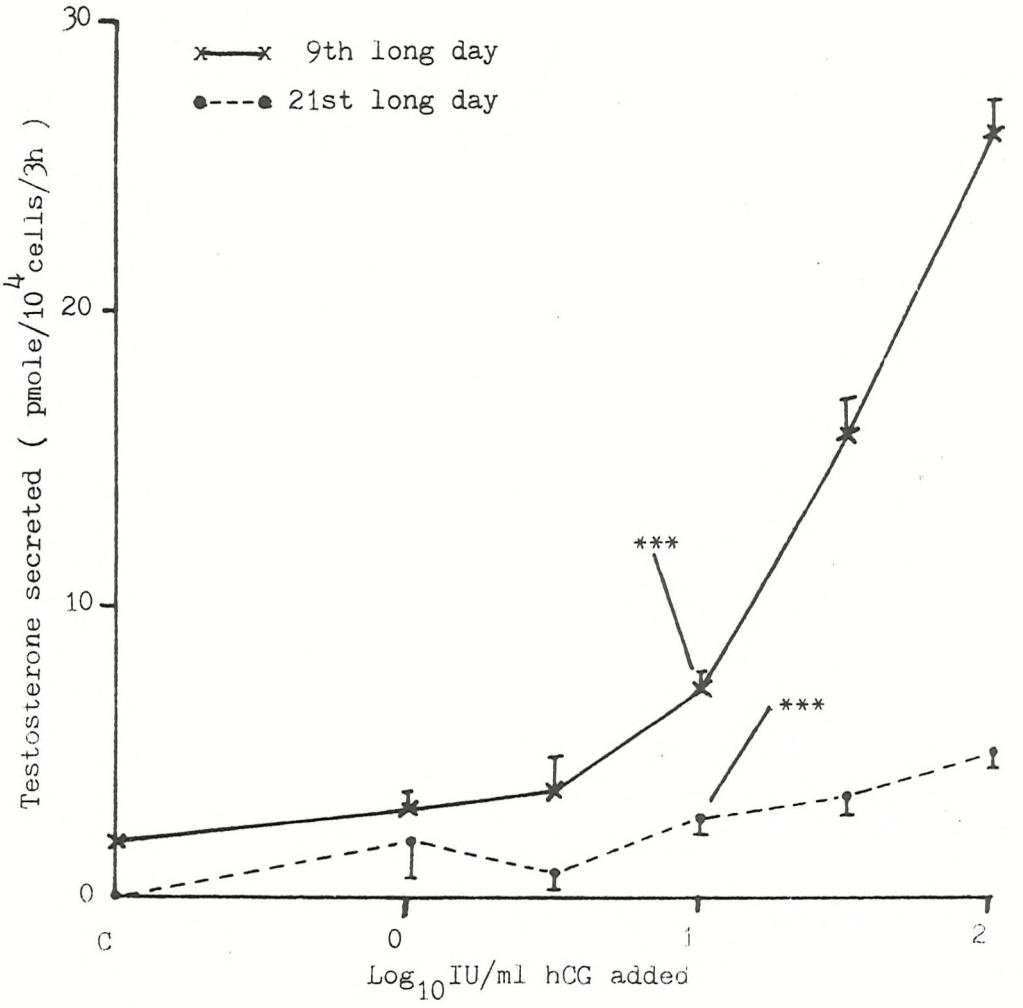

Figure 6.3

Testosterone Secreted by Leydig Cell Incubates with Increasing Doses of hCG on the 9th Long Day (x——x) or on the 21st Long day (o----o)

Isolated Leydig cells were prepared, and testosterone measured as described for Figure 6.1, but the quail used were raised from hatching on d70TLD. C indicates the incubation with no added hCG. Results are expressed as mean ± S.E.M. for 5 observations. The point at which the response was significantly greater than with no added hCG is shown: ***$p<0.001$

had the ability to secrete **oestradiol** in the absence of hCG. The ability of these dispersed testicular cells to secrete oestradiol in the absence of hCG, decreased with increasing exposure to the long photoperiod.

A significant increase ($p<0.01$) in this secreted oestradiol was seen after stimulation with 100 I.U/ml hCG in birds killed on the 2nd long day (from $1.35\pm0.15\times10^{-13}$ mole/10^4 cells/3h, n=5 to $3.88\pm0.59\times10^{-13}$ mole/10^4 cells/3h, n=5: Figure 6.4a). However, this hCG stimulated increase in oestradiol secretion was not seen at any other age.

There was no change in the testicular cell content of oestradiol, after the 3h incubation in the absence of hCG, in cells from the 2nd or 9th long day compared to the day of transferral. A significant decrease was seen by the 21st long day ($p<0.001$). Again hCG (100 I.U/ml) stimulated an increase in cellular content of oestradiol only in quail killed on the 2nd long day (from $3.51\pm0.45\times10^{-13}$ mole/10^4 cells/3h, n=5 to $4.94\pm0.68\times10^{-13}$ mole/10^4 cells/3h, n=5: Figure 6.4b).

As the birds matured, a transient increase in the cellular content of oestradiol in the absence of hCG was seen in cells isolated from birds killed on their 37th or 43rd long day. hCG (100 I.U/ml) stimulated the secretion of oestradiol from these cells, but there was no increase in the cellular content of oestradiol. By the 70th long day levels of both secreted oestradiol and cellular content of oestradiol were low.

There was no change in the level of **testosterone** secreted from these dispersed testicular cells in the absence of hCG with age (Figure 6.5a). Cellular content of testosterone showed the highest levels on the 9th long day (at 2.84 ± 0.29 pmole/10^4 cells/3h, n=5) and lowest levels on the 21st and 37th days (0.58 ± 0.24 and 0.68 ± 0.13 pmole/10^4

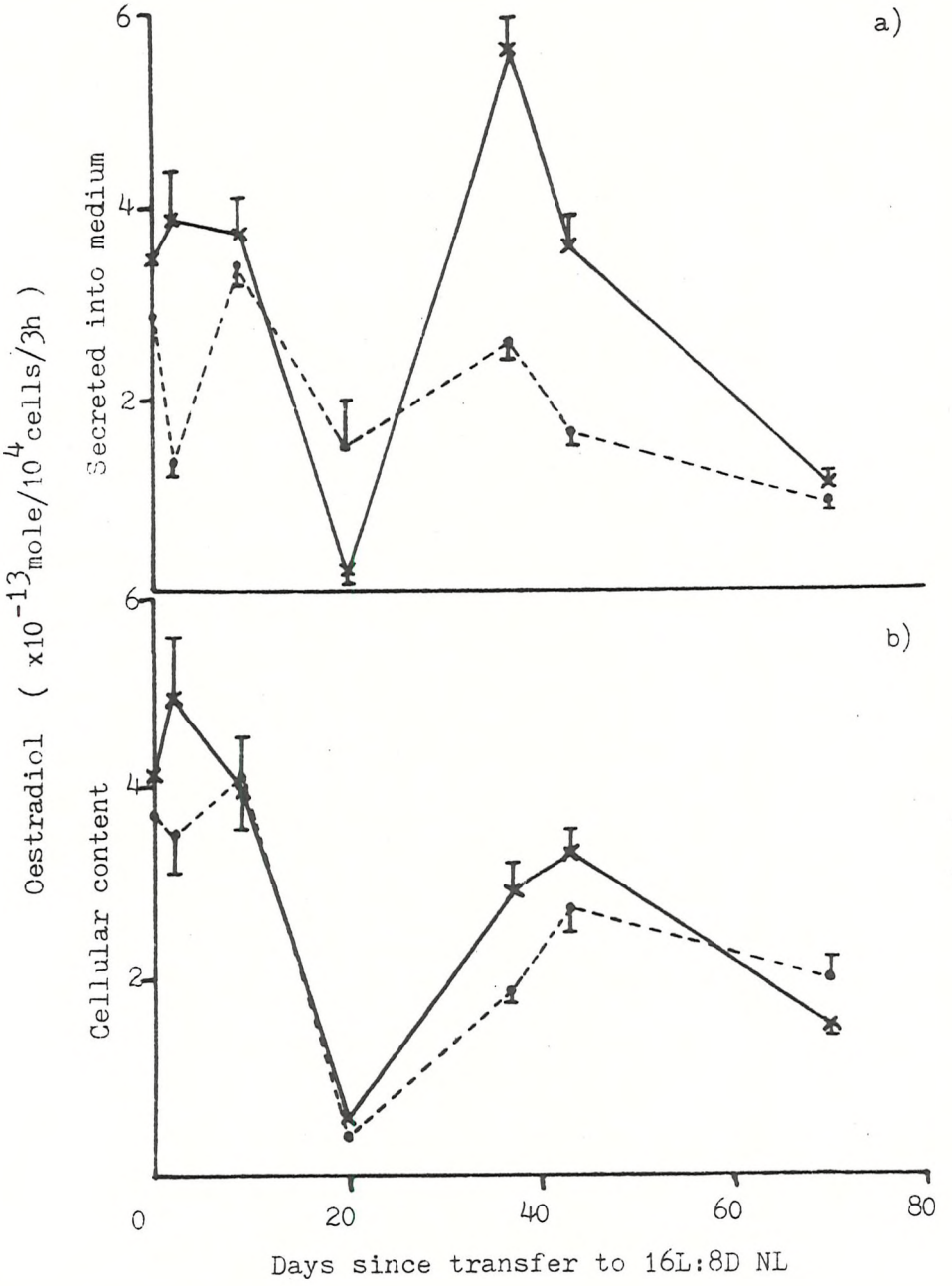

Figure 6.4

Effect of Increasing Exposure to Long Photoperiods (16L:8D NL) on:

a) the accumulation of oestradiol in the medium

b) the cellular concentration of oestradiol, in the presence (x——x) or absence (o----o) of 100 I.U/ml hCG, after a 3h incubation of dispersed whole testicular cells.

Dispersed testicular cells were obtained from quail, raised from hatching on d70TLD, with increasing age. Cells were dispersed using collagenase and incubated in Krebs-bicarbonate Buffer pH7.4+0.2% glucose. Oestradiol secreted into the medium was measured by RIA. Cellular content of oestradiol was determined, after ether extraction of the cells, also by RIA. Results are expressed as mean ± S.E.M for 5 observations.

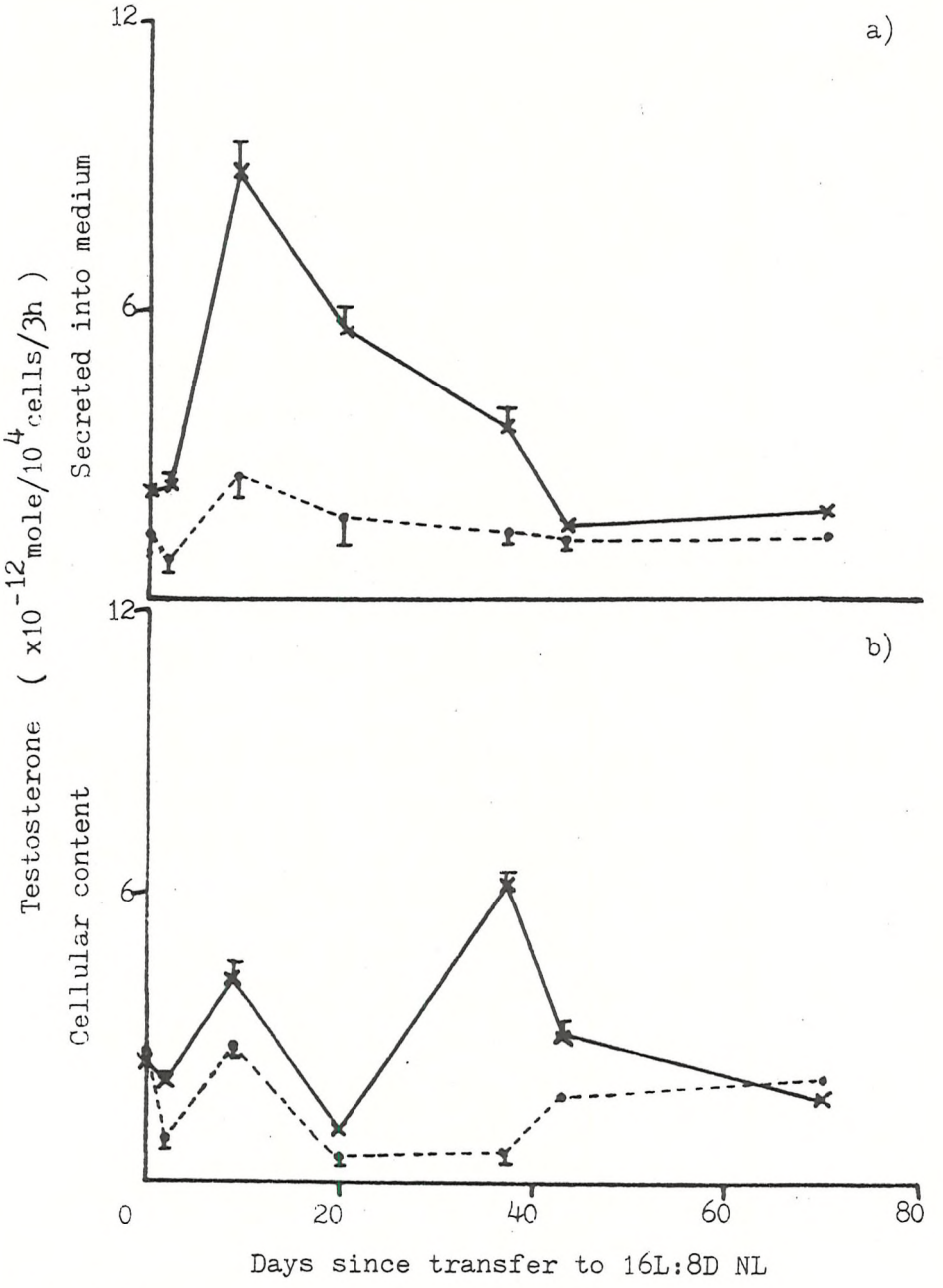

Figure 6.5

Effect of Increasing Exposure to Long Photoperiods (16L:8D NL) on:-

a) the accumulation of testosterone in the medium,

b) the cellular concentration of testosterone, in the presence (x——x) or absence (o----o) of 100 I.U/ml hCG, after a 3h incubation of dispersed testicular cells.

Dispersed testicular cells were obtained as described for Figure 6.4. Testosterone in the medium and cells were measured by RIA as for oestradiol (Figure 6.4). Results are expressed as mean ± S.E.M. for 5 observations.

cells/3h, n=5 respectively) in the absence of hCG (Figure 6.5b).

A significant increase in testosterone secreted by the testicular cells was seen, after the addition of hCG (100 I.U/ml), by cells isolated from birds killed either on their 9th long day (from 2.58±0.43 pmole/10^4 cells/3h, n=5 to 8.97±0.59 pmole/10^4 cells/3h, n=5 : $p<0.001$) or on the 21st long day (from 1.65±0.69 pmole/10^4 cells/3h, n=5 to 5.65±0.51 pmole/10^4 cells/3h, n=5 : $p<0.01$). The response on the 9th long day (8.97±0.59 pmole/10^4 cells/3h, n=5) was significantly greater than the response seen on the 2nd long day (2.37±0.20 pmole/10^4 cells/3h, n=5) ($p<0.001$).

Maximum stimulation of the cellular content of testosterone by hCG (100 I.U/ml) was seen on the 37th long day (from control 0.68±0.13 pmole/10^4 cells/3h, n=5 to stimulated 6.21±0.25 pmole/10^4 cells/3h, n=5 : $p<0.001$). Small increases were seen on the 2nd, 9th, 21st and 43rd long days. No stimulation was seen on the day of transferral or on the 70th long day.

6.4 Discussion

Semi-purified Leydig cells from quail testis secrete testosterone in response to hCG. A similar response was observed with isolated testicular cells which also secreted testosterone but in response to LH (Maung and Follett, 1977). The isolated testicular cells used by Maung and Follett contained approximately 32% cells which showed positive staining for Δ5-3β-hydroxysteroid dehydrogenase. These cells responded to both ovine and chicken LH with an increase in testosterone synthesis and release.

Isolated seminiferous tubule cells showed no such stimulation with either the ovine or chicken LH (Maung and

Follett, 1977). All the cells were isolated from testes of mature Japanese quail and no study was undertaken on the effect that the age of the bird might have on the response of isolated testicular cells.

The results reported in this thesis have shown that isolated Leydig cells show a changing responsiveness to hCG with age. hCG, has in mammals, LH-like actions and has been used as an indication of the LH-responsiveness of various tissues. The present study shows that avian testicular cells can respond to hCG **in vitro** and while an even greater response might have been obtained using avian LH or pituitary extract, the testosterone response obtained after hCG administration was measurable in our RIA system. Care must be taken in extrapolating the results obtained here with those that avian LH might induce **in vivo** or **in vitro** but they do show that the sensitivity of the avian testes to gonadotrophic stimulation changes during maturation.

In birds raised on the 16L:8D NL from hatching, Leydig cells from the immature 24 day old birds have the ability to secrete testosterone in response to low doses of hCG. With increasing age, a higher dose of hCG is required to stimulate testosterone production, although once stimulated, Leydig cells isolated at 29d and 35d secrete larger amounts of testosterone. A similar change in responsiveness is also found in the rabbit (Berger et al, 1976), and in the rat (Odell et al, 1974) where the increase in plasma testosterone in response to LH/hCG is less in the immature animal than in the adult. This shows that there is either an increase in the testicular sensitivity to LH/hCG during sexual maturation in these animals, or an increase in Leydig cell numbers - or a combination of these two.

The period of maximum secretion of testosterone by the isolated quail Leydig cells corresponds to the age when

plasma testosterone levels start to rise in each group and to when the testes start to increase rapidly in weight (see Chapter 3.3, Figure 3.2). Leydig cells isolated from the second group of birds (d70TLD) showed the greatest response on the 9th long day, while the ability to secrete increased testosterone in response to hCG was still present on the 21st long day, it was somewhat reduced. This was also seen in the incubations of dispersed testicular cells (containing both Leydig and tubule cells) where, although total testosterone secretion was less (in terms of the number of cells), cells could respond by the 2nd long day, although the maximal response was obtained on the 9th long day.

This correlates with the **in vivo** work of Maung and Follett (1978). They found that not only did the basal level of testosterone increase around the 6th and 7th long day, but that there was a significant increase in the magnitude of the increase in plasma testosterone levels caused by an injection of LH. Nicholls and Graham (1972) showed that by the 10th long day histologically 'mature' Leydig cells are present within quail testes. Since Leydig cells isolated at this age contained all the necessary organelles of a steroid secreting cell: a spherical nucleus, numerous mitochondria, large quantities of tubular smooth membrane, little granular endoplasmic reticulum and variable numbers of lysosomes and lipid droplets, when examined under the electron microscope.

It would appear, therefore, that in the quail, the increasing levels of testosterone seen during maturation result not only from increasing levels of plasma LH, but from an increase in the ability of the Leydig cells to secrete testosterone in response to LH.

Quail testes, under natural daylengths, undergo regression as the daylength shortens. Regression starts when the photoperiod drops below 17L:7D; a photoperiod which is

normally stimulatory to testicular growth (Follett and Maung, 1978), and regression is complete by a photoperiod of 14L:10D. Plasma testosterone and plasma LH levels start to decrease as gonadal regression occurs. The experiments reported in this thesis have shown that a decrease in the responsiveness of the testicular incubates to hCG occurs with increasing age, after sexual maturity has been reached; i.e after prolonged exposure to LH **in vivo**, a decreased testosterone response to hCG **in vitro** is seen.

Recently, Katikineni, Davies, Huhtaniemi and Catt (1980) have shown that LH/hCG-receptor complexes in rats become increasingly resistant to dissociation with time and that testicular cells once stimulated by LH or hCG show a decrease in binding of additional hormone. This is due not only to a lack of unoccupied receptors, but also to an actual loss of receptors (review: Catt, Harwood, Aguilera and Dufau, 1979; Sharpe, 1976; Hsueh, Dufau and Catt, 1976; Tsuruhara, Dufau, Cigorraga and Catt, 1977).

The decrease in responsiveness of the testicular incubates seen in these experiments, could have occurred as a result of a similar form of 'down regulation' by LH. The prolonged exposure to LH may have caused a loss of LH/hCG receptors. The loss of responsiveness to hCG, in the older birds, may contribute to the decreasing plasma testosterone levels seen in quail kept under natural daylengths as daylength shortens, which together with the decreasing photoperiod led to testicular regression.

The present results also indicated that hCG could stimulate the production of oestradiol from dispersed testicular cells **in vitro**, while semi-purified Leydig cells did not produce or secrete any oestradiol. This suggests that the source of testicular oestradiol in the quail, is in the seminiferous tubules, probably from the Sertoli cells. This is in agreement with work performed in mammals on

dispersed testicular cells, where the Sertoli cell has been shown to be the main source of oestradiol (De Jong, Hey and van der Molen, 1974; Dorrington, Fritz and Armstong, 1978; Rommerts, Kruger-Sewnarain, van Woekom-Blik, Grootegoed and van der Molen, 1978). However, the mammalian Leydig cell has also been shown to be capable of producing oestradiol, (Canick, Makris, Gunsalis and Ryan, 1979; van der Molen, Brinkmann, De Jong and Rommerts, 1980). This was not found to be so in avian incubations since no secretion of oestradiol from the semi-purified Leydig cells was found.

Leydig cell number increased as well as the thickness of the tubule wall (shown in Figure 3.4) with increasing exposure to long photoperiods. Therefore, the percentage of Sertoli cells within the dispersed whole testicular incubates, is likely to decrease with increasing age. The reduction in basal oestradiol production (cellular content and secretion) with age, from the dispersed whole testicular cells, may therefore have resulted from the decreasing percentage of Sertoli cells within the incubates, rather than in a decrease in Sertoli cell activity.

The oestradiol response of the dispersed whole testicular incubates to hCG is also dependent on the age of the quail used. Testicular cells isolated on the 2nd long day are stimulated by hCG to secrete oestradiol. This response is no longer apparent by the 9th long day. The ability of these testicular cells to respond to hCG on the 2nd long day, producing oestradiol, suggests that the transient rise in plasma oestradiol levels seen on the 2nd long day is of testicular origin. It is, therefore, possible that LH, which has been shown to rise within 20 hours of photostimulation (Follett, Davies and Gledhill, 1977), stimulates the testes to secrete oestradiol on the 2nd long day.

This response, **in vitro**, then decreases by the 9th long day correlating with decreasing plasma oestradiol levels in

these birds.

Oestradiol secretion from the testicular incubates decreases at the same time as testosterone secretion increases. This drop in oestradiol secretion may have resulted from a lack of sufficient available substrate (testosterone) for its production. Most of the testosterone synthesised is secreted into the medium **in vitro** and therefore would probably be secreted into the blood stream **in vivo**. It also appears that most oestradiol synthesised in the older birds remains within the cells and is not secreted into the medium **in vitro**.

Therefore, with increasing exposure to long photoperiods testosterone secretion from the testes increases, causing the increased plasma testosterone levels seen. At the same time, any oestradiol synthesised in the testes is not secreted from the testes, but remains within the cells and so plasma oestradiol levels within mature male quail are low.

Leydig cells isolated from quail show an increase in sensitivity to LH/hCG during sexual maturation, thus contributing to increased plasma testosterone levels. Once mature, the Leydig cells show a gradual decrease in sensitivity to LH/hCG. This is possibly due to a change in LH-receptor number and LH-receptor sensitivity due to prolonged exposure to LH. The transient rise in plasma oestradiol levels, which have been observed in photostimulated birds, is secreted by the testes, possibly by the Sertoli cells, in response to LH.

CHAPTER 7 - EFFECT OF *in vivo* ADMINISTRATION OF FOUR DIFFERENT OESTROGENS ON OVIDUCT WEIGHT, PLASMA CALCIUM AND ON VITAMIN D_3 METABOLIC ENZYMES IN IMMATURE FEMALE JAPANESE QUAIL

7.1 Introduction

Oestradiol-17β injected into Japanese Quail has been shown to cause an increase in the hepatic production of 25-hydroxycholecalciferol (25HCC) ***in vitro***, probably as a result of an increase in the activity of the cholecalciferol-25-hydroxylase (25-hydroxylase) converting cholecalciferol to 25HCC (Nicholson et al, 1979). A similar increase in the renal production of 1,25-dihydroxycholecalciferol (1,25DHCC) ***in vitro***, occurs after an injection of oestradiol benzoate, (Sedrani, 1979). This probably resulted from an increase in the activity of the 25-hydroxycholecalciferol-1α-hydroxylase enzyme (1α-hydroxylase). These effects have already been reviewed in this thesis (Chapter 1.2.5). The exact mechanism by which oestrogen acts has still to be elucidated, but recent developments are reported in the discussion of this section.

The high plasma oestradiol-17β levels found in sexually mature female Japanese quail compared with those seen in mature males or immature females, could explain the higher activity of the 1α-hydroxylase enzyme found in these female birds. However, the activities of the 1α-hydroxylase and 25-hydroxycholecalciferol-24R-hydroxylase (24-hydroxylase) enzymes start to change prior to the onset of sexual maturity. This change occurs at a time when plasma oestradiol-17β levels, although higher in female quail than in males, are not significantly different between the sexes.

Other oestrogens have also been found to be present in the plasma of avian species. Oestrone has been found in

the plasma of hens (O'Grady and Heald, 1965; Peterson and Common, 1972) as well as in the plasma of female Japanese quail (Doi et al, 1980; Onagbessan and Peddie, unpublished results). Chan and Common (1974) found radioactive oestradiol-17α in hens' plasma as well as oestradiol-17β and oestrone after an injection of ^{14}C-labelled oestrone. Oestriol has also been detected in hens' plasma (O'Grady and Heald, 1965).

Therefore, the changes in the activity of the renal hydroxylases during sexual maturation of Japanese quail may be influenced by changes in plasma levels of these other oestrogens, as well as changes in plasma oestradiol-17β levels. The presence of other oestrogens in quail plasma may contribute to a higher total plasma oestrogen content prior to the onset of egg-laying in this species. Total plasma oestrogen has been shown to increase as early as 3 weeks prior to egg-laying in the hen (Peterson and Webster, 1974; Senior, 1974).

We therefore decided to look at the effect which injections of four different oestrogens had on the activities of the renal 1α-hydroxylase and 24-hydroxylase enzymes. The oestrogens used were oestrone (E_1), oestradiol-17β (E_2-17β), oestradiol-17α (E_2-17α) and oestriol (E_3). Their comparable effects on oviduct weight and on total plasma calcium levels were also recorded. Oviduct weight was used as a criterion of oestrogen action since oestrogens have been shown previously to stimulate oviduct growth (as reported in Chapter 1.1.5).

This experiment was performed jointly with Mr. P.J. Williams who measured the relative activities of the 1α-hydroxylase and 24-hydroxylase enzymes in the same birds. (The results of which will form part of his PhD thesis).

7.2 Methods

31 female quail, 43 to 50 days old, raised on short photoperiods (8L:16D) were divided into five groups. These birds were somatically mature but sexually immature. Body weights were recorded. Quail were injected intramuscularly (i.m.) in their 2nd or 3rd hour of light with either oestrone, oestradiol-17β, oestradiol-17α, or oestriol with ethyl oleate as vehicle, or with the vehicle alone (see Materials and Methods, Chapter 2.3). Blood was collected from the wing vein (see Materials and Methods, Chapter 2.2) on days 1, 2, and 3. All quail were killed by decapitation on day 4, 24h after the last injection, and trunk blood was collected. Ovaries and oviducts were removed and their weights recorded. Some kidney tissue was also removed for the estimation of 1α-hydroxylase and 24-hydroxylase enzyme activities. Total plasma calcium was determined in all samples (see Materials and Methods, Chapter 2.10) and plasma oestradiol-17β and plasma oestrone in all samples were measured by specific RIA (see Materials and Methods, Chapter 2.12).

7.3 Results

While body and ovary weights remained unchanged after oestrogen treatment, oviduct weights increased with the administration of all four oestrogens (Table 7.1). The greatest response was seen in quail injected with oestradiol-17_{β} or oestrone where the weights of the oviducts increased significantly ($p<0.01$ and $p<0.001$ respectively). Oestradiol-17α and oestriol both caused smaller increases in oviduct weights ($p<0.01$ and $p<0.05$ respectively) than the other two oestrogens.

Total plasma calcium levels 24h after a single oestrogen injection were only significantly elevated from control

Table 7.1

Effect of Intramuscular (i.m.) Injections of 4 Different Oestrogens on Ovarian and Oviduct Weights

1.8μmole/kg BW of either
1) Vehicle alone or
2) Oestradiol-17α (E_2-17α) or
3) Oestradiol-17β (E_2-17β) or
4) Oestrone (E_1) or
5) Oestriol (E_3)

Injections were carried out daily for 3 days, using sexually immature female quail raised on 8L:16D. Ovaries and oviducts were removed and weighed 24h after the last injection. Body weights recorded at the beginning of the experiment are also shown. Results are expressed as mean ± S.E.M. with the number in each group given in parenthesis. Significant changes from control values are shown: ***p<0.001, **p<0.01 and +p<0.05.

GROUP	BODY WT (g)	OVARY WT (mg)	OVIDUCT WT (mg)
1. CONTROL (6)	100.7 ±2.4	35.3 ±3.4	17.3 ± 1.2
2. E_2 - 17α (6)	100.3 ±2.9	35.8 ±3.0	64.9 ±10.9 **
3. E_2 - 17β (6)	94.7 ±3.0	35.5 ±2.5	178.6 ±40.2 **
4. E_1 (7)	97.4 ±1.8	39.7 ±2.7	135.8 ±10.3 ***
5. E_3 (6)	99.7 ±3.2	37.0 ±3.2	42.5 ± 9.2 +

levels by oestradiol-17β ($p<0.01$), although injection of oestrone also caused a small, but not significant, increase in plasma calcium (Table 7.2). Plasma calcium continued to rise following the two subsequent injections in quail treated with oestradiol-17β and oestrone, reaching 40.3± 7.7mg% ($n=6$:$p<0.01$) and 26.9±3.6mg% ($n=7$:$p<0.01$) respectively, compared to control values of 11.2±0.5mg% ($n=6$).

Plasma calcium levels in quail treated with oestradiol-17α and oestriol remained low, showing only a small but not significant, increase to 12.9±0.6mg% ($n=6$) and 12.4±0.4mg% ($n=6$) respectively, compared with birds injected with vehicle alone on day 4. It was not possible to measure the changes in ionic plasma calcium.

Significant increases in the activity of the renal 1α-hydroxylase enzyme was seen after injections of oestradiol-17β or oestrone from 12.3±2.2pmole/g/h ($n=6$) to 36.9±7.7pmole/g/h ($n=6$:$p<0.02$) and 25.7±4.4pmole/g/h ($n=7$:-$p<0.05$) respectively (Table 7.2). Injections of oestradiol-17α or oestriol caused small, but not significant, increases in the activity of the 1α-hydroxylase enzyme. The mean relative activities of the 1α-hydroxylase enzyme induced by oestradiol-17α or oestriol were not significiantly lower than those induced by oestradiol-17β or oestrone. None of the four oestrogens had any significant effect on modifying the activity of the 24-hydroxylase enzyme although the mean level of the 24-hydroxylase enzyme activity was increased in the groups treated with oestradiol-17β or oestrone.

In order to determine that plasma levels of oestradiol-17β and oestrone had not been increased above physiological levels by the repeated injections, plasma levels of these two steroids were determined by RIA. The results are shown in Table 7.3. Each blood sample was taken prior to the next oestrogen injection and the last sample taken 24h after

Table 7.2

Effect of i.m. Injections of 4 Different Oestrogens on Total Plasma Calcium and on the Activities of the 1α- and 24-hydroxylase Enzymes

1.8μmole/kg BW of either
1) Vehicle alone or
2) Oestradiol-17α (E_2-17α) or
3) Oestradiol-17β (E_2-17β) or
4) Oestrone (E_1) or
5) Oestriol (E_3)

Injections were carried out daily for 3 days, using sexually immature female quail. Blood was collected daily, from the wing vein. Each sample (Day 2-4) was taken 24h after the previous injection; Day 1 is the control plasma level prior to any injections. Plasma was stored at -25°C until assayed. Plasma calcium was measured as described in Chapter 2.10. 1α- and 24-hydroxylase enzyme activities were measured in kidney homogenates on Day 4, by Mr. P.J. Williams. Results are expressed as mean ± S.E.M. with the number in each group given in parenthesis. Significant changes from control values are shown: $^{**}p<0.01$, $^{*}p<0.02$ and $^{+}p<0.05$.

GROUP	PLASMA CALCIUM mg%				1α-OHlase (pmole/g/h)	24-OHlase (pmole/g/h)
	DAY 1	DAY 2	DAY 3	DAY 4		
1. CONTROL (6)	11.4 ± 0.2	11.6 ± 0.3	11.3 ± 0.7	11.2 ± 0.5	12.3 ± 2.2	145.1 ± 33.0
2. E_2- 17α (6)	11.0 ± 0.8	11.9 ± 0.6	12.9 ± 0.4	12.9 ± 0.6	23.5 ± 10.3	93.6 ± 15.3
3. E_2- 17β (6)	11.6 ± 0.6	15.1 ± 0.7**	25.4 ± 4.2**	40.3 ± 7.7**	36.9 ± 7.7*	246.2 ± 90.3
4. E_1 (7)	11.6 ± 0.6	13.4 ± 1.0	17.4 ± 1.8*	26.9 ± 3.6**	25.7 ± 4.4+	162.9 ± 24.5
5. E_3 (6)	11.0 ± 0.3	12.0 ± 0.2	12.7 ± 1.0	12.4 ± 0.4	24.9 ± 8.4	144.1 ± 29.2

1α-OHlase = Renal 25HCC-1α-hydroxylase
24 -OHlase = Renal 25HCC-24R-hydroxylase

Table 7.2

Table 7.3

Plasma Oestradiol-17β and Oestrone Levels in Sexually Immature Female Quail before (Day 1) and after (Day 2-4) i.m injections of 4 Different Oestrogens

1.8μmole/kg BW of either
1) Vehicle alone or
2) Oestradiol-17α (E_2-17α) or
3) Oestradiol-17β (E_2-17β) or
4) Oestrone (E_1) or
5) Oestriol (E_3)

Injections were carried out, and blood samples collected, as described in the legend for Figure 7.2. Steroid levels were measured using specific RIAs (see Chapter 2.12). Results are expressed as mean ± S.E.M. and, where applicable, the number in each group is given in parenthesis. Significant changes from control values are shown: *$p<0.05$.

GROUP	OESTRADIOL-17β x 10^{-13} mole/ml plasma				OESTRONE x 10^{-12} mole/ml plasma			
	DAY 1	DAY 2	DAY 3	DAY 4	DAY 1	DAY 2	DAY 3	DAY 4
1. CONTROL	(3) 7.66 ± 0.77	(4) 5.41 ± 0.41	(3) 5.81 ± 0.74	(6) 6.00 ± 0.53	(4) 3.26 ± 0.48	(5) 2.92 ± 0.54	(3) 3.70 ± 0.30	(6) 3.28 ± 0.66
2. E_2-17α	(4) 6.43 ± 2.56	(5) 5.34 ± 0.90	(5) 4.69 ± 0.65	(6) 5.57 ± 1.87	(4) 4.33 ± 1.63	(6) 3.51 ± 0.60	(5) 4.58 ± 1.11	* (5) 1.51 ± 0.15
3. E_2- 17β	(4) 5.16 ± 0.53	(5) 7.88 ± 1.01	(5) 9.20 ± 2.08	* (6) 10.49 ± 1.41	(5) 2.94 ± 0.80	(5) 2.72 ± 0.99	(4) 4.52 ± 0.85	(5) 3.57 ± 0.34
4. E_1	(4) 7.81 ± 1.12	(4) 7.54 ± 1.21	(4) 4.85 ± 1.34	(7) 5.43 ± 0.65	(4) 4.85 ± 1.79	(4) 2.62 ± 0.81	(5) 3.81 ± 0.95	(7) 2.38 ± 0.66
5. E_3	(2) 4.31	(5) 6.00 ± 1.31	(2) 8.04	(6) 5.32 ± 0.89	(2) 5.51	(5) 2.69 ± 0.16	(3) 3.54 ± 0.76	(6) 2.47 ± 0.51

Table 7.3

the last injection. Oestradiol-17β was found to be slightly elevated only in the birds injected with oestradiol-17β. There were no other changes in oestradiol-17β levels in any of the other groups. Oestrone levels were unaltered by the administration of any of the four oestrogens until day 4 when oestrone levels fell in the birds injected with oestradiol-17α. However, it must be noted that these plasma levels only reflect the concentrations in the birds 24h after the last injection. Short term fluctuations in the plasma oestrogen levels were not measured at this stage.

7.4 Discussion

All four oestrogens showed the ability to induce an increase in oviduct weight when injected into immature Japanese Quail. As early as 1940 it was demonstrated that immature female starlings injected with oestradiol, oestrone or oestriol, showed an increase in oviduct weight (Witschi and Fugo, 1940). Since then oestrogen receptors have been isolated from chick oviduct (Harrison and Toft, 1975; Smith, Clarke, Zalta and Taylor, 1979) and from hen oviduct (Kon, Webster and Spelsberg, 1980). These receptors were shown to have a high affinity and low capacity for oestradiol-17β. Competitive binding studies showed that only oestrogenic compounds displaced the bound ^{3}H-oestradiol-17β; with oestradiol-17β and diethylstilbestrol most effective, followed by oestradiol-17α and then oestriol and oestrone. Progesterone, cortisol, testosterone and DHT showed no displacement of the bound ^{3}H-oestradiol-17β.

In these studies, oestradiol-17β and oestrone were not only the most effective of the oestogens tested in increasing oviduct weight, but were also the two oestrogens which significantly elevated plasma calcium levels. These birds also showed a signficantly higher production of 1,25DHCC

than the control group.

Oestrogens are important for egg-yolk production and endogenous and exogenous oestrogens stimulate the liver to produce egg-yolk phospholipoproteins (Griminger, 1976). This phospholipoprotein has a high calcium binding capacity. The increase in serum calcium in oestrogen-treated birds is principally due to an increase in the nonultrafilterable or protein-bound calcium fraction (Urist, Schjeide and McLean, 1958; Urist, Deutsch, Pomerantz and McLean, 1960), rather than in the free ionic calcium. This is in contrast with the effect of parathyroid hormone, which increases only the ultrafilterable (or ionised) calcium (Urist et al, 1960) by causing, in the presence of 1,25DHCC, resorption of medullary bone. 1,25DHCC stimulates the intestinal transport of calcium (Myrtle and Norman, 1971; Omdahl, Holick, Suda, Tanaka and DeLuca, 1971), as well as the reabsorption of calcium from the kidney (Puschett, Moranz and Kurnick, 1972) causing increased plasma levels of ionised calcium. Ionised calcium is important for bone and egg-shell formation.

Oestrogens, in particular oestradiol, have been shown to stimulate the production of 1,25DHCC **in vitro**, probably by increasing the activity of the 1α-hydroxylase enzyme (Baksi and Kenny, 1977; Sedrani, 1979). These workers, on injecting oestradiol benzoate **in vivo**, showed the stimulation of the 1α-hydroxylase **in vitro** in the absence of testosterone, while other workers claimed the oestrogen stimulation of 1α-hydroxylase only occurs in the presence of testosterone (Tanaka, Castillo and DeLuca, 1976; Castillo, Tanaka, DeLuca and Sunde, 1977). It now seems that oestrogens alone can stimulate the synthesis of 1,25DHCC as shown in the results presented here and by those of Sedrani (1979). This response can be potentiated by the addition of testosterone (Pike et al, 1978) although Sedrani failed to find this potentiation.

Nicholson (1977) showed that administration of oestradiol-17β, oestrone, oestriol, or the synthetic oestrogen, diethylstilbestrol **in vivo**, all increased the production of 25HCC **in vitro**. Oestradiol-17β stimulated the production of 25HCC to a slightly greater extent than the other oestrogens tested. It seems clear that other oestrogens in addition to oestradiol-17β could be involved in the regulation of Vitamin D_3 metabolism.

Oestrone seems equipotent to oestradiol-17β in stimulating the 1α-hydroxylase activity, although the oestrone-stimulated increase in total plasma calcium concentration takes longer than that for oestradiol-17β. The possibility that the oestrogens used in these experiments are metabolised prior to initiating their effect cannot be ruled out. Injecting radioactive oestrone into hens results in radioactive 17α- and 17β-oestradiol being found in the plasma (Chan and Common, 1974). Therefore, oestrone may be having its effect after conversion to oestradiol-17β. However, plasma levels of oestradiol-17β were only raised in the group injected with oestradiol-17β. These plasma samples were taken 24h after the oestrogen injections and further time course studies are now being carried out within this department to measure the levels of oestrogens throughout the 24h period. Any injected oestrone which has been converted to oestradiol-17β will most likely have been metabolised within 24h. Therefore high levels of oestrogens would not be expected within the plasma samples taken at 24h, if physiological doses of the oestrogens had been injected. Further studies are needed to determine what, if any, interconversions occur between the oestrogens and the time course of these interconversions. The relevance these interconversions may have on regulation of Vitamin D_3 metabolism can then be determined.

The relative affinity of oestrogen receptors for the

different oestrogens could also influence the response seen. As already mentioned in this discussion, using competitive binding studies, oestrogen receptors isolated from the hen and chick oviduct, show different specificities for the different oestrogens. The oestrogen-stimulated increase in 1,25DHCC production and thus in plasma calcium may also involve oestrogen receptors which have different specificities for the oestrogens used.

The mechanism of action of these oestrogens still remains unclear. Oestradiol-17β shows no direct action on kidney cells, since addition of oestradiol-17β failed to stimulate the 1α-hydroxylase enzyme in cultured chick kidney cells (Spanos, Barrett, Chong and MacIntyre, 1978). The oestrogen stimulated increase in 1α-hydroxylase activity may therefore be mediated indirectly via other stimuli, such as through parathyroid hormone (PTH) or prolactin. Oestrogen stimulated production of phospholipoproteins from the liver (Urist et al, 1958) results in ionised plasma calcium becoming bound to these phospholipoproteins and thus levels of free calcium decrease. This decrease in ionised plasma calcium could stimulate PTH release, thus elevating PTH levels.

Oestrogens may also stimulate the parathyroid gland directly to cause PTH release. A direct action of PTH on cultured kidney cells, in increasing intracellular c'AMP levels, has been shown (Henry, 1979). PTH has also been shown to cause increased production of 1,25DHCC by kidney cells after a 3h incubation (Bar, Hurwitz and Maoz, 1980). Recently PTH has been shown to have a direct action in stimulating increased 1,25DHCC production by chick kidney slices (Rost, Bikle and Kaplan, 1981). This PTH stimulation can be potentiated by the phosphodiesterase inhibitor, theophylline, and mimicked by c'AMP. Stimulation of 1,25DHCC production occurs at a lower concentration of PTH than that required for optimal stimulation of adenyl

cyclase, as is the case for many hormones whose actions are mediated through c'AMP.

It is possible, therefore, that the injected oestrogens elevate plasma PTH levels. The PTH will then directly stimulate 1,25DHCC production. Total plasma calcium levels would then be raised through the actions of 1,25DHCC by increasing intestinal absorption of calcium, renal reabsorption of calcium and, together with PTH, the resorption of medullary bone. These actions would ensure that ionised plasma calcium levels are maintained while the bound calcium levels increased. Oestrogen injections, as in this experiment, do increase production of 1,25DHCC as well as elevating total plasma calcium levels.

Another possible mediator of oestrogen action may be via the pituitary hormone, prolactin. Pituitary levels of prolactin, as well as plasma prolactin levels, are elevated 24h after an injection of oestradiol benzoate into ovariectomised rats (Chen and Meites, 1970; Ajika, Krulich, Fawcett and McCann, 1972; Caligaris, Astrada and Taleisnik, 1974). While oestrogens have been shown to stimulate prolactin production in mammals, a similar role in birds has yet to be found. If oestrogens do stimulate prolactin release in birds then this could result in an increase in 1,25DHCC levels and hence increased levels of plasma calcium, since circulating levels of 1,25DHCC are elevated after 5 days of prolactin treatment (100 g/day) in male chickens (Spanos et al, 1976).

Prolactin has been found to stimulate the **in vitro** renal production of 1,25DHCC and increase plasma calcium levels of Japanese Quail after 5 days of prolactin treatment (0.5mg/kg daily) (Baksi, Kenny, Galli-Gallardo and Pang, 1978). Recently, prolactin has been shown to have a direct effect on stimulating 1,25DHCC production by chick renal tubules and by chick kidney slices (Bikle, Spencer, Burke

and Rost, 1980). For 1,25DHCC production from the renal tubules a 3h-preincubation period was required for prolactin to exert an effect, perhaps due to a loss of prolactin receptors after the enzymatic preparation of the tubules. The prolactin effect on chick kidney slices was immediate. Since oestrogens can stimulate prolactin secretion, and prolactin has a direct action on the kidney to stimulate 1,25DHCC production, oestrogens could exert part of their effects on Vitamin D_3 metabolism via prolactin.

Further studies are still required to elucidate the exact mechanism of action of oestrogens and the relative contribution that the different oestrogens found within avian plasma have in the regulation of Vitamin D_3 and calcium metabolism.

CHAPTER 8 - SUMMARY AND CONCLUSIONS

The results presented in this thesis have increased our knowledge in several fields of avian endocrinology, but many gaps still remain to be filled in the future. Since each chapter presented in this thesis contains its own discussion, this section will only summarise the conclusions reached and suggest areas of interest for future study. With the use of specific RIAs for the gonadal steroid hormones; progesterone, testosterone and oestradiol-17β, detailed studies on the changes in their plasma levels during maturation in Japanese Quail were studied.

The steroid profiles were found to be dependent on the lighting regime on which the quail were raised, with lower levels found in the birds raised on 8L:16D which remained sexually immature. The plasma steroid levels in individual females were related to the time of onset of lay: it was found that progesterone and oestradiol levels rose just prior to the onset of lay. In the males testosterone levels increased at the time when the testes were developing rapidly. Work in this field could be extended by a further study of individually bled quail which have been raised under different lighting systems, enabling further studies on diurnal events, as well as on maturational changes to be carried out. It would also be of interest to relate the prepubertal changes in steroid levels to the subsequent egg-laying capacity in individual birds. If such a relationship exists then selection for birds with a high frequency of egg-laying might be possible prior to puberty. Some of this work is already being carried out within this department.

Recently studies within this department on plasma oestrone and oestradiol levels in female quail have shown that oestrone levels are always higher than oestradiol

levels (Onagbessan, unpublished results). This is in contrast to the results of Kumagi and Homma (1974) who found that in the laying quail oestradiol was always higher than oestrone in the plasma, despite follicular levels of oestrone being greater than follicular oestradiol levels. They suggested that the high follicular oestrone levels indicated that oestrone could be the main storage form of oestrogens in the avian ovary. It would be of interest to study the secretion patterns of oestrogens from the yolk-filled follicles and immature ovary, by incubating them **in vitro**, and measuring the oestrogens secreted into the medium in the presence and absence of gonadotrophins. If more oestrone is found to be secreted into the medium than oestradiol, then the high follicular content of oestrone in follicles from laying birds, may not be converted to oestradiol prior to secretion. This might explain why, in the present experiments, plasma oestrone levels have always been found to be higher than oestradiol levels (Williams, Brain, Peddie and Taylor, 1981).

It was found that both oestrone and oestradiol, if injected into immature female quail in equimolar amounts, had very similar effects on increasing oviduct weight, increasing renal 1α-hydroxylase activity and on increasing plasma calcium levels. If plasma oestrone levels are higher than plasma oestradiol levels, it may be that oestrone is the more important oestrogen in preparing the immature female for the egg-laying state. Furthermore, when both oestrone and oestradiol levels were measured in these oestrogen-injected birds, levels of oestrone were as high or higher than those of oestradiol following an intermuscular injection of oestradiol. The apparent 'interconversion' in the reverse direction was not so marked (Williams et al, 1981).

These observations may be interpreted to mean that secreted oestradiol can be rapidly converted to oestrone,

possibly at some extra-ovarian site such as the liver. In order to substantiate this hypothesis, it would be necessary to inject radioactive oestrogens and to trace the appearance of the oestrogen metabolites in the plasma, using initially, thin layer chromatography to separate the oestrogens, and subsequently recrystallisation of the products with authentic steroid to constant specific activity.

Diurnal changes in plasma progesterone levels were seen in the female quail, with peaks of progesterone found around the time of the expected LH surge, in quail which subsequently ovulated. This progesterone peak was absent in quail which did not ovulate. These results are similar to those seen in chickens, although the relative time within the light/dark cycle is different. Progesterone has yet to be shown to have a positive feedback effect in the Japanese Quail. In the laying hen, injected progesterone will stimulate the secretion of LH, often resulting in premature ovulation (Etches and Cunningham, 1976a). More support for progesterone as the hormone which stimulates the hypothalamus in chickens comes from the studies of Furr and Smith (1975), who were able to block ovulation with an antiserum to progesterone or to testosterone but not with one to oestradiol.

In the hen, progesterone appears to act on the hypothalamus, since the progesterone-stimulated LH surge can be blocked by an injection of an antiserum to LH-RH (Fraser and Sharp, 1978). In the ovariectomised hen, the hypothalamo-hypophyseal system needs to be 'primed' by oestradiol and progesterone for progesterone to stimulate LH release (Wilson and Sharp, 1976b). It remains to be seen by employing similar experiments if a similar system exists in the female quail. In particular, studies on the relationship between LH levels and progesterone levels within the daily cycle: does the timing of the progesterone peak precede that of the ovulatory LH surge? This could be

performed using individually bled quail whose laying record was known. It would also be necessary to establish the time between an endogenous LH surge and oviposition; this could be done by injecting LH-RH, measuring plasma LH levels, and observing the subsequent time of oviposition.

Equally, other steroids may be involved in the regulation of the LH surge. In hens, testosterone, deoxycorticosterone or corticosterone can also stimulate LH release (Etches and Cunningham, 1976a; Wilson and Sharp, 1976a; Croze and Etches, 1980) and whether they also stimulate LH release in quail remains to be seen. Plasma testosterone and oestrone levels have been found to peak 6h before an ovulation in laying quail raised on 14L:10D, i.e. at the time of the LH peak (which occurs 3-6h prior to ovulation) (Gulati, Nakamura and Tanabe, 1981), while in non-ovulating quail this testosterone peak was absent.

Testosterone, therefore, could be involved in regulating the LH surge in quail. A role for testosterone in stimulating LH release could be investigated using an antiserum to testosterone and, if testosterone is required, then addition of the antiserum should prevent or delay the LH surge and thus ovulation. Alternatively, antiandrogens might be employed, as has been done in chickens. Either of these options may still leave some doubt about the exact site of action of testosterone (hypothalamus, pituitary or ovary) and specific receptor sites for testosterone in these tissues have yet to be found. Testosterone-concentrating cells have been located in the hypothalamus of the male fowl using autoradiographic localisation (Barfield, Ronay and Pfaff, 1978) suggesting that this hormone may act 'centrally'. Testosterone and testosterone mediated regulation of ovarian follicular maturation are also likely to be equally important. Testosterone is the necessary precursor for oestrogens in the ovary, and its removal might therefore disrupt normal oestrogen secretion.

The control of the timing of the normal ovulatory cycle in quail and hens remains unclear. Fraps (1955) proposed that an 'open' period in hypothalamic sensitivity existed during an 8h period each day and that progesterone could initiate an LH surge only during these hours, should the steroid be present. However, progesterone has been shown to stimulate LH release even during the 'closed' period (Etches and Cunningham, 1976b).

An alternative hypothesis was propsed by Bastian and Zarrow (1955) which was modified by Williams and Sharp (1978) suggesting that the timing of the preovulatory LH release could be dependent on a diurnal rhythm of basal LH secretion, which would stimulate progesterone secretion, providing a mature follicle were present in the ovary. This triggers an LH surge through the positive feedback actions of progesterone on the hypothalamus. If this hypothesis is correct, administration of LH-RH or LH into quail or hens should stimulate progesterone secretion from an ovary containing a mature follicle. In experiments on isolated, dispersed hen granulosa cells, LH did stimulate progesterone secretion (Huang, Kao and Nalbandov, 1979) and the progesterone content of the granulosa cells was also stimulated after plasma LH had been raised by an injection of LH (Imai and Nalbandov, 1978; Culbert, Hardie, Wells and Gilbert, 1980). Likewise in quail, the secretion of progesterone from follicular cells can be stimulated by the LH-like hormone hCG.

Another possibility is that a daily rhythm in plasma corticosterone in laying hens may regulate the timing of the ovulatory cycle. Maximum levels of corticosterone in hens are found at night (Beuving and Vonder, 1977). This rise in corticosterone may trigger the small diurnal increase in plasma LH, which then stimulates progesterone secretion and the LH surge results. If corticosterone is involved,

maximum plasma levels of corticosterone in quail should be found in their first hours of light. This could be established using a RIA for corticosterone. Recently Wilson and Cunningham (1980) showed that plasma levels of corticosterone in chickens ovulating their first eggs of a sequence, remained low as LH rose at the onset of darkness, and only rose about the time of ovulation (shortly after dawn). This was in contrast to hens ovulating an egg later in the sequence where corticosterone levels did rise during the dark period, but was paralleled by a fall in LH levels. In both cases corticosterone increased rapidly as LH levels fell after the preovulatory LH surge, reaching maximum levels at the time of ovulation and oviposition.

Thus the contribution adrenal steroids may make in regulating the ovulatory cycle remains uncertain. Any measurements of adrenal steroids can only be made reliably in totally unstressed birds: the birds should therefore be killed in order to collect the blood, which would preclude any observation on their subsequent time of ovulation. Ovulation/oviposition in quail are not predictable events as there are no definitive 'clutch-sequence' patterns in their laying. Alternatively an inhibitor of adrenal steroid synthesis e.g. metirapone, might be used to inhibit adrenal function, and its effects on egg-laying investigated.

Any combinations of these models may well exist and further studies in which the light/dark cycles of both chickens and quails are manipulated may help to elucidate the correct model. By manipulating the light/dark cycles in hens, changes in the time of oviposition and ovulation should be paralleled by changes in the timing of any factors involved in controlling ovulation; for instance, the time of the progesterone peak relative to the LH surge. The changes in the time of oviposition and ovulation should also be paralleled by changes in the timing of any diurnal events involved in the regulation of ovulation. All these changes

should result in the time of ovulation and oviposition, relative to the onset of dark (since it is the onset of dark which appears to be the signal in chickens), remaining constant. Any hypothesis which applies to the chicken may also apply to the quail, but the timing of these events should reflect the different timing in ovulation and oviposition between these two species.

By using a histochemical technique, the localisation of two enzymes involved in follicular steroidogenesis were studied. This showed that the largest quail follicles, which secreted predominantly progesterone (Cole and Peddie, 1980), contained most 3β-HSD within the granulosa cells while the medium-sized follicles, which contained most of the oestrogens (Kumagi and Homma, 1974), contained most 17β-HSD within the thecal cells. This demonstration of the use of specific substrates to localise enzymes within different cell layers could be used to determine the types of cells present in dispersed cell incubations.

By using cell suspensions, the changes in response of the follicles during the egg-laying cycle can be studied more easily. Additions to the medium can be made to increase the understanding of the control mechanisms of steroidogenesis. For instance: how does LH exert its effect? Is it through c'AMP? If so addition of dibutyryl c'AMP to the medium should stimulate a similar response to that seen by LH and an inhibitor of phosphodiesterase, such as theophylline, should potentiate the effect of the LH. Preliminary results in this department indicate that this does seem to occur; the stimulation of progesterone secretion is particularly apparent in the F1/F2 follicles and may vary according to the time of day at which the tissues are collected. Is protein synthesis involved? If so, then addition of labelled amino acids e.g. ^{3}H-leucine to the medium should result in specific proteins becoming labelled after the addition of LH and addition of protein

synthesis inhibitors e.g cycloheximide, actinomycin D or puromycin should inhibit the LH-stimulated reponses.

Another factor which could be investigated is whether substrate availability affects the steroids secreted by follicles of different sizes. Does the largest follicle secrete predominantly progesterone, in preference to testosterone or oestradiol, because the enzymes involved in progesterone metabolism have become less active or is there little or no substrate present for them to act on? Addition of progesterone to the medium would not cause an increase in levels of further metabolites e.g testosterone or oestradiol, if these enzymes had become inactive.

Studies in rats have suggested that enzymes involved in the conversion of progesterone to androgens are inhibited following an LH surge (Uilenbroek, Woutersen and van der Schoot,1980). However, in quail, there is no marked decrease in testosterone production by F1 and F2 follicles compared with F3 and F4 follicles, suggesting that the aromatising enzymes necessary for oestrogen synthesis may be inhibited by LH. The histochemical study showed that there was little 17β-HSD enzyme activity in the largest follicles. It is most likely that enzymes involved in progesterone metabolism have become inactive in these follicles, resulting in a decrease in oestradiol production and secretion.

What triggers the change-over in the secretion pattern of the follicles as well as the timing of these events is unknown. Results using isolated cells from follicles of different sizes may help to increase our understanding of the control of ovarian steroidogenesis and why the type of steroids secreted depends on follicle size, which in turn results in only one follicle being capable of ovulating. Obviously, one might be able to increase the frequency of egg-laying if the rate of follicular development could be stimulated.

In male quail, changes in the response of the testes to hCG occurred during maturation: further developments in this field should investigate the effects of the gonadotrophins LH and FSH on the responses of the testes with maturation. As with ovarian steroidogenesis, **in vitro** studies could be performed to determine whether the mechanism of action of the gonadotrophins is via c'AMP, protein kinases and protein synthesis in experiments similar to those just described.

It may be that in birds, as in mammals, testosterone is not the major secreted androgen in the immature animal. Incubation of testicular cells with radioactively labelled precursors (progesterone, pregnenolone) followed by separation and identification of metabolities using thin layer chromatography would identify the major metabolites of the immature and mature testis. In rats, the 5α-reduced androgens,5α-dehydrotestosterone and 5α-androstane-3α,17β diol, as well as androsterone predominate in the plasma of immature animals, while testosterone predominates in adult animals (Folman, Ahmed, Sowell, Eik-nes, 1973; Moger, 1977 and 1979).

Another topic for future research is in discovering what possible role oestradiol plays in immature male quail. Plasma oestradiol levels and testicular secretion of oestradiol are increased in quail just after they have been transferred to the photostimulatory lighting regime of 16L:8D. At this time LH and FSH levels are also increasing (Follett, 1976; Gledhill and Follett, 1976). In mammals, it appears that oestradiol can exert a regulatory role on testosterone biosynthesis by decreasing the 17-20 desmolase enzyme, since oestradiol injected into rats resulted in a decrease in testicular testosterone content but increased progesterone and 17α-hydroxyprogesterone levels (Kalla, Nisula, Menard and Loriaux, 1980). However, it was not

clear in this work whether the decrease in testosterone levels was within the Leydig cells, Sertoli cells, or due to decreased androgen-binding protein in the seminiferous tubules.

The production of oestradiol by the immature testis could result in an intratesticular short-loop feedback, which may be important for the maintenance of normal testicular function. Whether oestradiol has a similar effect in quail has yet to be studied, but while oestradiol is secreted in the immature testis, testosterone production is low. This suggests oestradiol may well affect testosterone production in quail in a similar way to that seen in mammals.

In our experiments the site of oestradiol production was in the seminiferous tubule component of the testis. In the developing testis, from which oestradiol secretion was observed, the Sertoli cells may have been the major contributory cells for this secretion. This is similar to the findings in rats where again the Sertoli cells are capable of secreting oestradiol (Dorrington, Fritz and Armstrong, 1978).

Oestradiol binding to cytoplasmic receptors in rat testicular tissue has been demonstrated (Kato, Onouchi, Okinaga and Ito, 1974; Abney, 1976). Most binding protein was found in the interstitial tissue fraction (Brinkmann, Mulder, Lamers-Stahlhofen, Mechielsen and van der Molen, 1972). It seems likely, therefore, that oestradiol has a physiological role, possibly in the regulation of Sertoli cell function and spermatogenesis as well as in testosterone biosynthesis.

It has not yet been established in the avian testes whether oestradiol receptors are present in the Leydig cells or tubule cells. This could be investigated by incubating

the cells with radioactive oestradiol, and subsequently preparing the cytosol/nuclear fractions, layering them onto linear sucrose gradients and, after centrifugation, measuring the radioactivity in the fractions collected from the gradients. The specificity and affinity of the oestradiol-binding protein component could be established by parallel incubations in the presence of unlabelled oestrogens.

The effects of oestradiol on spermatogenesis and on Leydig cell function (LH-responsiveness, profile of steroid secretion, interaction with other putative agonists of testosterone secretion) have not been investigated. Indeed, there is little information concerning the regulation of steroidogenesis and spermatogenesis in avian species and demands further studies. A recent development in research in mammalian species which may apply in birds is the putative role of LH-RH-like peptides in regulating Leydig cell responses to LH. Furthermore, since work done recently has revealed that oestrone levels in male quail are higher than oestradiol levels, and that, as in females, injected oestradiol may appear in the plasma as oestrone (Williams et al, 1981), it would be interesting to measure the production of both these oestrogens by the testes and also the levels in the plasma. Some preliminary work in male birds, transferred from short to long photoperiods, shows that oestrone levels are also higher than oestradiol levels in these birds (Williams et al, 1981).

Lastly, this work has increased our understanding of the relationship between Vitamin D_3 metabolism and the sexual maturity of quail. Oestrogens are involved in the regulation of Vitamin D_3 metabolism, although their exact mechanism of action, whether directly on the kidney or indirectly via stimulating PTH or prolactin secretion, remains unclear. The presence or absence of oestrogen receptors in the kidney could be examined using radioactively labelled oestrogens, as briefly described above, and thus

help to establish whether or not a direct action on the kidney is possible.

It is likely that the increase in oestradiol levels seen prior to the onset of lay in female quail, does contribute to the increasing activity of the 1α-hydroxylase, while the low levels of oestradiol, together with increasing testosterone levels, contribute to the decrease in 1α-hydroxylase activity in male quail during sexual maturation. Since differences in plasma steroid levels between male and female quail are not very apparent until after the onset of lay, other factors are likely to be involved in the regulation of Vitamin D_3 metabolism.

One such factor is the possible role other steroids, in particular progesterone, may also play in Vitamin D_3 metabolism. Plasma progesterone levels in female quail are also elevated prior to the onset of lay and levels are higher than those found in males of the same age. Future studies in this field should therefore include measurement of the activities of the renal hydroxylases after **in vivo** injections of progesterone into immature male and female quail. It would also be desirable to measure plasma oestrone levels as well as oestradiol levels in maturing quail and to relate these changes to changes in Vitamin D_3 metabolism, occurring with sexual maturation.

By doing these further experiments, the roles that reproductive steroids play in the control of Vitamin D_3 metabolism and how their effects are exerted may be elucidated.

8.1 Conclusions

1. Plasma oestradiol, testosterone and progesterone levels are dependent on the sexual maturity of the bird, with

lowest levels found in sexually immature quail.

2. Oestradiol-17β and progesterone levels rose prior to the onset of lay and were always higher than levels in male quail. Plasma testosterone levels were higher in mature male quail than in mature females. The plasma steroid levels found help to explain the changes in Vitamin D_3 metabolism seen with sexual maturation, and the differences between the sexes.

3. Plasma progesterone and oestradiol levels were highest in quail on the day when an ovulation occurred, with maximum progesterone levels around the time of the expected LH surge. Progesterone could be the hormone involved in initiating the LH surge in quail as proposed for chickens.

4. The presence of 3β-HSD within the granulosa cells of the largest follicles and 17β-HSD within the thecal cells of the medium-sized follicles was demonstrated histochemically. These sites parallel the postulated sites of steroidogenesis observed in chicken follicles.

5. The testicular response to hCG changes with age, with most oestradiol secreted in the immature quail, while testosterone secretion increased with sexual maturation and then decreased.

6. Oestrone as well as oestradiol-17β can stimulate the 1α-hydroxylase enzyme and increase plasma calcium levels. Changes in plasma levels of oestrone during sexual maturation could also be important in the regulation of Vitamin D_3 metabolism.

CHAPTER 9

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