Hormonal stimulation of ovarian development, ovulation and oviposition in Japanese quail

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Abstract

Stimulation of ovarian development and ovulation leading to production of fertile offspring using exogenous hormones has been successful in mammals, but until recently this was not the case for avian species. These techniques would be useful for increasing the reproductive output of endangered birds such as the kakapo.

Pregnant mare serum gonadotropin (PMSG) was used to stimulate ovarian development in Japanese quail as it is readily available, easy to use, and equally effective as avian gonadotropins. The research examined the best method for administering PMSG, and the doses, duration and frequency of treatment required to stimulate follicular growth.

Treatment with PMSG can stimulate ovarian development, ovulation and oviposition in Japanese quail held under a short day photoperiod. However, there was considerable variation in ovarian response to PMSG between birds receiving the same treatment. In birds in which large yellow follicles developed, many follicles were similar in size and were not arranged in a hierarchy.

Doses of 20-80 IU PMSG were the most appropriate for stimulating ovarian development in Japanese quail. Doses lower than 20 IU PMSG stimulated little or no ovarian development in most birds, and doses higher than 80 IU PMSG led to overstimulation of follicular development in most birds. Continuous delivery of PMSG by osmotic pumps and daily treatment using injections were equally effective in stimulating ovarian development in Japanese quail. The use of daily injections is a more practical method of delivering PMSG to birds, as it does not involve surgery and allows more control over dosage and timing of treatment. Treating birds with injections of PMSG every two days rather than daily led to a rate of ovarian growth similar to that of long day birds. Treatment every four days was not sufficient to stimulate ovarian development in quail. Restricting the feed intake of quail did not have any affect on the ovarian response to PMSG treatment.

Although PMSG can stimulate ovarian development and ovulation in Japanese quail, further work is required to increase the number of birds that respond to treatment, increase the number of eggs produced by an individual, and improve egg quality.

Statement

This is to certify that the work on which this thesis is based was carried out by the undersigned, and has not been accepted in whole or in part for any other degree or diploma. Assistance is specifically recorded in Acknowledgements section and within each chapter.

A section of the work presented in Chapter 3 has in published as follows:

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New Zealand Veterinary Journal, vol. 50, pp 115-121.

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1 Literature Review and General Introduction

The use of exogenous reproductive hormones to facilitate breeding programs of endangered and threatened species has already been implemented for a number of mammalian species. However, such techniques have not been used to increase the reproductive output of an avian species. Severely endangered birds such as kakapo (*Strigops habroptilus*) would benefit greatly from such techniques. The aim of the research presented in this thesis was to find a hormone treatment regime that will stimulate development of the reproductive tract through to ovulation, oviposition and hatching of fertile offspring in avian species.

1.1 Kakapo

A total population of 87 kakapo remains on islands around New Zealand's coastline, and although intensive conservation and management efforts are ongoing, extra measures need to be taken to increase population numbers. The Kakapo is a large, long-lived, ground dwelling parrot, endemic to New Zealand. It exhibits an unusual lek mating system in which the males build a track and bowl system, and attract females using deep booming noises and wing flapping displays (Merton *et al.*, 1984). Kakapo, like many avian species, are seasonal breeders; that is, they become reproductively active when conditions are optimum for successful mating, nesting and chick rearing. This usually occurs in the spring and summer months in temperate species, when temperatures are warmer, climatic conditions more settled, and food sources are abundant. Female kakapo have bred in successive years (Cockrem, 1999), but usually only breed once every 3-4 years. Successful breeding seasons are rare, and often a summer will pass with little or no copulatory or nesting behaviour, the reasons for which are unknown. The 2001/2002 breeding season was the most successful in the last 20 years, with 25 chicks fledging. Although the kakapo conservation effort is intense, alternative methods for improving the success of each breeding season may be required

to significantly increase population numbers. Reproductive output in this species could potentially be increased by hormonal stimulation of the reproductive system as used in mammalian breeding programs. A successful treatment would lead to the stimulation of ovarian development followed by the induction of ovulation, oviposition and hatching of reproductively viable offspring.

1.2 Photoperiodism in birds

Most birds are seasonal breeders, regardless of latitude, with the time of year in which reproduction occurs being driven by food availability for successful rearing of offspring (Follett, 1984). In temperate regions breeding occurs during the spring and summer when food sources are abundant. Changes in daylength provide the first predictive information leading to physiological changes in preparation for breeding to occur. Supplementary information, such as presence of a mate, suitable breeding territory and nesting sites, is also required for the birds to reach full breeding condition. Much of the study of photoperiodism in birds has been done on temperate zone species. As daylength increases, a critical daylength will eventually be reached when gonadotropin-releasing hormone (GnRH) neurons are stimulated to produce GnRH (Sharp, 1996). Critical daylengths differ between species and are dependent on genotype. Photoperiod has both a stimulatory and inhibitory effect on the reproductive axis. The intensity of the inhibitory influence, and the rate of onset, is dependent on the sensitivity of the hypothalamus and pituitary gland to feedback from gonadal steroids (Sharp, 1996). A strong photoinduced inhibitory effect can override a photostimulatory effect, leading to the development of absolute refractoriness and regression of the reproductive tract. In birds exhibiting absolute refractoriness, reproductive activity can only be stimulated again after exposure to short days (i.e. the response to critical daylength needs to be "reset"). Relative refractoriness occurs when the photoinduced inhibitory input is weak, and has no effect on reproductive activity until daylength decreases. The photostimulatory effect is weakened by decreasing daylength, allowing it to be overridden by the photoinhibitory influence, resulting in regression of the reproductive tract. In birds that show relative photorefractoriness, a period of short daylength is not

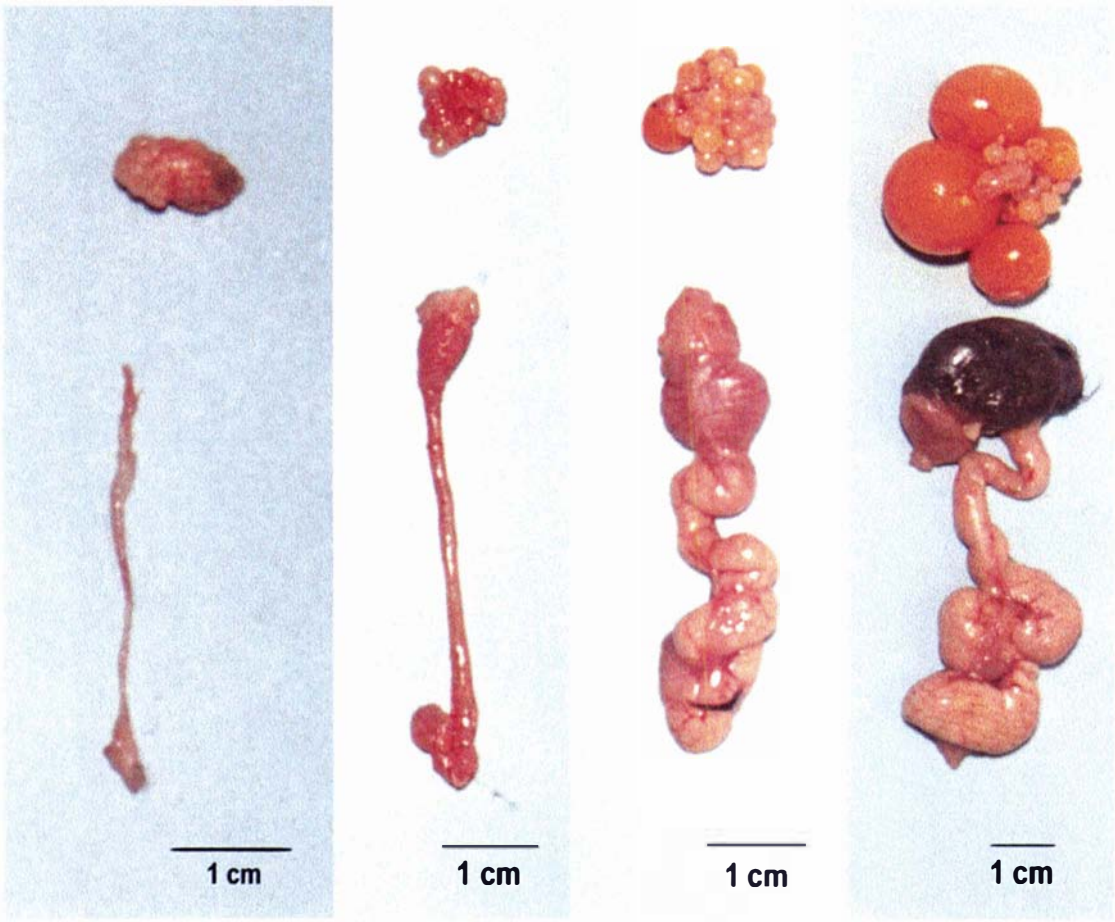

Figure 1.1. Changes in the morphological appearance of the Japanese quail ovary and oviduct from a reproductively inactive state to sexual maturity. The change from a completely regressed reproductive system (A) to a fully developed ovary and oviduct (D) occurs in approximately two to three weeks in response to increasing day length.

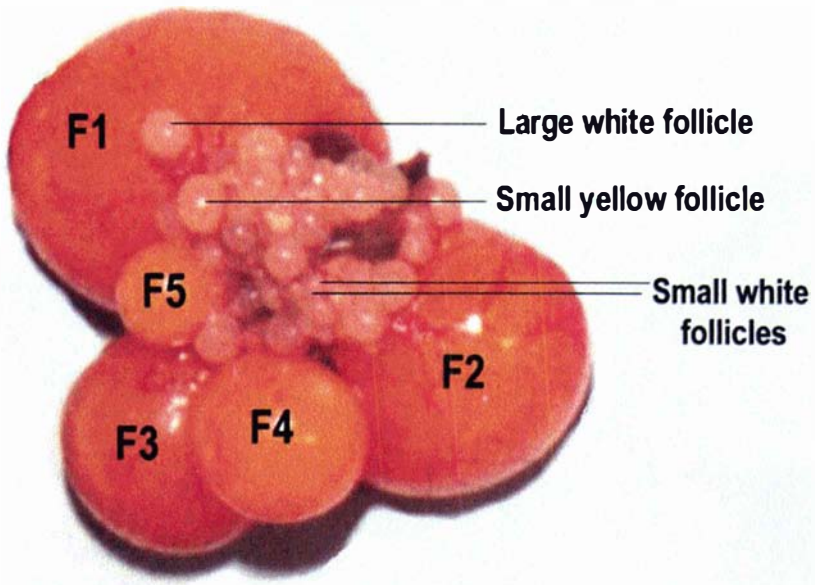

Figure 1.2. The ovary of a laying quail consists of a hierarchy of large yellow follicles, identified as F1, F2, F3, F4 and F5, and several thousand small follicles from which hierarchical follicles are recruited. The smaller follicles are classified according to their diameters as small yellow follicles (4-8 mm), large white follicles (2-4 mm) and small white follicles (>1 mm).

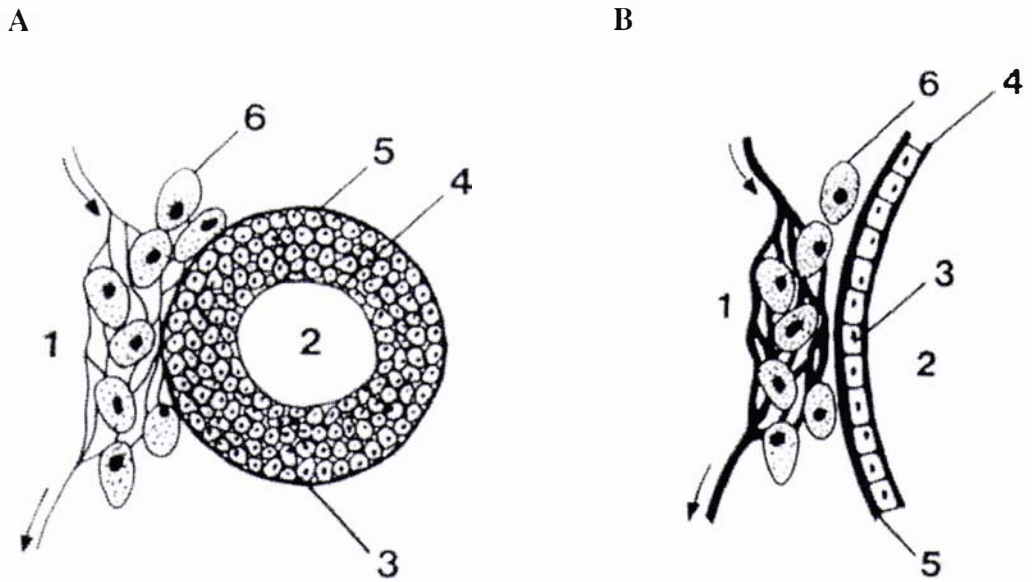

Figure 1.3. A diagrammatic comparison of the structure of a small white follicle (A) and a large yellow follicle (B) from the ovary of a chicken. Small follicles contain less yolk, several layers of granulosa cells and have a less well developed vascular system. 1. capillary network; 2. yolk; 3. granulosa cells; 4. vitellogenin membrane; 5. basement membrane; 6. theca cells. From Etches, 1996.

required, and development of the reproductive system can be stimulated again by increasing daylength (Sharp, 1996).

For domesticated birds such as chicken and quail, the timing of reproduction can be easily manipulated to advance the onset of lay, maximise egg production, and increase egg size and quality, simply by providing the appropriate lighting regime (Etches, 1996).

1.3 The avian ovary

1.3.1 Structure of the ovary

The ovary of a juvenile bird lies in the cranial part of the body cavity, is triangular in shape, brownish red in colour and weighs 0.3 – 0.5 g in chickens (*Gallus domesticus*; Amin and Gilbert, 1970) and 0.06 - 0.09 g in *Coturnix* quail (Tanaka *et al.*, 1965). The juvenile ovary consists of a central medulla surrounded by the cortex, the surface of which is granular in appearance due to the large number of small white follicles embedded in the parenchymatous tissue. Each follicle contains an ovum that is confined by a vitelline membrane that is surrounded by several layers of granulosa cells.

When the bird becomes sexually mature the ovary increases in weight to approximately 60 g in the chicken (Romanoff and Romanoff, 1949) and 10–12 g in the Japanese quail (personal observations). There is some increase in the mass of the medulla and cortex, but the increase in weight is due primarily to the development of large, yolky ovarian follicles (Figure 1.1). The mature ovary of domestic chickens consists of a hierarchy of 5 - 7 yolk filled follicles (Figure 1.2). The largest follicle, which will be the next follicle to ovulate, is referred to as the F1 follicle, the second largest F2, and so on. A mature F1 follicle is ovulated each day throughout a laying sequence and the F2 follicle then becomes the F1 follicle that will ovulate the following day. The hierarchy is maintained by recruitment of small follicles into the hierarchy. The maturing follicle consists of several tissue layers, as shown in Figure 1.3. As follicles grow, the granulosa tissue is reorganised into a single layer. The vascular system within the

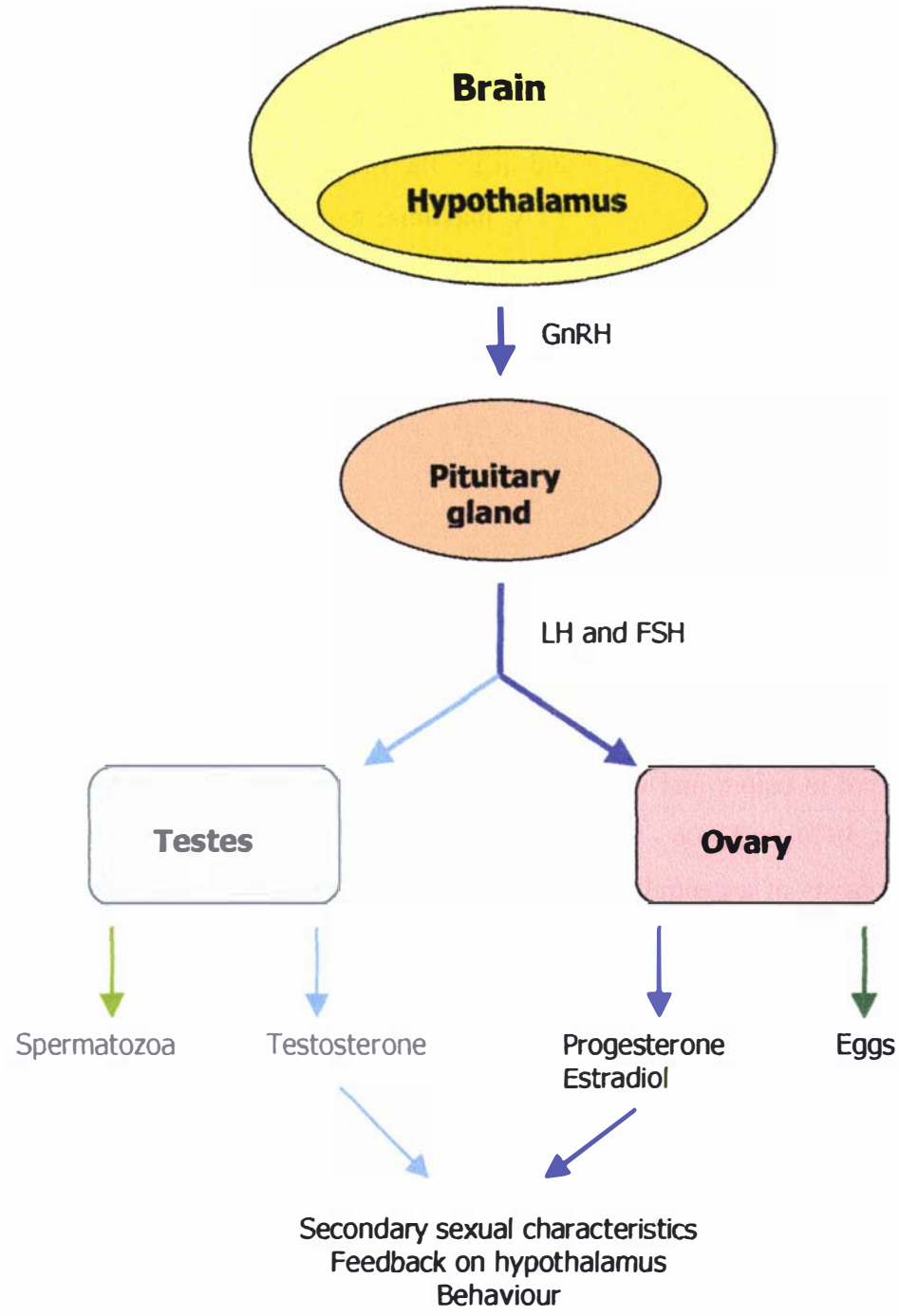

Figure 1.4. Diagram of the hypothalamic-pituitary-gonadal axis. In response to stimulatory photoperiodic conditions, GnRH neurons release GnRH, which acts on the pituitary to stimulate synthesis and release of LH and FSH. LH and FSH bind to receptors within the ovary (or testes) and stimulate production of progesterone and estradiol from the ovary (or testosterone from the testes). The reproductive steroids participate in the control of gonadotropin secretion through feedback systems, and support development of secondary sexual characteristics.

thecal layer increases in size and capacity in order to facilitate transport of yolk precursors into the developing follicle from the liver. The vascular network also allows dispersal of steroidogenic products from the ovary to target tissues. The steroidogenic thecal tissue also contains extensive innervation, the function of which is unknown.

1.3.2 Development and maintenance of the follicular hierarchy

The ovary is part of the hypothalamic-pituitary-gonadal axis (Figure 1.4). Two forms of GnRH have been isolated from the chicken hypothalamus, GnRH-I and GnRH-II (Miyamoto *et al.*, 1984). Only GnRH-I appears to be involved in stimulating the release of gonadotropins from the pituitary, whereas GnRH-II is thought to function as a neurotransmitter (Maney *et al.*, 1997). GnRH is transported to the anterior pituitary via the hypothalamic portal system, where it stimulates the synthesis and release of the gonadotropins, luteinising hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH regulate follicular growth and are involved in maintenance of the follicular hierarchy. LH is the most active stimulator of steroidogenesis in both hierarchical and non-hierarchical follicles, and is also involved in the induction of ovulation of the largest yellow follicle. LH and FSH bind to receptors within the ovarian follicles, which stimulates androgen and estrogen synthesis in the thecal layer of small follicles, and progesterone production from granulosa cells of the pre-ovulatory follicle (Gilbert, 1971). The small yellow follicles are also capable of producing progesterone, but in these follicles progesterone may be converted to androgens or estrogens in the theca layer. The avian reproductive steroids (androgens, estrogen and progesterone) participate in the control of gonadotropin secretion through negative and positive feedback systems, and support development of secondary sexual characteristics and growth of the reproductive tract.

Only a very small number of the total population of small follicles will enter the rapid growth phase, whilst the vast majority become atretic. The biological and physiological processes controlling the entry of follicles to the hierarchy are not clear, although it appears that the gonadotropins play a key role. The time at which follicles enter into the hierarchical subclass is not well defined but is limited to a 4-8 hour period of the day.

In general, one follicle is recruited into the hierarchy for each ovum released by ovulation, thereby maintaining between 7 and 10 follicles in the hierarchy (Etches, 1990; 1993). There are several hypotheses concerning the factors controlling follicle selection and maintenance of the follicular hierarchy, and these will be discussed below.

Steroid production in the thecal layer is thought to be primarily under the control of LH, and isolated thecal cells from 3-8 mm follicles of chickens express LH receptor mRNA and contain measurable levels of cytochrome P_{450} side-chain cleavage (P_{450}SCC) and P_{450} 17α-hydoxylase (P_{450} 17α-OH) mRNA (Kowalski *et al.*, 1991; Johnson *et al.*, 1996). P_{450}SCC and P_{450} 17α-OH are enzymes in the steroid synthesis pathway. However, high does of FSH can stimulate steroid production by thecal cells *in vitro* (Kowalski *et al.*, 1991). In contrast, the granulosa cells from 3-8 mm follicles have very low levels of P_{450}SCC mRNA, are devoid of LH mRNA and are consequently unable to produce steroids (Tilly *et al.*, 1991). *In vitro* incubation with LH therefore fails to stimulate cAMP formation or steroid production. However, human (*Homo sapiens*) FSH and vasoactive intestinal peptide (VIP) can stimulate cAMP production in granulosa cells, but fail to stimulate progesterone production due to the lack of P_{450}SCC enzyme activity. After an 18-24 hour incubation period both FSH and VIP, acting via cAMP, can increase P_{450}SCC mRNA levels and induce progesterone production (Tilly *et al.*, 1991; Johnson *et al.*, 1994, cited in Johnson, 1996). These results suggest that FSH and also VIP may have a critical role either in directly signalling follicle selection, or in promoting differentiation once a follicle has entered the rapid growth phase.

Insulin-like growth factors I and II (IGF-I and IGF-II) are peptides that stimulate cell division and differentiation *in vitro* (Rotwein, 1991, cited in Armstrong and Hogg, 1996) and are thought to be involved in the control of ovarian function. The ovary is a major site for IGF production (Adashi and Roban, 1992) and IGF-I has been shown to enhance the action of gonadotropins on granulosa and thecal cells *in vitro* (Onagbesan *et al.*, 1999a). Armstrong and Hogg (1996) found an IGF-II variant in the granulosa cells of only a proportion of small yellow follicles that is expressed for a short period during follicular development. The expression of this variant coincides with the period during which small follicles are selected for recruitment into the follicular hierarchy. Therefore, it has been suggested that only follicles expressing the IGF-II variant are recruited into the hierarchy, and if the variant is not expressed, the follicle becomes

atretic, indicating that the IGF-II variant may have a major role in development of the follicular hierarchy.

The highest levels of ovarian inhibin are produced by the largest preovulatory follicles (F4 to F1), and circulating levels of inhibin are negatively correlated with the rate of lay. Inhibin may act as a paracrine or autocrine factor within the ovary, or may influence pituitary FSH secretion in order to regulate the rate of follicular differentiation (Wang and Johnson, 1993).

Urokinase is an enzyme involved in cellular remodelling, reorganisation and extra- and intra-cellular matrix turnover. There is increased expression of urokinase in 6-8 mm follicles from chickens compared to 3-5mm follicles. Urokinase mRNA levels and activity decline abruptly once a follicle has entered the hierarchy (Tilly *et al.*, 1992), indicating that urokinase may be required to facilitate selection of a follicle into the rapid growth phase.

Yoshimura *et al.* (1994) showed that the germinal disc region of the developing follicle is involved in maintenance of the follicular hierarchy. Destruction of the germinal disc region can initiate atresia in preovulatory follicles. It has been suggested that the germinal disc region provides paracrine signals to the outer granulosa layer that are important for maintaining follicle viability (Yoshimura *et al.*, 1994).

It has been suggested that inhibitory or stimulatory effects of follicles on adjacent follicles may be involved in follicular recruitment (Johnson, 1996), as is the case in mammalian ovaries. However, there is currently no evidence to support this theory.

1.3.3 Ovulation

Once a follicle has reached full maturity (F1 stage) it is ready to ovulate. Chickens ovulate and lay eggs in sequences. Each ovulation occurs approximately 24-27 hours after the previous ovulation, and a sequence will usually end when an ovulation occurs

approximately eight hours after the first in the sequence. There is generally a 38-40 hour gap before the first ovulation of the next sequence occurs (Etches, 1993).

There are substantial changes in steroid production and responsiveness to gonadotropins and reproductive steroids as the F1 follicle approaches the time of ovulation. The production of androgen and estrogen in the thecal layer, which has an inhibitory effect on progesterone production, decreases markedly as the follicle reaches the final stages of maturation (Mori *et al.*, 1985). During the last few hours prior to ovulation, progesterone is the only steroid produced by the F1 follicle. The increase in plasma progesterone concentrations triggers an increase in the secretion of GnRH from the hypothalamus. GnRH, in turn, initiates secretion of LH. The increased LH concentration in the blood perfusing the F1 follicle stimulates further secretion of progesterone, which continues to drive secretion and release of LH. This positive feedback loop generates the preovulatory LH surge, which eventually causes rupture of the F1 follicle (Etches, 1996).

The mechanisms controlling the changes in ovarian steroidogenesis in follicles approaching ovulation are not fully understood. The number of FSH receptors decreases gradually as the follicle matures (Etches and Cheng, 1981; Ritzhaupt and Bahr, 1987), but the decrease is not abrupt enough to explain the major shift in steroidogenesis. Kikuchi and Ishii (1992) found that the affinity of the LH receptor in the thecal layer increased three-fold between stages F2 and F1, whilst the number of LH receptors decreased significantly. Consequently, there was a decrease in LH binding to about one fourth that of smaller follicles. There was no change in LH receptor number or affinity in the granulosa tissue of F1 or F2 follicles. The decrease in LH binding in the thecal layer corresponds precisely with the change in steroidogenic activity (Doi *et al.*, 1980; Mori *et al.*, 1985; Mori and Kantou, 1987), suggesting that the change in the number of LH receptors is somehow related to the change from estrogen to progesterone production. Kikuchi and Ishii (1992) hypothesised that the decrease in LH receptor number and affinity decreases the sensitivity of thecal tissue to LH, which in turn decreases the production of estrogen and androgen. This reduces the inhibitory effect of estrogen and androgen on progesterone production, and as a consequence, increased progesterone production and subsequent ovulation occur in the F1 follicle.

It is not clear what function the avian postovulatory follicle has in relation to ovulation. Unlike the mammalian corpus luteum, the avian post-ovulatory follicle is only active for approximately 24 hours (Gilbert, 1979). The postovulatory follicle must be involved in the oviposition process, as excision of the post-ovulatory follicle results in delayed oviposition of the egg derived from that follicle (Rothchild and Fraps, 1944; Gilbert, 1979). When the postovulatory follicle was left intact and the oldest maturing follicle removed, oviposition occurred at the predicted time (Rothchild and Fraps, 1944).

The mechanisms involved in ovarian development, follicular maturation and ovulation have now been discussed, but what is the best way of inducing growth of the reproductive tract in reproductively inactive birds using exogenous hormones? Treatment with GnRH should stimulate release of LH and FSH from the pituitary, and set in motion the normal cascade of events leading to ovulation of a viable egg. Initiating the release of pituitary gonadotropins could be bypassed by treating birds with LH and FSH in order to have a direct effect on ovarian tissue. The structure and function of avian and mammalian gonadotropic hormones will now be discussed and compared in Sections 1.3 and 1.4 to assist in planning an approach to stimulating reproductive activity in birds using exogenous hormones.

1.4 Comparison of avian and mammalian gonadotropins

In order to stimulate ovarian growth, ovulation and oviposition in birds using exogenous reproductive hormones, it would be logical to use avian pituitary gonadotropins. However, synthetic avian gonadotropins are not available commercially and considerable time, effort and expense are associated with collecting chicken pituitaries and extracting the glycoprotein fraction. Avian gonadotropins are not used in the commercial poultry industry, so there is little demand for them. The extraction of thousands of chicken pituitaries yields less than 2 g of dried glycoprotein powder, which is enough to treat less than 20 quail with a single injection. Recombinant avian LH and FSH could be produced, but this has not been done as yet.

Mammalian gonadotropic hormones are more readily available, and have been used in much of the research involving stimulation of reproduction in vertebrates. Gonadotropins of mammalian origin have been used to stimulate ovarian and follicular growth and induce ovulation in commercial livestock such as cattle (*Bovis domesticus*; González *et al.*, 1994b; Fricke *et al.*, 1997), sheep (*Ovis aries*; Ryan *et al.*, 1991; McNatty *et al.*, 1993), goats (*Capra hircus*; Saharrea *et al.*, 1998) and pigs (*Sus scrofa*; Bolamba *et al.*, 1992), and in various fish species (Hodson and Sullivan, 1993; Hassin *et al.*, 1997). Human chorionic gonadotropin (hCG) and pregnant mare serum gonadotropin (PMSG, also known as equine chorionic gonadotropin) are used more often than mammalian LH or FSH for this purpose, as they are readily available and easily extractable sources of potent gonadotropic hormone. PMSG, unlike the other reproductive gonadotropins, is not produced by the pituitary gland, and is instead secreted by endometrial cups of the placental tissue during pregnancy. It is unusual amongst the gonadotropins as although it consists of only one molecule, it has both LH- and FSH-like activities (Allen and Stewart, 1992; Hoppen, 1994).

For mammalian gonadotropins to stimulate ovarian development and induce ovulation in non-mammalian vertebrates, there must be considerable structural and functional similarity between the gonadotropins from different vertebrate classes. LH and FSH receptors must also share some common characteristics among the higher vertebrates. The structural and functional properties of the gonadotropins are coded for by the nucleotide sequence of the LH, FSH and CG cDNA. The nucleotide and amino acid sequences of each component of LH, FSH and PMSG cDNA, as well as their receptors will be discussed below. Particular emphasis will be placed on the gonadotropic hormones from chickens, quail and equids where information is available.

1.4.1 Structure of the gonadotropins

LH, FSH and PMSG are all heterologous dimer glycoproteins that consist of non-covalently bound α- and β-subunits. The α-subunit of these hormones, as well as thyroid-stimulating hormone (TSH), is common within a species, whilst the β-subunit is different and confers the biological specificity of each particular heterodimeric protein.

1.4.1.1 The common α-subunit

The common α-subunit is thought to confer little or no biological activity to the gonadotropins, and therefore analysis of its molecular structure has received little attention compared to the β-subunits. In all species examined so far, the α-subunit has been shown to be encoded by a single gene. Complementary DNA encoding the common α-subunit from the chicken pituitary gland was first cloned and sequenced by Foster *et al.* (1992). The deduced amino acid sequence of the common α-subunit is 100% homologous between chicken, quail and turkey (*Meleagris gallopavo*), and consists of a signal peptide of 24 amino acid residues and an apoprotein region of 96 residues. Predicted amino acid sequences of chicken, quail and turkey α-subunits can be compared with those of other vertebrates (see Table 1.1 for signal peptide region and Table 1.2 for apoprotein region).

Table 1.1. Homologies (percentage) for amino acid sequence of signal peptide for the common α-subunit (From Ishii *et al.*, 1993; Ando and Ishii, 1994).

Human	67	71	75	71	71	71	4	17
	Rat	71	71	67	67	67	4	22
		Bovine	92	75	75	75	9	26
			Porcine	75	75	75	4	22
				Quail	100	100	4	17
					Chicken	100	4	17
						Turkey	4	17
							Salmon	22
								Carp

There is 100% homology of the amino acid sequence between avian species in the signal peptide region. The deduced amino acid sequence of the signal peptide region of the avian α-subunit shows high homology with the sequences of mammals (67-75%), and is very similar to the intra-mammalian homology values (67-92%). The sequence

of the teleost signal peptides showed low homology values to birds (4-22%) and mammals (4-30%) and these values reflect the large phylogenetic distance between teleosts and tetrapods.

Table 1.2. Homologies (percentage) for amino acid sequence of apoprotein for the common α-subunit (From Ishii *et al.*, 1993; Ando and Ishii, 1994).

Human	80	71	71	69	69	70	59	68
	Rat	93	96	80	80	70	65	74
		Bovine	97	85	85	72	66	72
			Porcine	84	84	72	66	72
				Quail	100	75	66	71
					Chicken	75	66	71
						Bullfrog	61	66
							Salmon	74
								Carp

The apoprotein region consists of 96 amino acid residues in all avian and mammalian α-subunits examined so far, including the horse (*Equus caballus*; Bousfield *et al.*, 1996). In contrast to the signal peptide region, homology values between vertebrate classes are high (59-85%) in the apoprotein region. The values do become smaller as phylogenetic distances increase, but the overall values suggest this region is more highly conserved than the signal peptide region. Once again, there is good homology between avian and mammalian amino acid sequences in this region (69-85%).

Foster *et al.* (1992) found amino acid homology between chicken, cattle, human, mouse (*Mus musculus*) and rat (*Rattus norvegicus*) α-subunits in several regions that they believed were important in gonadotropin-receptor interactions, non-covalent binding of the α-subunit to the β-subunit, and the secondary structure of the α-subunit. Two putative glycosylation sites were found at positions 56-58 (Asn-X-Thr) and 82-84 (Asn-His-Thr) in the chicken α-subunit (Foster *et al.*, 1992). The number and position of these sites has been conserved in all species studied. Bielinska and Biome (1989)

showed that amino acids 37-40 are essential for recombination of the α- and β-subunit of hCG, and this region is strictly conserved in all mammalian and avian species, which have been analysed. Cysteine residues are involved in structural folding and maintain stable conformation of the proteins. Ten cysteine residues were found in the chicken α-subunit (Foster *et al.*, 1992) in exactly the same positions as had been reported for all other species, including the horse (Hoppen, 1994).

The α-subunit is believed not to contribute to the binding action of the gonadotropins to their receptors. Therefore it is not surprising that the primary and secondary structure of the α-subunit has been highly conserved throughout evolution. The equine α-subunit, however, may differ quite substantially from the α-subunit of other mammals. PMSG is an unusual member of the gonadotropin family as it is a single molecule that possesses both LH- and FSH-like activities in a rat bioassay (Moore and Ward 1980, cited in Sugino *et al.*, 1987). Homology between the amino acid sequences of the equine α-subunit and those of other mammals is 68-80% (Stewart *et al.*, 1987). Interestingly, there are five amino acids (positions 33, 70, 87, 93 and 96) that are conserved in all mammals studied except the horse. Although it is believed that the α-subunit is not involved in receptor-binding specificity, it has been suggested that the α-subunit does interact with the receptor (Milius *et al.*, 1983, cited in Stewart *et al.*, 1987). It is possible that the anomalous binding of PMSG to both LH and FSH receptors (Combarnous *et al.*, 1984) is, in part, due to the unusual amino acid substitutions within the α-subunit.

1.4.1.2 The LH/CG β-subunit

Chicken pituitary cDNA encoding the LH β-subunit was first cloned and sequenced by Noce *et al.* (1989). This was the first time that the molecular structure of an avian LH β-subunit had been investigated. The LH β-subunit cDNA has been cloned and sequenced for several other vertebrates, and the homologies between the primary protein structures for each group are shown in Table 1.3 for the signal peptide region and Table 1.4 for the apoprotein region.

Table 1.3. Homologies (percentage) for amino acid sequence of signal peptide for the LH β-subunit (From Ishii *et al.*, 1993; Ando and Ishii, 1994).

Human	70	70	75	40	35	20	10
	Rat	70	75	40	30	20	15
		Bovine	85	45	35	15	15
			Porcine	35	25	20	15
				Quail	54	26	15
					Chicken	22	7
						Salmon	4
							Carp

The signal peptide region of LH β -subunit cDNA consists of 39 amino acid residues in the chicken (Noce *et al.*, 1989), 47 in the Japanese quail (Ando and Ishii, 1994) and 39 in the turkey (You *et al.*, 1995). This region is considerably longer in these birds than the LH β -subunit signal peptide region of mammalian species such as the rat (20 amino acid residues; Chin *et al.*, 1983, cited in Noce *et al.*, 1989) and human (20 amino acid residues; Talmadge *et al.*, 1984), and the long leader sequence may be a characteristic of the avian β-subunit. The signal peptide sequence is thought to be involved in maturation and secretion of hormones, and may confer some species specificity to the activity of the LH molecule.

In the signal peptide region, the homology between quail and mammals is 10% higher than the homology between chicken and mammals, suggesting greater functional similarity of the LH β-subunit between quail and mammalian species.

There is a relatively constant interclass homology between the different vertebrate groups in the amino acid sequence of the apoprotein region. Ando and Ishii (1994) explained that the LH β-apoprotein molecule consists of two portions, one of which has been conserved through all vertebrate classes and occupies approximately 45% of the molecule. The other 55% is completely divergent among vertebrate classes, hence the similar homologies between species for the entire apoprotein region. Most protein and

peptide molecules consist of a variable portion, but this usually differs only partially from class to class. Ando and Ishii (1994) suggested that the variable portion of the LH β-apoprotein evolved rapidly, so that as new vertebrate classes appeared, no common sequences were conserved in this region. It is believed that the conserved portion is required for expression of LH activity, and the variable portion for the expression of species specificity. Ishii (1988) reported that the interaction of LH and its gonadal receptor is specific to animal class, and that in higher vertebrates the specificity of the LH-LH receptor interaction may be due to the highly variable amino acid sequence of the LH β-subunit molecule.

Table 1.4. Homologies (percentage) for amino acid sequence of apoprotein for the LH β-subunit (From Ishii *et al.*, 1993; Ando and Ishii, 1994).

Human	72	70	74	48	48	42	44	49
	Rat	82	89	45	45	44	39	45
		Bovine	85	44	43	44	39	42
			Porcine	45	44	44	40	43
				Quail	92	39	45	47
					Chicken	41	45	48
						Bullfrog	48	50
							Salmon	77
								Carp

The apoprotein region of the LH β-subunit consists of 119 residues in the Japanese quail (Ando and Ishii, 1994), 121 residues in most mammalian species (Bousfield *et al.*, 1996), and 149 residues in the horse (Bousfield *et al.*, 1994). The amino acid sequence of the pregnant mare serum gonadotropin β-subunit is identical to the equine LH β-subunit, so it can be readily compared with the LH β-subunit of other vertebrates. The significance of the C-terminal extension in the equid LH and PMSG β-subunit apoprotein region is still unclear. It may be related to expression or secretion of the hormones in the placental tissue, or simply serve to increase the biological half-life of

the hormones (Allen and Stewart, 1992). Despite the C-terminal extension, the horse β-subunit still shows 70% homology with β-subunits from other mammals.

The PMSG β-subunit is also highly glycosylated, containing the highest amount of carbohydrate (consisting of N- and O- linked chains) of all the mammalian pituitary or placental gonadotropins (Allen and Stewart, 1992; Hoppen, 1994). The high degree of glycosylation is thought to extend the half-life of the hormone as well as contributing to receptor binding affinity (Allen and Stewart, 1992). Glycosylation is essential for the biological functioning of most gonadotropins. It has therefore been suggested that the "unusual" glycosylation exhibited by the equine β-subunit leads to "unusual" functions of the gonadotropin, and may be involved in the dual LH- and FSH-like activity of equine LH and PMSG (Hoppen, 1994).

Several functional regions in the amino acid sequence have been compared between vertebrate groups. Chicken LH β has a large number of codons ending in G and C (approximately 70%). The incidence of codons ending in G and C is also high for mammals (68-85%), and may represent a common feature of the gonadotropin β-subunit gene among vertebrates (Noce *et al.*, 1989). The polyadenylation signal sequence, AATAAA, of chicken LH-β precursor cDNA has a dual function as it consists of part of the last codon and the termination codon, as well as the polyadenylation signal (Noce *et al.*, 1989). Similar dualism in this region has been reported for quail LH-β (Ando and Ishii 1994), turkey LH-β (You *et al.*, 1992), hCG (Fiddes and Goodman, 1980) and bovine FSH (Esch *et al.*, 1986, cited in Ishii *et al.*, 1993).

There are several secondary structural features of the LH β-subunit that have been conserved in vertebrate groups, suggesting functional similarities between gonadotropins from different species. The sequence between positions 84 to 88 (Val-Ala-Leu-Ser-Cys), believed to be necessary for the binding of LH receptors or the expression of LH activity, and the receptor binding domain (positions 56-59, Val-Cys-Thr-Tyr) are conserved in the LH β-subunits in all tetrapod vertebrates and in most gonadotropin β-subunits in teleosts (Ishii *et al.*, 1993). Position 13, Asn, is an attachment site for the carbohydrate moiety, and is also conserved in all vertebrate species for which the LH β-subunit has been sequenced (Ishii *et al.*, 1993). The twelve

cysteine residues at positions 9, 23, 26, 34, 57, 72, 88, 90, 93, 100 and 110 are conserved in all vertebrate species for which sequences are known (Noce *et al.*, 1989; Ishii *et al.*, 1993; Ando and Ishii, 1994; Hoppen, 1994).

Noce *et al.* (1989) determined that there are 15 proline residues within the chicken LH β-subunit. The position of these residues coincides with the 15 residues present in the quail LH β-subunit (Ando and Ishii, 1994), and 10 of these are also in the same position as Pro residues of other vertebrates for which the secondary cDNA LH β structure has been determined. There are a total of 20 Pro residues present in the LH β-subunit of mammals (Ishii, 1993), and only seven in the bullfrog (*Rana catesbeiana*; Ishii, 1993). Proline residues stabilise the native structure of the protein, as the proline side chain is fixed by a covalent bond to the main chain, and therefore provides more rigidity to the protein structure than any other residue. High rigidity increases the specificity of the protein-receptor interaction, therefore the relatively high numbers of Pro residues in the avian and mammalian LH β-subunits are considered to enhance LH-LH receptor binding specificity (Ishii *et al.*, 1993). This supports the hypothesis of Ishii (1988) that the number of proline residues in the LH β-subunit increases as the vertebrate class becomes phylogenetically higher.

The LH β-subunit is responsible for species specificity of the LH molecule, and has therefore undergone a greater number of nucleotide mutations and amino acid substitutions throughout evolution than the common α-subunit, as shown in the homology tables. However, many of the regions that are involved in receptor-binding interactions and formation and stabilisation of protein structure have been conserved among the vertebrate classes.

1.4.1.3 The FSH β-subunit

Cloning of cDNA encoding the FSH β-subunit has, until recently, been limited to mammalian species. Kikuchi *et al.* (1998) were the first to sequence the FSH β-subunit in a non-mammalian tetrapod vertebrate (the Japanese quail), although bullfrog and ostrich (*Struthio camelus*) FSH β-subunits have been chemically isolated and their

primary structures reported (Hayashi *et al.*, 1992; Koide *et al.*, 1996). Consequently, there is considerably less information available about the structure and function of the FSH β-subunit than the LH β-subunit.

The putative quail FSH β-subunit consists of 20 amino acids in the signal peptide region and 111 residues in the apoprotein region (Kikuchi *et al.*, 1998). The homologies between the FSH β-subunit of the few species that have been analysed are shown in Table 1.5.

Table 1.5. Homologies (percentage) for the overall amino acid sequence of the FSH β-subunit (data from Kikuchi *et al.*, 1998).

Mammals*	**61-70**	**-**	-
	Quail	**86**	58
		Ostrich	-
			Bullfrog

* Combined data for horse, human, sheep, cow, rat and pig.

The homology of the primary structure of the FSH β-subunit between avian and mammalian species (61-70%) is similar to the homology of the amino acid sequence in the common α-subunit (67-75%). The primary structure of the LH β-subunit is far less conserved among species: 43-48% between Japanese quail and mammals. Ishii (1988) and Ishii *et al.* (1993) have hypothesised that the highly conserved amino acid sequence in the FSH β-subunit indicates comparatively slow evolution of the FSH β-subunit when compared with the LH β-subunit, and consequently, the LH-LH receptor interaction shows a higher species specificity than the FSH-FSH receptor interaction.

There are several structural similarities between the FSH β-subunits of the vertebrates species so far examined. The number and position of 11 cysteine residues are common in the Japanese quail, ostrich, bullfrog and mammals (Kikuchi *et al.*, 1998). The FSH

β-subunit of the Japanese quail contains two potential glycosylation sites (Asn-X-Thr) and these are common among vertebrates. The sequence for interaction with the α-subunit (Cys-X-Gly-Tyr-Cys) at positions 26-30 has been conserved in birds, mammals and the bullfrog (Kikuchi *et al.*, 1998).

There are seven proline residues in the quail FSH β-subunit (Kikuchi *et al.*, 1998), which is similar to the number of Pro residues found in other vertebrates (5-6; Ishii, 1993). The similar number of Pro residues in the FSH β-subunit across vertebrate groups supports the theory of Ishii *et al.* (1993) that the interaction of FSH with its receptor is less species specific than the LH-LH receptor interaction.

1.4.2 Structure of the gonadotropin receptors

The receptors for LH, FSH and TSH are G protein-coupled hormone receptors that contain seven transmembrane domains and activate adenylyl cyclase after binding (Mizutani *et al.*, 1998). Information on the characteristics of gonadotropin receptors provides valuable insights into various aspects of gonadotropin function. For mammalian hormones to initiate appropriate responses in non-mammalian vertebrates, it is crucial that the receptors are sufficiently similar between vertebrate groups to allow binding of the gonadotropins. This section will compare the amino acid sequences and secondary structures of LH and FSH receptors in different vertebrate groups.

1.4.2.1 The LH receptor

Complementary DNA sequences for the LH receptor have been determined for many mammals (Mizutani *et al.*, 1998). Akazome *et al.* (1994) and Johnson *et al.* (1996) reported partial DNA sequences for the quail and chicken LH receptors respectively, and it was not until 1998 that a complete DNA sequence for the LH receptor in chickens was reported by Mizutani *et al.* (1998). The mature receptor protein of the chicken LH

receptor consists of an extracellular domain of 366 amino acid residues and a 76-amino acid C-terminal intracellular domain (Mizutani *et al.*, 1998).

The LH receptor homologies between birds and mammals (67-69%, see Table 1.6) are lower than the intra-mammalian homologies (85-88%). However, differences in the primary structure do not necessarily lead to differences in the functioning of the receptor. In fact, the binding properties of ovarian LH receptors of the Japanese quail, such as temperature dependency and saturability, are similar to those reported in the gonads of other vertebrates (Kikuchi and Ishii, 1992).

Table 1.6. Homologies (percentage) for amino acid sequence of the LH receptor (data from Akazome *et al.*, 1994; Mizutani *et al.*, 1998).

Human	86	88	68	67
	Rat	85	67	69
		Porcine	69	69
			Quail	-
				Chicken

Mizutani *et al.* (1998) found 25 cysteine residues in the LH receptor molecule, and all but two of these are conserved between mammalian and chicken LH receptors. Three of the four potential N-linked glycosylation sites encoded within the chicken LH receptor are conserved compared to the mammalian LH receptor amino acid sequence (Johnson *et al.*, 1996). The glycosylation at Asp^{173} is important for high-affinity ligand binding and cAMP production in response to interaction with the ligand in the rat LH receptor (Zhang *et al.*, 1997), and this Asp^{173} site corresponds to Asp^{72} of the quail and chicken PCR product (Johnson *et al.*, 1996). Cysteine sites are known to maintain stable conformation of the proteins and N-linked glycosylation sites are responsible for specific ligand binding activity of the human LH receptor (Minegishi *et al.*, 1989). The

fact that the cysteine and glycosylation sites are highly conserved among all LH receptors suggests that these sites are important for ligand binding activity.

1.4.2.2 The FSH receptor

The mature chicken FSH receptor protein sequence (693 amino acids) is similar in size to that previously reported for the rat (675 amino acids) and bovine (678 amino acids) species (You *et al.*, 1996). The predicted amino acid sequence of the chicken and quail FSH receptor show 68% homology with human FSH receptors, and 67% homology with the rat and horse (Akazome *et al.*, 1996). These values are lower than homologies amongst mammalian FSH receptors, but are similar to the homology between birds and mammals for the LH receptor amino acid sequence, even though the LH receptor shows greater species-specificity in hormone receptor binding than the FSH receptor (Ishii and Kubokawa, 1984).

There is a leucine-rich repetitive motif at exons 2-9 in the rat, and this motif has also been observed in the quail FSH receptor at exons 4-9 (Akazome *et al.*, 1996). It is believed that the leucine rich region contributes to the hormone-receptor interaction (Akazome *et al.*, 1996). The repetitive motif is also found in porcine, equine and primate LH receptors, suggesting that LH, FSH and TSH receptors are derived from a common ancestral molecule (Moyle *et al.*, 1994).

The chicken FSH receptor contains seven transmembrane domains, four of which are well conserved between chickens and mammals (Wakabayashi *et al.*, 1997). Ten of the 11 cysteine residues are conserved in the chicken FSH receptor compared to the rat and bovine FSH receptor cDNA (You *et al.*, 1996). As mentioned previously, the cysteine residues are important for maintaining a stable structure required for biological function, and it is therefore not surprising that they are conserved among vertebrates.

There is some variation between avian and mammalian FSH receptors in the number and position of glycosylation sites. There are five potential glycosylation sites in the chicken FSH receptor, and those at positions 191 and 199 are conserved in all FSH

receptors that have been sequenced. However, the N-linked glycosylation residues at positions 47 and 268 are unique to the chicken (Wakabayashi *et al.*, 1997), quail (Akazome *et al.*, 1996) and horse (Bousfield *et al.*, 1987) FSH receptors. Equine and chicken LH bind to both LH and FSH receptors in various mammals. However, in both of these species, FSH receptors show high specificity for their ligands (Bousfield *et al.*, 1987). The glycosylation sites that are the same in avian and equine FSH receptors indicate considerable structural and functional similarity between avian and equine gonadotropin receptors.

1.4.3 Binding of mammalian hormones to avian receptors

There appear to be sufficient functional and structural similarities between the gonadotropins and gonadotropin receptors from different vertebrate species to indicate that mammalian gonadotropins should bind to avian LH and FSH receptors and have biological activity. Unfortunately, relatively few papers have examined the ability of mammalian hormones to bind to avian LH and FSH receptors and there are no papers examining binding of PMSG to avian receptors.

The testes of the Japanese quail, domestic fowl, mallard duck (*Anas platyrhynchos*), white-crowned sparrows (*Zonotrichia leucophrys*) as well as rats show both specific (saturable) and non-specific (nonsaturable) binding of rat FSH (Ishii and Farner, 1976, Licht and Midgley, 1976a; 1976b; Adachi and Ishii, 1977; Tsutsui and Ishii, 1978; Gordon *et al.*, 1989). Ishii and Farner (1976) showed that radioiodinated rat FSH was able to bind to a testis homogenate from white-crowned sparrows. Competition experiments showed that the specific binding sites bound only FSH and not LH or other contaminants in the gonadotropin preparation. They also showed a marked increase in binding capacity of the testis two to four weeks after the beginning of photostimulation (which is also the case in rat testis), which correlates with the increase in plasma FSH during early testicular development (Follett, 1976).

Mammalian, avian and chelonian FSH receptors also exhibit a high FSH specificity in the binding of radioiodinated human FSH (Licht and Midgley, 1976a). Purified rat LH

showed little competition for the binding of radioiodinated human or rat FSH. The very low potencies of mammalian LH preparations in the radioligand assays in both turtles and birds suggest that mammalian LH does not bind to the same site as FSH.

Equine FSH exhibits a binding affinity to chicken and rat FSH receptors at least one order of magnitude greater than human or porcine FSH (Gordon *et al.*, 1989). The degree of specific binding in the absence of any competing unlabelled FSH was greater for equine FSH (14%) than for porcine FSH (9%).

There is considerable variation in the relative potencies of the different preparations of mammalian FSH, and this may be due to variation in the degree of purity of the preparation, or to the phylogenetic specificity of the receptors to "recognise" mammalian hormones. One of the ovine preparations used by Licht and Midgley (1976a) was known to be relatively impure, but was more potent in receptor binding than the more pure preparations. This discrepancy may be related to the properties or contaminants of the hormone that alter *in vivo* activity but are not required for binding. FSH and LH specificity of binding sites depends on the sources of both the gonads and the hormones.

The FSH binding sites in Japanese quail, chicken, mallard and sparrow testis are similar to those in mammalian testis in that they show high affinity and low capacity, are saturable, and have a low affinity for LH-like hormones (Licht and Midgley, 1976a; Ishii and Adachi, 1977; Gordon *et al.*, 1989). The incubation time required for maximum FSH binding, and the effect of tissue concentration on binding in the Japanese quail and the domestic fowl are similar to those reported in other animals (Means and Viatukaitis, 1972; Ishii and Farner, 1976; Ishii and Adachi, 1977). These results indicate that the biological characteristics of the avian FSH receptor are similar to those of the mammalian gonadotropin receptors.

Bona Gallo and Licht (1979) were the first to demonstrate binding of ^{125}I-labelled turkey LH to avian gonads, as previous attempts had been unsuccessful (Licht *et al.*, 1977). Unfortunately, there is no published research that focuses on the binding of mammalian LH or hCG to avian LH receptors. However, several authors have shown an increase in steroidogenesis in the avian ovary in response to mammalian LH. Ovine

and bovine LH are potent stimulants of progesterone production in chicken granulosa cells *in vitro* (Hammond *et al.*, 1980; Wells *et al.*, 1980; Zakar and Hertelendy, 1980; Asem *et al.*, 1983; Ogawa *et al.*, 1985) and *in vivo* (Wells *et al.*, 1983, Li *et al.*, 1993). These results indicate that exogenous mammalian LH does bind to LH receptors within the avian ovary and stimulates steroid production. It is therefore logical to conclude that administration of either mammalian or avian hormones to non-breeding birds has the potential to stimulate reproductive activity.

1.5 Hormonal stimulation of reproduction in birds

Treatment with exogenous gonadotropic hormones has been used to increase the reproductive output of numerous domesticated mammalian species. Treatment with mammalian gonadotropins can induce superovulation in sheep (Naqvi and Gulyani, 1998) and cattle (Alfuraiji *et al.*, 1993; González *et al.*, 1994b), cause out-of-season breeding activity in sheep (Naqvi and Gulyani, 1998) and goats (Saharrea *et al.*, 1998), increase litter size in pigs (Sechin *et al.*, 1999), sheep (Ryan *et al.*, 1991) and cattle, and induce follicular growth in pigs (Duanyai and Srikandakumar, 1998). GnRH has been widely used to synchronise estrus and induce ovulation in cattle (Martinez *et al.*, 2000; Barros *et al.*, 2000; Yavas and Walton, 2000). Exogenous mammalian gonadotropins have also been used to stimulate reproductive activity in non-domesticated mammalian species such as the white-tailed deer (*Odocoileus virginianus*; Waldhalm *et al.*, 1989), red-ruffed lemur (*Varecia variegala rubra*; Karesh *et al.*, 1985), dolphin (*Tursiops truncatus*; Sawyer Steffan *et al.*, 1983) and clouded leopard (*Neofelis nebulosa*; Howard *et al.*, 1996).

Few experiments in which birds have been treated with exogenous reproductive hormones have been designed specifically to stimulate ovarian development and increase reproductive output. Much of this work has focused instead on the physiology of ovarian function, particularly ovarian steroidogenesis, pituitary and hypothalamic function, and the mechanisms controlling the follicular hierarchy. Nonetheless, useful information can be gained from collating and interpreting the results from the relatively

few papers in which ovarian growth and ovulation have been stimulated in birds by the action of exogenous hormones.

1.5.1 Stimulation of ovarian development

1.5.1.1 Gonadotropin-releasing hormone

Gonadotropin-releasing hormone (GnRH) acts on the pituitary gland and stimulates the release of endogenous LH and FSH. Two types of GnRH have been isolated from the chicken hypothalamus; the first variant is known as cGnRH-I and the second as cGnRH-II. The amino acid sequence of both variants as well as mammalian GnRH (mGnRH) are shown in Table 1.7.

Table 1.7. Structure of mammalian and avian GnRH variants.

	Amino acid number									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
mGnRH	Pyro-Glu	His	Trp	Ser	**Tyr**	Gly	**Leu**	**Arg**	Pro	Gly-NH_2
cGnRH-I	Pyro-Glu	His	Trp	Ser	**Tyr**	Gly	**Leu**	**Gln**	Pro	Gly-NH_2
cGnRH-II	Pyro-Glu	His	Trp	Ser	**His**	Gly	**Trp**	**Tyr**	Pro	Gly-NH_2

(Data from Miyamoto *et al.*, 1984 and Delobelle *et al.*, 1995).

Most studies in which exogenous GnRH has been administered to birds have investigated the effect of GnRH on the production of LH and FSH, with relatively few examining the effects of GnRH on ovarian stimulation. A single injection of 5-20 μg of synthetic cGnRH-I is able to significantly increase LH levels in chickens (Bonney *et al.*, 1974; Wilson *et al.*, 1989), turkeys (Burke and Cogger, 1977) and Japanese quail (Davies and Collins, 1979; Hattori *et al.*, 1986). However, the extent and duration of

the response is variable between species and reproductive stages. Superactive GnRH analogues, with modifications of amino acid sequence in certain positions, cause a greater increase in endogenous LH concentrations, as well as resulting in a more sustained release of LH (Bonney and Cunningham, 1977; Sterling and Sharp, 1984). Johnson and van Tienhoven (1981) treated laying hens with synthetic GnRH-I, and showed that progesterone, testosterone and LH all rose to pre-ovulatory levels in response to a single GnRH injection. The pituitary response to exogenous GnRH is greatest in sexually immature birds, and diminishes during sexual development (Bonney *et al.*, 1974; Knight *et al.*, 1985; Wilson *et al.*, 1989). This may be related to the change in the functionality of feedback control systems in the HPG axis approaching sexual maturity. Chicken GnRH-II is up to six times more potent than cGnRH-I in releasing LH in chickens (Millar and King, 1984; Wilson *et al.*, 1989; Delobelle *et al.*, 1995), has a more prolonged action (Sharp *et al.*, 1987; Wilson *et al.*, 1989) and is more active in birds than mammalian GnRH (Sharp *et al.*, 1986; Delobelle *et al.*, 1995). In contrast, Hattori *et al.* (1986) found no differences between chicken GnRH, mammalian GnRH or GnRH analogues in their ability to stimulate LH release in Japanese quail either *in vitro* or *in vivo*.

Few papers have reported attempts to stimulate ovarian growth in birds with exogenous GnRH, and none of these attempts was successful. Reeves *et al.* (1973) found no difference in ovarian weight or follicular development between mammalian GnRH treated and saline treated birds after two weeks of daily treatment. Non-laying turkeys were treated twice daily for six days with 10-30 μg of cGnRH-I, but showed no increase in ovarian, oviductal or pituitary weight (Burke and Cogger, 1977). Sterling and Sharp (1984) treated laying chickens daily for 12 days with 1-100 μg of porcine GnRH or a GnRH analogue, buserelin, and reported no change in weight or morphology of the ovary or oviduct. Minoia *et al.* (1984) claim to have stimulated follicular development in pheasants and partridges with injections of mammalian GnRH every eight hours, but results were confounded by the fact that all birds were kept under a long day lighting regime during treatment. Endogenous release of GnRH is known to be pulsatile, and in chickens recurs at intervals of 1-3 hours with each pulse spanning 15–60 minutes (Sharp and Gow, 1983). It has proved to be very difficult to artificially mimic the pattern of GnRH release that occurs in birds. The LH response to single injections of exogenous chicken, mammalian or synthetic superactive GnRH is immediate and relatively short

lived in birds and presumably is not long enough to initiate the early stages of ovarian growth. Longer acting analogues (Sharp *et al.*, 1986) and slow release implants (Burke and Cogger, 1977) have been used in an attempt to overcome this problem. In both cases, LH levels returned to baseline after several days of treatment, which may indicate a loss of pituitary sensitivity to exogenous GnRH with prolonged use. Pulsatile GnRH administration has been used successfully in cows to stimulate luteal activity, increase plasma LH and FSH levels and induce ovulation (Hamilton *et al.*, 1999; Vizcarra *et al.*, 1997; 1999). Vizcarra *et al.* (1997) found that pulsatile GnRH release was more effective than continuous GnRH release, and continuous release actually led to a decrease in plasma LH and FSH concentrations. In contrast, Porter *et al.* (1997) showed that LH concentrations in mares increased and remained elevated in response to both pulsatile and continuous GnRH treatment, whereas there was little or no effect of either treatment on LH secretion in sheep. Methods for delivering pulsatile GnRH to birds need to be investigated, as there may be potential for this technique to stimulate ovarian development and ovulation in birds.

1.5.1.2 Avian gonadotropins

Early experiments with avian gonadotropins involved crude pituitary preparations that were thought to contain LH and FSH. In order to attribute ovarian development to hormone treatment, rather than a response to external conditions, it is important to start with a group of birds in which the reproductive system is inactive. This has been achieved in various ways, including hypophysectomy, treatment with methallibure (a pituitary inhibitor) and fasting. Taber *et al.* (1958) simply started with sexually immature birds and treated them with various doses of a ground chicken pituitary powder. Female hens were treated from hatch up until 90 days of age, and follicular development was stimulated in all but the youngest group of birds (35 days old). Although the ovarian weight of treated birds did not differ significantly from non-treated birds, there were more medium-sized follicles present in treated ovaries, and the yellow follicles were considerably larger than those of controls.

Imai *et al.* (1972) treated moulting hens (presumably with quiescent ovaries) with a chicken anterior pituitary powder (CAP) to stimulate ovarian growth. All treated hens responded with ovarian growth, but overstimulation of follicular development was evident in some birds. Small atretic follicles were present in only two hens, and large atretic follicles were not found in any treated birds.

Hypophysectomy has been used numerous times (Das and Nalbandov, 1955; Opel and Nalbandov, 1961a; Mitchell 1967a, 1967b, 1970) to ensure ovarian growth is in response to hormonal treatment, rather than pituitary hormone release. Das and Nalbandov (1955) induced follicular growth in the ovaries of immature, hypophysectomised hens with a preparation of air-dried chicken pituitaries. Chicken pituitary powder had the greatest effect on hypophysectomised hens close to sexual maturity compared with immature birds, causing a significant increase of cortical tissue and stimulating follicular development. The pituitary preparation had the greatest effect on follicle size and number in older (110 days) hypophysectomised hens. It increased comb size and restored the medullary area to the size of that found in normal laying birds. Taber *et al.* (1958) found no change in the medullary tissue of the ovary of immature birds when treated with a crude anterior pituitary suspension. In ovaries of treated hens, there was an increase in the number of interstitial cells and a slight increase in the thickness and density of the cortical tissue. Opel and Nalbandov (1961a) and Mitchell (1967a, 1967b) were able to stimulate ovarian development in hypophysectomised adult hens using daily treatment with CAP, but found considerable variation in the gradation of follicles in the hierarchy. However, there was little or no accumulation of follicles in the smaller size classes (Opel and Nalbandov, 1961a; Mitchell, 1967b) as had been found in younger CAP treated birds (Das and Nalbandov, 1955). Mitchell (1967a) showed that daily treatment with 50 mg of CAP for eight days led to little or no overstimulation of follicles, and resulted in the formation of a normal follicular hierarchy. Lower doses stimulated some follicular development in a few birds, with higher doses usually leading to overstimulation of the ovary (Mitchell, 1967a). Many of the large yellow follicles had become atretic in overstimulated ovaries (Opel and Nalbandov, 1967a; Mitchell 1967a); a condition rarely found in normal laying birds. Despite the breakdown of the follicular hierarchy, and a large percentage of yellow follicles becoming atretic, a few birds did ovulate, sometimes resulting in oviposition of a hard-shelled egg (Mitchell, 1967a).

Opel and Nalbandov (1961a) and Mitchell (1967a) allowed a sufficient period of time following hypophysectomy, onset of fasting conditions or treatment with pituitary inhibitor for the ovary to completely regress prior to treatment with CAP. However, Mitchell (1970) also treated laying hens with a chicken pituitary fraction immediately after hypophysectomy and was able to maintain the reproductive tract in an active state with daily injections of CAP, although there was considerable variation between individuals. Birds were treated for 10–12 days, with most birds ceasing egg-laying after five days. Egg size decreased during treatment, and on autopsy, regression of both the ovary and oviduct was evident in most birds, although ink tests showed that the rate of yolk deposition did not differ significantly from expected during the first half of the experiment. In birds that were allowed to regress, treatment with CAP failed to stimulate significant ovarian or thyroid growth.

Mitchell (1966; 1967b) and Imai (1972) administered the pituitary inhibitor methallibure (also referred to as I.C.I. compound 33828) to chickens to maintain ovaries in a regressed state before gonadotropin treatment. Birds were treated with an acetone-dried pituitary powder (Mitchell, 1966), a chicken gonadotropin precipitate (Mitchell, 1967b) or a chicken pituitary extract (Imai, 1972). Doses of 25 mg or less of CAP or the pituitary extract were inadequate to stimulate development of the ovary, or an increase in comb size (Mitchell, 1966; Imai, 1972). Recently formed yellowish atretic follicles were found in hens treated with low doses, but these were not present in any control birds (Imai, 1972). The increase in comb size, oviductal and ovarian weight, as well as an increase in the degree of follicular development, correlated with the increase in the treatment dose (Mitchell, 1966; Imai, 1972), with the highest level of ovarian development occurring at the highest dose of 200 mg (Imai 1972). Slightly overstimulated follicular development occurred in the ovaries of a number of hens, but a graded series of follicles was still present in some hens at this dose. In contrast to the CAP and pituitary extract treated birds, hens treated with the gonadotropin precipitate showed substantial follicular overstimulation in response to low doses (1.5 mg), with many of the large yellow follicles becoming atretic (Mitchell, 1967b). The ratio of LH to FSH may be critical for normal development of the follicular hierarchy, and the relative proportions of these gonadotropins may vary between different pituitary preparations.

In methallibure treated hens, relatively high doses of CAP were required to stimulate ovarian development (Mitchell, 1966) when compared with similar experiments on hypophysectomised birds (Das and Nalbandov, 1955; Opel and Nalbandov, 1961a; Mitchell 1967a). In hypophysectomised birds, daily treatment with 10–20 mg of CAP was able to stimulate some follicular growth (Mitchell 1967a), but this was not the case for methallibure treated hens (Mitchell, 1966). It is possible that this compound may have an inhibitory effect on the ovarian response to exogenous gonadotropins, and is therefore not the best means of maintaining the ovary in a regressed state.

Imai (1972) also used 3-5 days of fasting to maintain a regressed reproductive system prior to hormone treatment. Results were very similar to those from methallibure treated hens, with CAP treatment inducing follicular development, although a breakdown in the follicular hierarchy was noted in some birds. No large yellow follicles were observed on the ovaries of hens treated with low doses.

Extracts of CAP were not used to stimulate ovarian development again until the early 1990s. Work by Wakabayashi and colleagues concentrated on the development of a method for stimulating reproduction in endangered birds, in the hope of using this technology to increase population numbers of the severely endangered Japanese ibis (*Nipponia nippon*). Wakabayashi *et al.* (1992; 1996) employed Alzet mini-osmotic pumps to deliver gonadotropins continuously over the treatment period. A dose of 12.5 μg/h of chicken pituitary glycoprotein extract was sufficient to stimulate growth of follicles, and deposition of yolk into follicles in Japanese quail. Although follicular growth was stimulated, the treatment failed to induce a normal follicular hierarchy. There was a greater number of large yellow follicles in treated birds than in non-treated, and an abnormally large number of F3 follicles. The breakdown of the hierarchy may be attributable to an excessive dose being administered, or to inappropriate proportions of FSH and LH in the glycoprotein fraction (either excessive amounts of FSH or insufficient LH). Plasma FSH concentrations were measured at the end of the experiment, and were found to be higher in treated birds than in controls. Atretic follicles were found only in the ovaries of female Japanese quail that had failed to ovulate in response to exogenous CAP glycoprotein (Wakabayashi *et al.*, 1996). These follicles probably stopped growing before they had reached full maturity, or

started to regress as soon as they reached full maturity. In an attempt to explain why some individual birds ovulated in response to exogenous hormones and others did not, specific LH binding to both granulosa and theca tissue in the hierarchical follicles was measured in treated and non-treated females. In a normal laying female, LH binding in the granulosa of the follicle increases as it moves from the F3 to F1 stage. However, in glycoprotein treated females, there was little difference in LH binding between the F3 and F1 follicles. LH binding in the thecal tissue of avian follicles increases slightly from F3 to F2, but then drops rapidly once the follicle reaches the F1 stage due to the down regulation of receptors initiated by the preovulatory LH surge. A similar pattern occurred in the follicles of treated females, but the changes were not as marked as in normal laying females.

It is clear that preparations from chicken anterior pituitaries are capable of stimulating ovarian and follicular growth in avian species, although there is considerable variation in responses between birds. Treatment with exogenous chicken gonadotropins often leads to overstimulation of follicular development. As discussed earlier, little is known about the control mechanisms involved in establishing and maintaining a follicular hierarchy in the avian ovary, so it is difficult to form conclusions about why the hierarchy often breaks down in response to exogenous gonadotropins. It is likely that many of the pituitary preparations do not contain all the components necessary to stimulate and maintain normal ovarian growth. Adrenocorticotropin (ACTH) and TSH have been found in some pituitary preparations (Taber *et al.*, 1958; Opel and Nalbandov, 1961a) and these may act on the ovary.

Although there has been considerable variation in ovarian responses to avian gonadotropin treatment (Opel and Nalbandov, 1961a; Mitchell, 1966, 1967a, 1967b; Imai, 1972; Imai *et al.*, 1972), oviduct development has been relatively consistent (Opel and Nalbandov, 1961a; Mitchell, 1966, 1967b; Imai *et al.*, 1972). The oviduct of treated birds appears morphologically similar to non-treated controls, and usually reaches a final weight within the range of a normal laying bird. Often, the size and weight of the oviduct will increase in treated birds, with little or no change in size or follicular development of the ovary (Mitchell, 1966). This suggests that although treatment with exogenous gonadotropins does not always stimulate ovarian growth, it can stimulate estradiol secretion from the ovary, leading to oviduct and comb growth.

1.5.1.3 Mammalian pituitary gonadotropins

Studying the effects of mammalian hormones on the avian reproductive system can be useful for two reasons. Firstly, it provides the opportunity to compare the potency of avian and mammalian gonadotropins in stimulating avian reproduction, and to make further inferences about the evolution of these hormones and the homology between them. Secondly, if exogenous gonadotropins were used to increase reproduction in avian species, either commercially or for conservation purposes, mammalian gonadotropins would be less expensive and are much more readily available than avian equivalents.

The first attempts to stimulate ovarian development in birds using mammalian hormones were designed to determine the role of the hypophysis in the seasonal reproductive cycle. Pregnancy urine was injected daily in an attempt to induce ovarian growth in immature or non-breeding doves (*Streptopelia capicola*), pigeons (*Columba livia*; Riddle and Polhemus, 1931, cited in Witschi and Keck, 1935), ducks (Schockaert, 1933, cited in Witschi and Keck, 1935) and house sparrows (*Passer domesticus*; Witschi and Keck, 1935), but failed to stimulate reproductive activity in any of the birds. Witschi and Keck (1935) also treated a small number of sexually inactive house sparrows with extracts from equine and bovine anterior pituitaries. The thyroids of all treated birds enlarged to a size three times greater than that of controls. There was marked variation in ovarian responses to the pituitary extract. Half of the treated birds showed some oviductal development, but little or no ovarian development. Marked oviductal and ovarian development occurred in the remaining treated females, with some ovaries containing significantly more large yellow follicles than would be expected in a normal reproductively active sparrow. Although the mammalian pituitary extracts were capable of stimulating ovarian growth, a normal follicular hierarchy did not develop.

A commercial mammalian gonadotropic extract, known as Ambinon, obtained from the anterior lobe of the mammalian pituitary gland was used by Phillips (1943) to treat

laying hens. In most cases injections of Ambinon led to extreme overstimulation of developing follicles, and often resulted in decomposing follicular yolk material being found in the body cavity at autopsy. Phillips described the degenerating follicles as being "covered in tiny blisters" due to the occurrence of yolk-coloured fluid between the theca and germinal epithelial layer. Injections for six days lead to a decrease in egg production, and caused some hens to stop laying.

In contrast to previous studies, Das and Nalbandov (1955) found that mammalian gonadotropins had no effect on the ovaries of immature hypophysectomised birds, and also had no effect on the area of the ovarian medulla of hypophysectomised hens approaching sexual maturity, but did lead to an increase in the cortical area of the ovary (Das and Nalbandov, 1955).

Opel and Nalbandov (1961a) treated hypophysectomised adult hens with daily injections of porcine FSH. FSH treatment resulted in considerable increases in the populations of small follicles and follicles in the rapid growth phase. When compared to control birds, a three-fold increase in ovarian size was evident in treated birds after only eight days of treatment. However, porcine FSH was not capable of stimulating growth of the F1 follicle to the same size found in control birds (maximum weight of approximately 9 g in treated birds compared with up to 15 g in normal laying hens).

Palmer and Bahr (1992) found that high doses of porcine FSH (400 µg administered daily for five days) increased the number of small yellow follicles and large white follicles, and decreased the number of atretic follicles in aging hens. The ovaries of treated birds contained numerous follicles that were greater than 10 mm in diameter, but were smaller than the F5 follicle. Plasma estradiol showed a linear dose-dependent increase in response to increasing doses of FSH, but plasma progesterone increased only in response to the highest doses (200 and 400 µg) and not to doses of 12.5 µg or 50 µg FSH. Body weight and liver weight were not affected by treatment with porcine FSH, but there was an increase in yolk deposition in the hierarchical follicles. Porcine FSH stimulated and supported the growth of small follicles. It appears to be effective in the initial stimulation of ovarian tissue, but is unable to stimulate normal ovarian growth and follicular development.

Ovine FSH had little or no affect on serum vitellogenin levels, ovarian growth or oviductal development in fasting pullets (Imai, 1972). Bovine anterior pituitary extract and ovine LH did increase serum vitellogenin levels slightly, but not as markedly as in groups treated with chicken pituitary extracts. Only half of the birds treated with bovine pituitary extracts and ovine LH showed signs of ovarian development. The number of white follicles and atretic follicles increased on stimulated ovaries, but yellow follicles were not present. Mixtures of ovine LH and FSH increased serum vitellogenin to within the normal range, but the oviducts remained small. A small number of birds in this group did have small yellow follicles on the ovary (Imai, 1972).

Mammalian pituitary extracts from various sources can induce ovarian and follicular growth in birds. However, there has been considerable variation in response between birds, with overstimulation of the ovary a common problem. Follicles in each of the different size classes may require different concentrations or combinations of gonadotropins in order for development to continue. Exogenous mammalian pituitary gonadotropins are able to stimulate development of the lower size classes, but are unable to sustain a rate of development in the hierarchy found in a normal laying hen.

Placental gonadotropins differ in both structure and function compared to mammalian pituitary hormones. Equine gonadotropins show a significantly higher binding affinity to chicken receptors, and exhibit a substantially extended half-life within the circulatory system. These facts indicate that pregnant mare serum gonadotropin may be more suitable for stimulating reproduction in avian species than mammalian pituitary gonadotropins.

1.5.1.4 Pregnant mare serum gonadotropin

PMSG (also known as equine chorionic hormone) is able to stimulate follicular development in birds, but sometimes treatment with this gonadotropin leads to overstimulation of all size classes of follicles and a breakdown in the follicular hierarchy (Phillips, 1943; Opel and Nalbandov, 1961a; Zadworny and Etches, 1988; Palmer and Bahr, 1992, Wakabayashi *et al.*, 1996). Data presented in Taber (1948)

indicated that treatment with PMSG produced pronounced medullary hyperplasia, leading to a large cystic, abnormal ovary. Some researchers have, however, reported development and maintenance of a normal hierarchy in PMSG treated birds (Petitte and Etches, 1989). In sexually immature hens treated with 75 IU or 375 IU PMSG daily for one week, a hierarchy containing 2-5 yolky follicles was present on each ovary (Petitte and Etches, 1989). Palmer and Bahr (1992) chose not to use a dose as high as 75 IU PMSG, assuming that this would lead to disruption of the hierarchy. Instead, birds were treated with doses of either 25 IU or 50 IU PMSG, but in both cases overstimulation of follicles and breakdown of the hierarchy was still evident. Treatment of incubating or out-of-lay turkeys with 400 IU or 2 000 IU PMSG daily stimulated follicular growth, but large yellow follicles were not arranged in a hierarchy (Zadworny and Etches, 1988). It is generally accepted that small follicles rely on FSH to develop and enter the hierarchy (Opel and Nalbandov, 1961a; Armstrong, 1994). It has been suggested that inhibitory factors prevent response to endogenous FSH in small follicles, and the inhibitory influence is only removed in those follicles that are to enter the follicular hierarchy (Johnson, 1996). It may be that PMSG is able to override the inhibitory effect in the small ovarian follicles of some birds, causing numerous follicles to enter the hierarchy together.

In laying hens, PMSG leads to an increase in progesterone and estradiol concentrations and a decrease in plasma LH (Johnson, 1983; Johnson and Leone, 1985; Palmer and Bahr, 1992). Plasma estradiol concentrations increased markedly during the first day of treatment with 400 IU PMSG in incubating or out-of-lay turkeys, but did not increase further in response to subsequent injections (Zadworny and Etches, 1988). Plasma estradiol concentrations in female turkeys treated daily with 2 000 IU PMSG, however, continued to increase for the duration of the experiment. LH concentrations did not change in the laying control group, or in response to PMSG in either of the treatment groups. Prolactin levels were four times higher in incubating hens than in out-of-lay hens, and this may have inhibited steroid production by the ovary in response to 400 IU PMSG. Both groups showed marked ovarian and oviductal growth in response to 2 000 IU PMSG, so the high dose of PMSG may have been overriding the effects of prolactin in the incubating turkeys.

Imai *et al.* (1972) treated moulting hens with daily injections of PMSG. After 6 days of treatment large yellow follicles were present in only 40% of the treated birds, and some of these large follicles had become atretic. Large atretic follicles have been found on ovaries of PMSG treated birds in several experiments (Hosoda *et al.*, 1955; 1956; Opel and Nalbandov, 1961a; Imai, 1972; Imai *et al.*, 1972; Wakabayashi *et al.*, 1996; personal observations). Atresia usually occurs in small follicles prior to recruitment into the follicular hierarchy, but under normal physiological conditions is rarely seen in large, preovulatory ovarian follicles (Gilbert *et al.*, 1983). The mechanisms involved in follicular atresia are poorly understood, but it is widely recognised that atresia is mediated via a specific type of programmed cell death or apoptosis (Johnson, 1996). When several follicles enter the hierarchy at once, as sometimes seen in PMSG treated birds, it may be that apoptosis has already been initiated in several of these follicles, causing them to become atretic after becoming large yellow follicles.

Palmer and Bahr (1992) noted that administration of PMSG to laying hens led to an increase in yolk deposition into the F1 and F2 follicles of the ovary. This suggests an increase in the synthesis of yolk precursors by the liver. An increase in serum vitellogenin indicates production of vitellogenin by the liver, which is likely to reflect an increase in estrogen production by the ovary. Experiments by Hosoda *et al.* (1955) and Imai *et al.* (1972) focused on the ability of PMSG to maintain or restore serum vitellogenin levels in moulting or fasted hens. Imai *et al.* (1972) treated hens with 100 IU PMSG daily for 6 days. Serum vitellogenin rose to a level found in laying birds within four days, and was maintained at this level for the duration of treatment. Daily treatment of fasting hens for seven days with only 5 IU PMSG was sufficient to delay the drop in serum vitellogenin associated with starvation, but did not prevent it (Hosoda *et al.* 1955). This dose was also insufficient to promote growth of new follicles, although daily treatment with 20 IU PMSG maintained serum vitellogenin at a level similar to that found in normal laying birds. They interpreted their findings to indicate that starvation decreases the production of gonadotropins by the pituitary, which decreases the amount of estrogen produced. The decrease in estrogen concentration, in turn, leads to a reduction in the formation and release of serum vitellogenin by the liver.

Measurement of ornithine decarboxylase activity (ODA) has been used successfully to monitor the response of target tissues to exogenous gonadotropins such as PMSG

(Janne *et al.*, 1978). Ornithine decarboxylase acts as a catalyst in the biosynthesis of polyamines, and plays an important role in the structure and function of nucleic acids (Russell and Synder, 1968). The activity of this enzyme therefore increases in response to cell proliferation and differentiation (Martin and Morris, 1987, cited in Armstrong 1994), and correlates with the rate of tissue growth (Russell and Snyder, 1968). Armstrong (1994) found that ODA in the theca of small, non-atretic follicles in hens increased in response to treatment with PMSG. However, the theca of F1, F3 and F5 follicles was not affected by PMSG, and there was no increase in ODA. After treatment with PMSG, ODA in the granulosa tissue of F1 follicles was significantly higher than other follicular size classes, all of which remained unaffected by the treatment. ODA levels in granulosa tissue increased as follicles approached ovulation, whereas they decreased in thecal tissue. Granulosa tissue showed a decrease in ODA in response to 75 IU PMSG, and increased to a maximum in response to 300 IU PMSG.

PMSG can stimulate steroidogenesis and ovarian growth in avian species. However, as with avian and mammalian pituitary extracts, PMSG treatment does sometimes lead to overstimulation of ovarian follicular development. Lower doses of PMSG, alternate delivery methods or other hormones administered in conjunction with PMSG may be required to stimulate normal ovarian development in birds.

1.5.2 Induction of ovulation

Ideally, successful stimulation of ovarian development should lead to ovulation of the F1 follicle. Ovarian and follicular growth has been achieved in birds using both exogenous avian and mammalian gonadotropins, often resulting in the development of an F1 follicle. However, relatively few papers have reported stimulation of ovarian development as well as the induction of ovulation (Taber *et al.* 1958; Mitchell 1967a, 1967b; Zadworny and Etches 1988; Wakabayashi *et al.* 1992, 1996). Hormone treatment to induce ovulation may be required in addition to hormone treatment that stimulates ovarian growth and the development of the follicular hierarchy.

Many papers have focused on inducing premature ovulation in laying birds (e.g. Fraps *et al.*, 1942a, 1942b; Kamiyoshi and Tanaka, 1972; Onagbesan and Peddie, 1988a) or in birds in which ovulation has been blocked using restricted feeding, hypophysectomy or exogenous hormones (e.g. Hosoda *et al.*, 1956; Opel and Nalbandov, 1961b; Nakada *et al.*, 1994). These studies have generally been conducted to learn more about the mechanisms involved in the process of ovulation. A number of hormones have been used to induce ovulation, including LH, FSH, GnRH, progesterone, corticosterone, ACTH and hCG.

1.5.2.1 Luteinising hormone

Preovulatory surges of both LH and progesterone occur in avian species, so these hormones are most commonly used in ovulation induction experiments. Fraps and Riley (1942) and Fraps *et al.* (1942a) were the first to induce ovulation in laying hens using an LH preparation extracted from horse pituitaries. Some hens ovulated in response to each of three successive treatments. As many as seven multiple ovulations could be effected in individual hens following a single LH injection, but only a single egg containing a single yolk was found in any of the oviducts, with any subsequent ovulated follicles being found in the body cavity.

Opel and Nalbandov (1961a) also induced successive ovulations with injections of mammalian LH in birds that had previously been treated with PMSG, FSH or CAP to stimulate ovarian development. Nearly all of the treated birds ovulated in response to the first injection, but the number of birds responding to each subsequent injection dropped significantly over the next eight days, by which time as few as 10% of the hens were ovulating. The inability of a bird to ovulate in response to LH treatment was attributed to the lack of an F1 follicle on the ovary, as the exogenous gonadotropins were unable to develop the follicles to maturity as quickly as they were being ovulated. Two or more consecutive ovulations occurred in 77% of treated hens.

Hosoda *et al.* (1956) attempted to induce ovulation in fasted and PMSG treated hens using mammalian LH. Doses of 0.1 mg and 0.5 mg LH were able to induce ovulation

of F1 follicles, but low doses of 0.05 mg LH had no effect. Even large doses of 2 mg LH were unable to induce ovulation of small (< 2 cm), immature follicles. This is in contrast with the results of Opel and Nalbandov (1961b) who were able to force ovulation of immature follicles not due to ovulate for 3-4 days in hypophysectomised birds. Ovulation could be induced in hypophysectomised hens up to 12 hours after hypophysectomy. Follicles remaining without hormonal support for longer than this became atretic, and ovulation could not be stimulated (Opel and Nalbandov, 1961b). There was an increase in follicular sensitivity to LH with increasing interval from hypophysectomy to LH injection, and this may explain why immature follicles could be induced to ovulate.

Premature ovulation has been induced in hens with ovine LH injections up to 21 hours before the next expected ovulation (Johnson and Leone, 1984; Sekiguchi and Imai, 1987; Onagbesan and Peddie, 1988a). There was a significant rise in plasma progesterone in the first hour following the injection, followed by a decline to almost initial levels nine hours post-injection (Onagbesan and Peddie, 1988a). The follicles were less responsive 18 hours before expected ovulation than 12 hours before, and this is consistent with observations of changing sensitivity of ovarian granulosa cells to LH during follicular maturation.

1.5.2.2 Follicle-stimulating hormone

Only Kamiyoshi and Tanaka (1972) have examined the ability of FSH to stimulate ovulation in hens. They compared the ability of mammalian LH alone, mammalian FSH alone, and various combinations of LH and FSH to induce premature ovulation in the hen. FSH was unable to stimulate ovulation in any of the treated birds. Injection of 0.02 mg mammalian LH induced ovulation in 34% of hens, but when this dose was combined with 0.5 mg mammalian FSH, ovulation was induced in 71% of treated hens. FSH may augment the ovulation inducing action of LH in the hen. They also found that FSH exhibited this augmentative effect if injected at the same time as, or one hour after the LH injection, but showed no effect if injected 15 minutes after the LH injection.

This suggests that the stimulatory effects of FSH vary throughout the ovulatory cycle, which may be due to changes in follicular sensitivity to the hormone.

1.5.2.3 Gonadotropin-releasing hormone

Both mammalian and avian GnRH have been used successfully to induce normal and premature ovulations in chickens. Doses of 5-10 µg induce ovulation before or at the expected time in almost all treated birds, but with higher doses (20-40 µg), almost all birds will ovulate prematurely up to six hours before expected ovulation (van Tienhoven and Schally, 1972; Reeves *et al.*, 1973; Tanaka and Kamiyoshi, 1976; Onagbesan and Peddie, 1988a). However, Bonney *et al.* (1974) were unable to stimulate ovulation in laying hens using 20 µg synthetic mammalian GnRH.

Johnson *et al.* (1984a; 1984b) used both mammalian and chicken GnRH-I to induce premature ovulation in laying chickens, and found them to be equally successful. It was also noted that when birds were treated prior to the first ovulation in a sequence, all birds ovulated prematurely. However, when treated prior to the second ovulation in a sequence, almost all ovulations would occur at the expected time. Several reports have noted that hypothalamo-hypophyseal and ovarian sensitivity to ovulation inducing hormones is different 8-15 hours before the first ovulation in a sequence (C1) compared to the same time prior to a mid-sequence ovulation (C2; Fraps and Dury, 1943; Etches *et al.*, 1983, Johnson, 1983, Johnson *et al.*, 1984a). This may be related to the inability of the preovulatory follicle to initiate progesterone production and secretion. The endogenous release of progesterone is also greater prior to a C1 ovulation, compared to a C2 ovulation (Johnson, 1983). The pituitary response to mGnRH is the same at both times, but ovarian response to LH may be different (Johnson *et al.*, 1984a).

1.5.2.4 Progesterone

Progesterone exerts a positive feedback effect on secretion of LH that can lead to a preovulatory surge of both hormones (Nakada *et al.*, 1994). In chickens, there are

significantly higher levels of LH and progesterone in the plasma 4-7 hours prior to ovulation than at any other time during the ovulatory cycle. Premature ovulation has been induced using progesterone, provided that the injection was administered less than 18 hours before the next expected ovulation (Wilson and Sharp, 1976; Sekiguchi and Imai, 1987; Onagbesan and Peddie, 1988a). Progesterone was able to induce ovulation in intact female hens, but when the pituitary was removed within 2 hours following injection of progesterone, ovulation did not occur (Rothchild and Fraps, 1949). Progesterone failed to induce ovulation in birds that were hypophysectomised more than 10 hours prior to the next expected ovulation (Nakada *et al.*, 1994). At this time, the F1 follicle may be insufficiently mature to respond to progesterone, or pituitary hormone secretion may be inadequate for progesterone to initiate an LH surge. These results suggest that progesterone acts on the hypothalamus or pituitary to induce ovulation and has no direct action on the ovary.

Exogenous progesterone causes abundant release of LH from the pituitary gland (Ralph and Fraps, 1960), and the temporal discrepancy between LH and progesterone in their ability to induce ovulation may be due to the low responsiveness of the hypothalamo-pituitary axis to progesterone 18-21 hours before ovulation.

1.5.2.5 ACTH and corticosterone

The potential involvement of adrenal hormones in the ovulatory cycle was recognised by Fraps *et al.* (1947) who induced premature ovulation in hens with an ACTH-rich pituitary extract. Since then, several researchers have investigated the ability of ACTH and corticosterone to induce ovulation in birds. Etches *et al.* (1982) injected hens with ACTH after the last oviposition in a sequence, and found that the number of birds that ovulated increased with increasing dose. In contrast, Etches and Cunningham (1976) found that only the highest doses of corticosterone (1 000 μg) could induce premature ovulations in hens. Etches and Croze (1983) treated birds with corticosterone or ACTH either six hours after a mid-sequence ovulation, or 14 hours before the first ovulation in a sequence. None of the birds ovulated in response to corticosterone or ACTH when

administered six hours after a mid-sequence ovulation. However, plasma corticosterone levels increased and plasma LH concentrations significantly decreased in response to both corticosterone and ACTH delivered at this time. The same pattern of corticosterone and LH release was evident when birds were treated before the first ovulation in the sequence, but over half of the birds ovulated in response to both ACTH and corticosterone. An injection of progesterone given six hours after a mid-sequence ovulation will lead to an increase in plasma LH concentrations (Wilson and Sharp, 1976), although exogenous ACTH or corticosterone has to be administered after this time to induce a release of LH (Etches and Croze, 1983).

1.5.2.6 Human chorionic gonadotropin

Human chorionic hormone (hCG), like FSH, has rarely been used to stimulate ovulation in birds. Mitchell (1967b) stimulated ovarian development using chicken pituitary extracts, and the attempted to induce ovulation on the final two days of treatment using hCG. None of the birds responded, but on autopsy no mature F1 follicles were found.

Wakabayashi *et al.* (1996) also used hCG to induce ovulation in birds which had previously been treated with PMSG or a chicken pituitary extract. Only three out of 21 birds were induced to ovulate. Apart from one successful ovulation reported by Mitchell (1967a), this was the first experiment in which birds with fully regressed ovaries from the beginning of the experiment were treated with exogenous gonadotropins to stimulate follicular development, and then to induce ovulation. There are no published studies in which hCG has been used to induce premature ovulation in normal laying hens, so it is difficult to compare the effectiveness of hCG with other hormones.

The ability to induce ovulation with exogenous hormones is dependent on several factors. Timing of the injections is probably the most important, i.e. the stage of the ovulatory cycle at which injections are administered, and the most suitable time prior to expected ovulation will vary depending on the hormone used. If treatment with mammalian gonadotropins initiates and sustains development of the ovary and

reproductive tract, then ideally, further treatment to induce ovulation will not be required.

1.5.3 Comparison of mammalian and avian gonadotropins

Mammalian and avian gonadotropins differ in their ability to stimulate ovarian development and induce ovulation in birds. Das and Nalbandov (1955) found chicken pituitary gonadotropins to be more capable of inducing follicular growth than mammalian gonadotropins. The chicken preparation was able to stimulate follicular development in the ovaries of immature, hypophysectomised hens, whereas the mammalian preparation had no effect. Opel and Nalbandov (1961a) found the response of ovaries to CAP was more variable than to PMSG or mammalian FSH in that some birds showed little response, some exhibited a normal follicular hierarchy, and others showed overstimulation of follicular growth in the middle size classes. Mammalian LH used to induce ovulation led to progesterone secretion in chickens (Huang *et al.,* 1979) and quail (Asem *et al.,* 1985), but with less potency than avian preparations (Scanes and Fagioli, 1980). Large atretic follicles are found on the ovaries of CAP treated birds (Phillips, 1943; Mitchell, 1967a and 1967), but occurred more frequently in PMSG treated birds (Phillips, 1943; Opel and Nalbandov, 1961a; Imai *et al.,* 1972).

Comparing the effectiveness of mammalian and avian gonadotropins for stimulating reproductive activity is confounded by the fact that so many factors vary from one experiment to another. This may explain some of the conflicting results that exist between supposedly similar studies. One of the major inconsistencies between the various experiments reported in the literature is the physiological state of the birds prior to treatment. The main difference between experiments is often the age of birds at the start of the experimental period. Most studies have utilised birds that are sexually mature, and are, or have been, reproductively active (Phillips, 1943; Hosoda *et al.,* 1955, 1956; Opel and Nalbandov, 1961a; Mitchell, 1966; 1967a; 1967b; 1970; Imai, 1972; Imai *et al.,* 1972; Kamiyoshi and Tanaka, 1972; Reeves *et al.,* 1973, Zadworny and Etches, 1988; Petitte and Etches, 1989; Palmer and Bahr, 1992). Relatively few researchers have used birds that are sexually immature or have never laid (Phillips,

1943; Das and Nalbandov, 1955; Taber *et al.,* 1958; Wakabayashi *et al.*, 1992; 1996). Only recently have birds been held on short day lengths that prevent ovarian growth before hormone treatment (Wakabayashi *et al.,* 1992; 1996). The failure to stimulate ovarian growth in birds treated from hatch to 35 days old suggests that the ovary is not able to respond to gonadotropins at this time (Taber *et al.,* 1958). Sexually immature birds may require different hormones, different doses of hormones or different combinations of hormones to stimulate ovarian development compared to mature birds that have never laid, or birds that have previously laid, but have undergone complete regression. It is likely that the endogenous ratio of LH:FSH will vary between birds of different reproductive states, which may account for some of the differences in response to exogenous hormones.

The methods used to extract the pituitary fractions vary from experiment to experiment. Mitchell (1967) and Imai (1972) found that chicken pituitary preparations from different stages of the extraction process do differ in their ability to stimulate ovarian development. Mitchell (1970) found that more highly purified extracts were unable to stimulate ovarian growth in hypophysectomised females with fully regressed ovaries. In those extracts that did activate ovarian development, egg size was found to decrease during treatment. The gonadotropin content of the extract may be too little to sustain adequate steroid production or release to maintain the normal level of yolk deposition. Different extracts may vary in their LH and FSH content without these differences being detected by mammalian bioassays. The variation in ovarian response to treatment with pituitary extracts may be due to differences in purification techniques, variation among the recipients of injections, or result from different doses being administered in each experiment.

Other factors that have varied between experiments, which make useful comparisons difficult, include variation in the timing, frequency, duration and delivery of hormones, and the use of numerous species of birds, and various strains of an individual species. Despite this, it is clear that both mammalian and avian gonadotropins are capable of stimulating growth of the ovary and reproductive tract. The choice of hormone for studies of the hormonal stimulation of breeding in birds therefore depends on factors such as the cost, availability and ease of use of different hormones and hormone preparations.

1.6 Outline of Thesis

The overall aim of the research described in this dissertation was to develop methods to stimulate ovarian development, ovulation and egg-laying in Japanese quail. A successful treatment protocol should result in oviposition of viable eggs, which if fertilised, would develop into healthy, fertile offspring. The protocol could then be used to stimulate reproduction in endangered and threatened birds such as kakapo.

The Japanese quail (*Coturnix coturnix japonica)* was an ideal model species for this research for several reasons. It was available from a nearby commercial source, and could be housed and maintained with relative ease. Japanese quail reach sexual maturity at 4-6 weeks of age, and females will lay year round in response to a stimulatory photoperiod. Reproductive activity in Japanese quail can be manipulated readily with changes in photoperiod and temperature. The New Zealand strain of Japanese quail requires cool temperatures in conjunction with a short day photoperiod to inhibit reproduction (unpublished observations, Chua and Hessell, 1998). Under these conditions, the reproductive system will either regress, if previously active, or will remain inactive.

PMSG was used to stimulate ovarian development and ovulation in Japanese quail. PMSG was chosen because it was readily available, relatively inexpensive, required no preparation prior to use, and was easily administered to the birds.

The following questions were addressed:

1. Will PMSG stimulate ovarian development in the New Zealand strain of Japanese quail?
2. Which is the best method for administering PMSG to the quail?
3. What is the duration of treatment required to stimulate development of an ovuable follicle?
4. Which dose, or combination of doses, is required to stimulate normal ovarian development?

5. What frequency of treatment is necessary to initiate and maintain follicular growth?
6. What is the effect of a restricted feeding regime on the ovarian response to treatment?

Chapter 2 describes two experiments in which ovarian development was stimulated in Japanese quail using PMSG, and ovulation induced with hCG or an avian pituitary extract. Chapters 3 and 4 describe a series of experiments that were designed to answer questions two through to five outlined above. The final experimental chapter (Chapter 5) addresses question six. In Chapter 6 (General Discussion), the results and ideas from all previous chapters, as well as future directions for this research, are discussed.

2 Effect of continuous PMSG treatment by osmotic pump on ovarian development

2.1 Introduction

The successful use of mammalian and avian gonadotropins to induce reproductive activity in birds has already been discussed in Chapter 1. Birds have been treated with exogenous gonadotropins to examine the effect on oviductal and ovarian development in numerous studies, but very few experiments have been designed specifically to devise a treatment schedule suitable for inducing reproduction in endangered or threatened avian species such as the kakapo (*Strigops habroptilus*). Recently, work by Wakabayashi and colleagues (1992; 1996) focused on stimulating ovarian and oviductal development, ovulation and oviposition in the Japanese quail, with the idea that similar techniques could be applied to the severely endangered Japanese ibis (*Nipponia nippon*; Ishii *et al.*, 1994). Ovarian development was stimulated in Japanese quail using chicken pituitary extract or PMSG. Ovulation and oviposition were then induced in approximately 30% of the treated quail using chicken pituitary extract or hCG. Hatchability of the eggs was high, and all newly hatched chicks were fertile (Wakabayashi *et al.*, 1996).

Similar techniques have also been used in mammals with successful results. PMSG treatment has been used in pigs to stimulate follicular development, followed 72 hours later by injection of hCG to induce ovulation (Duayani and Srikandakumar, 1998). Similarly, in the house musk shrew (*Suncus murinus*) and the vesper mouse (*Calomys musculinus*), PMSG was used to stimulate folliculogenesis, and 48 or 72 hours later, a single injection of hCG was administered to induce ovulation and over 80% of the treated females responded with an ovulatory or superovulatory response (Matsuzaki *et al.*, 1997; Lasserre *et al.*, 1998). These techniques have also been used in endangered mammalian species to stimulate reproduction and increase population numbers.

Clouded leopards (*Neofelis nebulosa*) have been successfully treated with PMSG to stimulate follicular growth, followed by an injection of hCG to induce ovulation (Howard *et al.*, 1996).

The New Zealand strain of Japanese quail has not previously been treated with PMSG to stimulate reproductive development. The aim of the current experiments was to stimulate ovarian and oviductal development by administering PMSG, and to induce ovulation with a chicken pituitary extract or hCG. The experimental protocol was similar to that employed by Wakabayashi *et al.* (1996) in which female Japanese quail were implanted with osmotic pumps containing PMSG and treated with injections of hCG to induce ovulation.

2.2 Methods and Materials

2.2.1 Animals

Three week old male and female quail were purchased from a commercial source (Rangitikei Game Birds). Prior to purchase, birds were reared on a long-day photoperiod (16L:8D). From three weeks of age, birds were maintained under either a long day photoperiod (16L:8D) at 20°C, or a short day photoperiod (8L:16D) at 10°C. Previous experiments have shown that short days as well as low temperatures are necessary to suppress gonadal development in the strain of Japanese quail available to us (Chua and Hessell, unpublished data). Birds were held individually in cages measuring 35 x 25 x 20 cm, with water and food available *ad libitum*.

2.2.2 Hormone preparation

All the hormones used in the following experiments were kindly donated by Professor Susumu Ishii, Waseda University, Tokyo. PMSG and hCG were originally purchased from Teikoku Zoki, Tokyo, Japan.

A pituitary glycoprotein fraction was prepared from 2.00 g of powdered acetone dried chicken pituitary glands also donated by Professor Ishii. The powder was placed in a siliconised beaker, dissolved in a solution of 40% ethanol containing 6% ammonium acetate and the pH adjusted to 5.1. The solution was homogenised overnight on a magnetic stirrer at 4°C, and then transferred to a polyethylene centrifugation tube. The homogenate was centrifuged for 10 minutes at 12 000 rpm using a SS34 rotor in a Sorvall RC 5C Plus centrifuge at 4°C. The resulting supernatant was measured precisely using a 500 µl Hamilton syringe, and transferred to a clean teflon centrifugation tube. The extraction procedure was repeated twice, and the supernatants combined. One hundred percent ethanol was gradually added to the supernatant, until a final concentration of 80% ethanol was reached. The solution was stored overnight at -20°C and then centrifuged for 10 minutes at 12 000 rpm. The supernatant was discarded, and the pellet rinsed with 5 ml of 100% ethanol. Centrifugation was repeated (10 minutes at 12 000 rpm), the supernatant was discarded, and the remaining pellet was rinsed in 1 ml of diethyl ether. The pellet was broken using a spatula, and placed in a vacuum flask until completely dry. The glycoprotein powder was dissolved in sterile saline before injection.

2.2.3 Hormone administration

Two experiments were conducted in which PMSG was administered using Alzet mini-osmotic pumps, models 2001 and 2002. The model 2001 pump has a delivery rate of 1.0 µl/hour, and delivers hormone continuously for one week. For both experiments, the low dose 2001 pump was loaded with a solution of PMSG so that 40 IU of PMSG would be delivered daily to the bird. The model 2002 pump delivers continuously for two weeks at a rate of 0.5 µl/hour. In experiment 1, model 2002 pumps were loaded with a PMSG solution enabling 160 IU to be administered to the bird daily, and this dose was decreased to 120 IU for experiment 2. Pumps were implanted subcutaneously on the underside of the body beside the thigh following local anaesthesia of a small area of skin using Lopaine (lignocaine hydrochloride) at 10 µg per gram of body weight. A small incision was made in the abdominal skin beside the thigh, and the pump inserted.

The incision was then closed using a surgical wound clip (Autoclip, Becton Dickinson, USA), and a topical antibiotic cream applied (Bactroban, Smith Kline Beecham).

The glycoprotein preparation and hCG were administered via intraperitoneal injections. Both hormones were dissolved in 0.9% sterile saline (7.5 mg of glycoprotein /1 ml saline and 10 000 IU hCG /1 ml saline), and each treated bird received a volume of 200 µl of hormone solution per injection.

2.2.4 Experimental design

2.2.4.1 Experiment 1

Forty-two female birds were divided into five groups as follows:

PMSG and hCG 1 Twelve females were held on short days at 10°C from three weeks of age. At six weeks of age, each bird was implanted on the right-hand side of the body with a model 2001 osmotic pump (low dose pump delivering 40 IU PMSG/day). A model 2002 osmotic pump (high dose pump delivering 160 IU PMSG/day) was implanted on the left-hand side of the body at seven weeks of age; at this time, the model 2001 pump was empty, but was not removed from the body. Five days after implantation of the second pump, birds received intraperitoneal injections of 2 000 IU hCG daily for two days, and then received a final injection of 6 000 IU hCG on the third day. A sexually active male was placed in the cage with each female for 30 minutes before each injection, allowing enough time for at least one successful mating to occur. Any eggs that were laid were incubated in an Ovo-Lux 100 electronic incubator (SP. R. L. Humblet, Belgium) at a constant temperature of 37.5°C and 60% humidity. Eggs were incubated for 17 days, and turned through a 90° angle four times a day. Birds in this group were euthanased at nine weeks of age using a standard method of stunning followed by decapitation.

PMSG and hCG 2 Twelve females were held on short days at 10°C from three weeks of age, and treated with PMSG in the same manner as the previous group. Injections of

hCG started four days after implantation of the second pump and were administered daily for four days. The first three injections contained a dose of 2 000 IU hCG, and the final injection was increased to a dose of 4 000 IU hCG. Birds were euthanased at nine weeks of age.

PMSG Six females were held on short days at 10°C from three weeks of age. Birds were implanted with model 2001 and 2002 pumps as described above, but did not receive any injections. All birds in this group were euthanased at eight weeks of age in order to determine the state of the reproductive system at the same time as the treatment with hCG started in the other groups of birds.

Long day and short day Six females were held on long days at 20°C and six on short days at 10°C from three weeks of age. Birds received no implants or injections, and were euthanased at nine weeks of age for comparison with hormone treated groups.

2.2.4.2 Experiment 2

Twenty-three female quail were divided into three groups as follows:

PMSG Nine females were held on short days at 10°C from three weeks of age. At six weeks of age, each bird was implanted with a low dose (40 IU/day) model 2001 osmotic pump on the underside of the body, beside the right thigh. At seven weeks of age, the low dose pump was removed, and replaced with a model 2002 osmotic pump delivering 120 IU PMSG/day. This ensured that the left-hand side of the body remained clear so the ovary could be easily viewed using ultrasound. The ovary of each bird was observed with an Ultramark 9 ultrasound machine (ATL Ultrasound, Australia) every four days after implantation of the second pump. If a follicle greater than 15 mm in diameter (pre-ovulatory F_1 follicle) was visible, then the bird was given daily injections of pituitary glycoprotein extract for at least two days. Birds with ovaries that did not meet this criterion were checked again using ultrasound every three to four days for up to two weeks following implantation of the second pump. Injections were administered when follicles greater than 15 mm in diameter were present. A sexually active male was placed in the cage with each female for 30 minutes before each injection, allowing

enough time for at least one successful mating to occur. Any eggs that were laid were incubated in an incubator. Birds were euthanased at nine weeks of age.

Long day Six female quail were held on long days at 20°C (LD 16:8) from 3 weeks of age to serve as control birds. These birds were euthanased at nine weeks of age.

Short day Eight female quail were held on short days at 10°C (LD 8:16) from 3 weeks of age to serve as control birds. These birds were euthanased at nine weeks of age.

All birds in experiments 1 and 2 had *ad libitum* access to food and water. Body weight and the diameter of the cloacal opening were measured in all birds every second day from three weeks of age (except treated birds in experiment 1 which were measured and weighed every two days from the start of treatment). The cloacal opening diameter increases as the oviduct develops, so this is a useful external indicator of reproductive tract growth. Blood samples were collected following decapitation, and plasma was stored at -20°C until assayed for estradiol and PMSG concentration. Ovaries and oviducts were excised, and weights of each recorded. The numbers of large yellow, small yellow, and large white follicles present on each ovary were recorded, and the diameter of each largest follicle in each size class measured. The number of large atretic follicles on the ovary was also recorded. Ovaries and oviducts were immediately fixed in Bouin's solution for histological studies carried out by Dr. Jane Girling and Mr. Guy Hessell.

All experimental procedures were approved by the Massey University Animal Ethics Committee.

2.2.5 Radio- and enzyme-immunoassay of plasma hormones

Plasma estradiol concentrations in plasma samples were measured by radioimmunoassay, and plasma PMSG concentrations by enzyme-immunoassay.

2.2.5.1 Extraction of estradiol from plasma

To separate lipid from the plasma, each sample was thawed and spun at 14 000 rpm for 5 minutes (IEC Micromax ventilated microcentrifuge OM3590) in a 1.5 ml Eppendorf tube. A 200 µl aliquot of clear plasma from below the lipid layer was transferred to a glass screw-top extraction tube (13 x 100 mm) and 2 ml of distilled dichloromethane (Analar, BDH) was added using a Merck bottle-top dispenser. The plasma and dichloromethane were vortexed together for 10 seconds, centrifuged at 1 900 g (Beckman TJ-6 centrifuge) for five minutes to ensure none of the plasma was left on the sides of the tube and shaken for one hour on an orbital shaker (Chiltern Scientific SS70). Samples were then centrifuged at 1 900 g for 10 minutes to separate the organic and aqueous phases. A 1 600 µl aliquot from the organic phase was removed from each tube, placed into an open-top glass tube (Kimax, 13 x 100 mm) and dried under a stream of air in a heating block at 37°C. The extract was reconstituted in 350 µl phosphate-buffered saline with gelatine (PBSG), vortexed three times at five-minute intervals, shaken for one hour at room temperature and left overnight at 4°C. Two 100 µl aliquots of this extract were then transferred into polystyrene assay tubes (12 x 75 mm) for immediate assay.

The recovery of estradiol during the extraction process was measured by adding 20 µl of tritiated estradiol solution (5 000 cpm) to 30 samples in a single extraction. The percentage recoveries from plasma samples were calculated for birds held on short days, birds held on long days and birds treated with PMSG. The mean percentage recovery for estradiol in quail plasma was 90.2 ± 3.1% for short day samples (n=10), 81.3 ± 3.1% for long day samples (n=10), and 78.5 ± 3.1% for samples from PMSG treated birds (n=10).

2.2.5.2 Radioimmunoassay of estradiol

One hundred microlitres of reconstituted plasma extract in PBSG was incubated with 100 µl of antibody (Etches, Canada; estradiol antiserum 41-12 raised in sheep) and 100 µl of tritiated estradiol (^{3}H-estradiol TRK.332, Amersham, UK; 5 000 cpm) overnight at

4°C. Five hundred microlitres of dextran-coated charcoal (2.5 g/l charcoal [Sigma], 0.25 g/l dextran [Dextran T70, Amersham Pharmacia] in PBSG) was added to each sample using an Eppendorf multi-pipetter and was incubated with the sample for 15 minutes at 4°C to separate bound and free estradiol. Samples were then centrifuged at 2 000 g for 15 minutes at 4°C (Beckman GS-6R refrigerated centrifuge), and the supernatant poured off into a 5 ml polypropylene scintillation vial. A Merck bottle-top dispenser was used to add 3 ml of scintillant (5 g/l PPO [2,3-diphenyl-oxazole, Sigma], 0.3 g/l dimethyl POPOP [1,4-bis-{methyl-5-phenyl-2-oxazoly}-benzene, Sigma] in toluene [Mobil]) to each vial, then the samples were shaken for one hour on an orbital shaker. The samples were left at room temperature for one hour, then were counted in a Wallac 1409-411 liquid scintillation counter for five minutes each.

The sensitivity of the radioimmunoassay for estradiol was determined as the hormone concentration at the mean minus two standard deviations from the percentage bound of the zero hormone tubes. The sensitivity was 17.6 pg/ml on the standard curve, which was equivalent to an estradiol concentration in quail plasma of 40.7 pg/ml (n=5 assays). Serial dilutions of extracted quail plasma in assay buffer (PBSG) were parallel to the estradiol standard curve (n=3). Recovery of estradiol added to quail plasma was 90.8 ± 8.8%, 92.9 ± 8.9% and 92.3 ± 13.3% for three different samples (W.H. Chua, pers. comm.).

Solutions of estradiol in PBSG at concentrations that gave approximately 20, 50 and 80% binding on the standard curve were used as high, medium and low quality controls in every assay. The mean concentrations of these standards were 1908.5 ± 30.7, 496.9 ± 23.0 and 105.4 ± 6.9 pg/ml respectively. Intra-assay coefficients of variation for estradiol were determined by conducting an assay with ten duplicates of each quality control. The intra-assay coefficients of variation for estradiol were 9.2%, 4.5% and 11.4% for high, medium and low quality controls respectively. Inter-assay coefficients of variation were calculated from duplicates of the quality controls included at the beginning and end of each assay. The inter-assay coefficients of variation for seven assays were 1.7%, 4.6% and 6.5% for high, medium and low quality controls respectively.

The cross-reactivity of the estradiol antibody was reported by Etches *et al.* (1981) as follows: estradiol 17-α (<1.0%), estriol (<1.0%) and estrone (4.3%).

2.2.5.3 Enzymeimmunoassay of PMSG

The concentration of PMSG in plasma samples from all treated birds was measured by Dr. Keith Henderson (AgResearch, Wallaceville Animal Research Centre, Upper Hutt, New Zealand) using a competitive enzymeimmunoassay (EIA) utilising reagents purchased from Biogenesis Ltd., Poole, England, UK. The antibody was a rabbit polyclonal antibody generated against PMSG. Its relative cross-reactivities with PMSG, eLH and eFSH were 100%, 30% and 10% respectively. The purified PMSG preparation used as the assay standard had a potency, as provided by the supplier Biogenesis Ltd., of 10 000 IU per mg powder, expressed relative to the second international PMSG reference standard 62/1. A less purified preparation of PMSG (also obtained from Biogenesis) was used as the coating antigen in the EIA, which was performed similarly to that previously described by Henderson *et al.* (1998). Wells of microtitre plates (Nunc, Maxisorp C12) were coated with the PMSG antigen by overnight incubation at 4°C with 0.25 IU of PMSG prepared in 0.1 ml of coating buffer (50 mM carbonate-bicarbonate buffer, pH 9.6). The wells were emptied by inversion, and remaining active sites saturated by incubation for 30 minutes at room temperature with 0.25 ml blocking buffer (coating buffer containing 0.5% gelatin). Wells were emptied and washed three times with washing buffer (10 mM phosphate buffer containing 0.8% NaCl and 0.05% Tween 20, pH 7.4). Replicate wells then received an aliquot of quail plasma (0.02 ml) or PMSG standard diluted to 0.1 ml with assay buffer (washing buffer containing 0.1% gelatin and 0.01% thimerosal, pH 7.4). This was followed by 0.1 ml of the PMSG antibody (1/10 000 dilution), and the plates were incubated for 2 h at 37°C. After emptying and washing, the wells received 0.1 ml of anti-rabbit IgG-horseradish peroxidase conjugate (1/1 000, Sigma Chemical Co., Missouri, USA) diluted in assay buffer, and the plates were incubated for 1 h at 37°C. After emptying and washing the wells three times, the peroxidase activity in each well was quantified by the addition of 0.1 ml of substrate-chromogen solution (50 ml of 0.05 M phosphate citrate buffer, pH 5.0, containing 20 mg o-phenylenediamine and 0.02 ml 30% H_2O_2). Colour was allowed to develop for 15 to 30 minutes at 37°C before the

reaction was stopped with 2M H_2SO_4 (0.05 ml/well) and the colour intensity read at 490 nm using a Bio-Tek ELX800 automated microplate reader. The concentration of PMSG in the quail plasma samples was calculated by interpolation from the standard curve, which had a working range from 1 IU/ml to 50 IU/ml. For the purpose of analysis, samples with non-detectable levels of PMSG were recorded as having 1 IU/ml (the minimum detectable concentration of the assay). Intra- and inter-assay coefficients of variation were <9%. Dose response dilutions of quail plasma (0.005 to 0.02 ml) were parallel to the standard curve.

2.2.6 Statistics

All variables were tested for homogeneity of variance using Levene's test. When variances were homogeneous across groups for a single variable, ANOVA followed by Bonferroni's post hoc tests were used to analyse data. When variances were not homogeneous across groups, Kruskal-Wallis non-parametric ANOVA followed by Mann-Whitney U tests were performed to determine differences between groups. One-way repeated measures ANOVA was used to analyse changes in cloacal diameter over time. Relationships between variables were investigated using linear regression to calculate r^2 values.

ANOVAs and non-parametric equivalents were carried out using Systat Version 8.0 (SPSS Inc., 1988), and relationships between variables (linear regression) were analysed using GraphPad Prism Version 3.0 (GraphPad Software Inc., 1999). Data were transformed to logarithms where necessary before analysis. Data are shown as mean ± standard error.

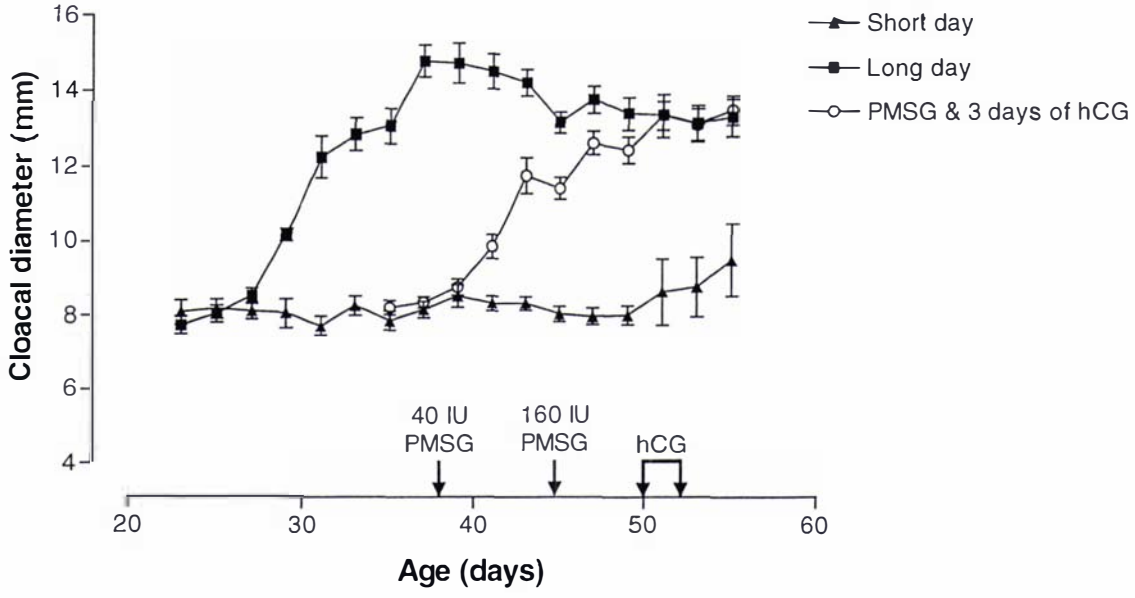

Figure 2.1. Changes in cloacal diameter of birds on short days, long days, and short days plus PMSG and hCG treatment (experiment 1). Days of implantation of low dose (40 IU PMSG) and high dose (160 IU PMSG) pumps, and the period of daily hCG injections are indicated by arrows on the x axis.

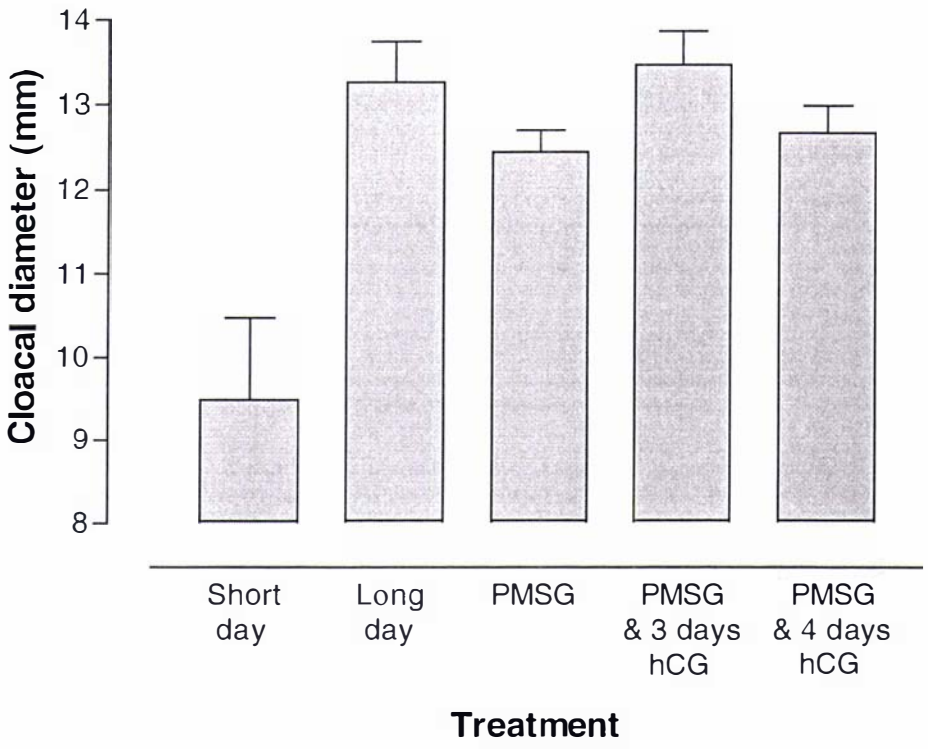

Figure 2.2. Mean cloacal diameter of birds on short days, long days, and short days plus treatment with 40 IU PMSG daily for one week followed by 160 IU PMSG daily for two weeks administered by osmotic pump (experiment 1). Two groups also received injections of hCG for three or four days.

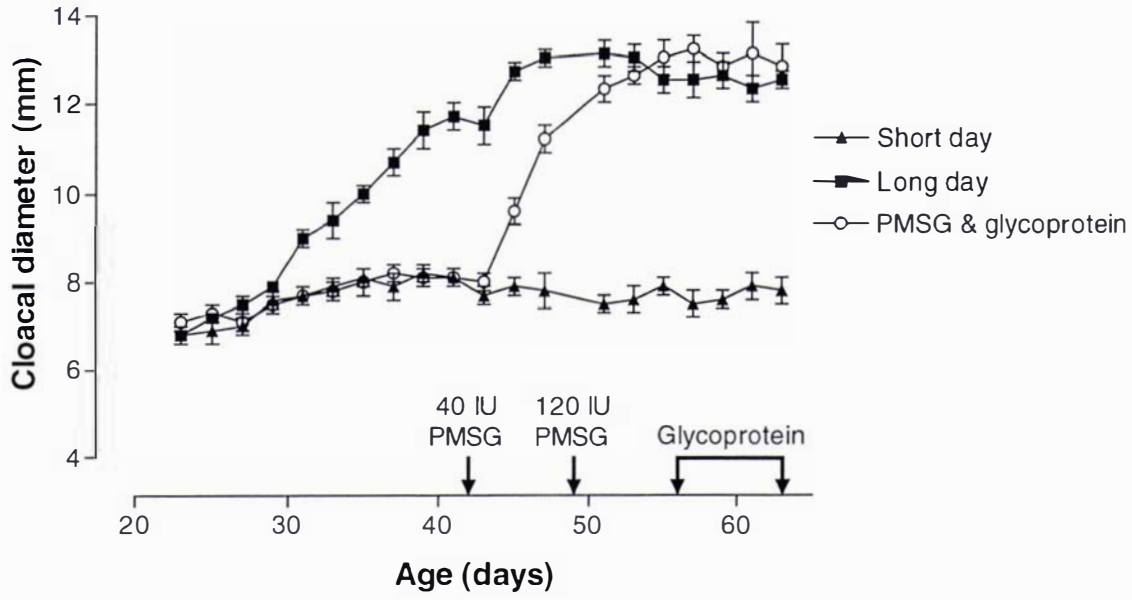

Figure 2.3. Changes in cloacal diameter of birds on short days, long days, and short days plus PMSG and glycoprotein treatment (experiment 2). Days of implantation of low dose (40 IU PMSG) and high dose (120 IU PMSG) pumps, and the period of glycoprotein injections are indicated by arrows on the x axis.

2.3 Results

2.3.1 External measurements and observations

2.3.1.1 Cloacal diameter

In both experiments 1 and 2, the cloacal opening of all PMSG treated birds increased after three weeks of treatment to a diameter similar to that of long day control birds (Figures 2.1, 2.2 and 2.3 (only one PMSG group shown in Figure 2.1), see Tables 2.1-2.4 for statistics). The cloacal diameter of birds held on short days at 10°C remained small and showed no significant change throughout either experiment. The first three days were excluded from the analysis of short day birds from experiment 2, as cloacal diameter increases slightly during this time as the birds are growing. Long day control groups and all of the PMSG treated groups had significantly larger cloacal diameters at the end of both experiments than at the start (Figures 2.1 and 2.3; see Tables 2.1 and 2.2 for statistics). Birds kept on short days had significantly smaller cloacal diameters than the long day birds or any of the treated groups at the end of both experiments. All PMSG treated birds had cloacal diameters similar to birds held on long days (see Tables 2.3 and 2.4 for statistics).

Table 2.1. Summary of one-way repeated measures ANOVA for changes in cloacal opening diameter across days for each group in experiment 1.

Group	F	df	*p*
Short day	0.965	16, 80	0.502
Long day	36.241	16, 64	0.000
PMSG	48.681	7, 28	0.000
PMSG & 3 days hCG	64.670	10, 100	0.000
PMSG & 4 days hCG	75.493	10, 100	0.000

Table 2.2. Summary of one-way repeated measures ANOVA for changes in cloacal opening diameter across days for each group in experiment 2.

Group	F	df	*p*
Short day (without first 3 days)	1.445	16, 112	0.134
Long day	64.943	19, 95	0.000
PMSG & glycoprotein	79.354	19, 114	0.000

Table 2.3. Summary of one-way ANOVA and Bonferroni's posthoc tests comparing cloacal opening diameter between groups at the end of experiment 1.

ANOVA	F	df	*p*
All groups	9.450	4, 30	0.000
Bonferroni's posthoc tests			***p***
Short day vs long day			0.000
Short day vs PMSG			0.010
Short day vs PMSG + 3 days hCG			0.000
Short day vs PMSG + 4 days hCG			0.002
Long day vs PMSG			1.000
Long day vs PMSG + 3 days hCG			1.000
Long day vs PMSG + 4 days hCG			1.000
PMSG vs PMSG + 3 days hCG			1.000
PMSG vs PMSG + 4 days hCG			1.000
PMSG + 3 days hCG vs PMSG + 4 days hCG			0.991

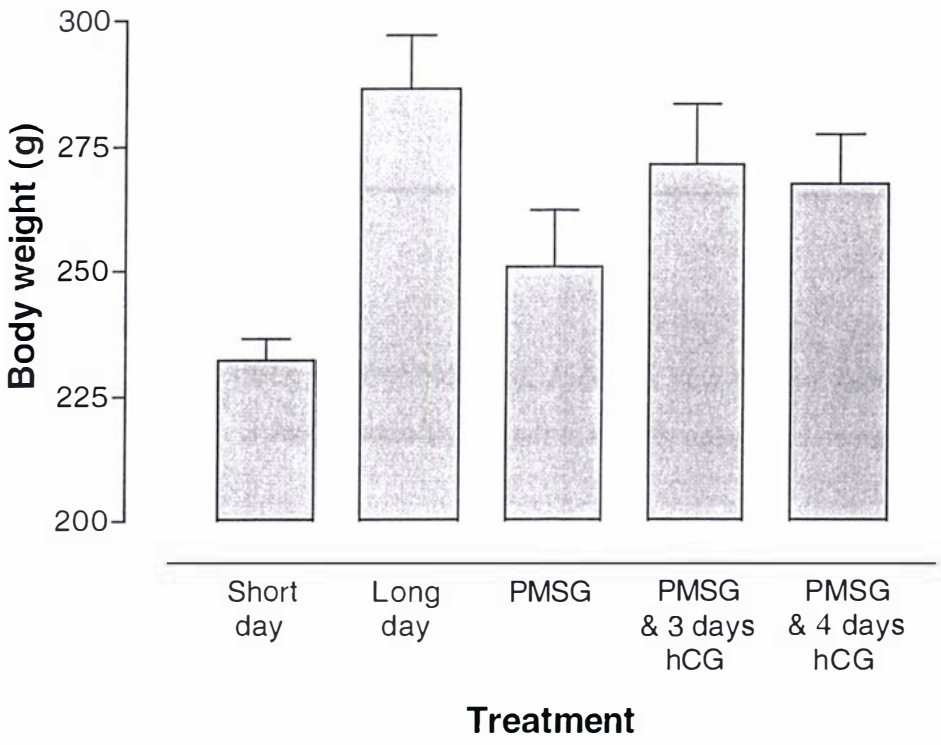

Figure 2.4. Mean body weight for birds on short days, long days, and short days plus treatment with 40 IU PMSG daily for one week followed by 160 IU PMSG daily for two weeks administered by osmotic pump. Two groups also received injections of hCG for three or four days.

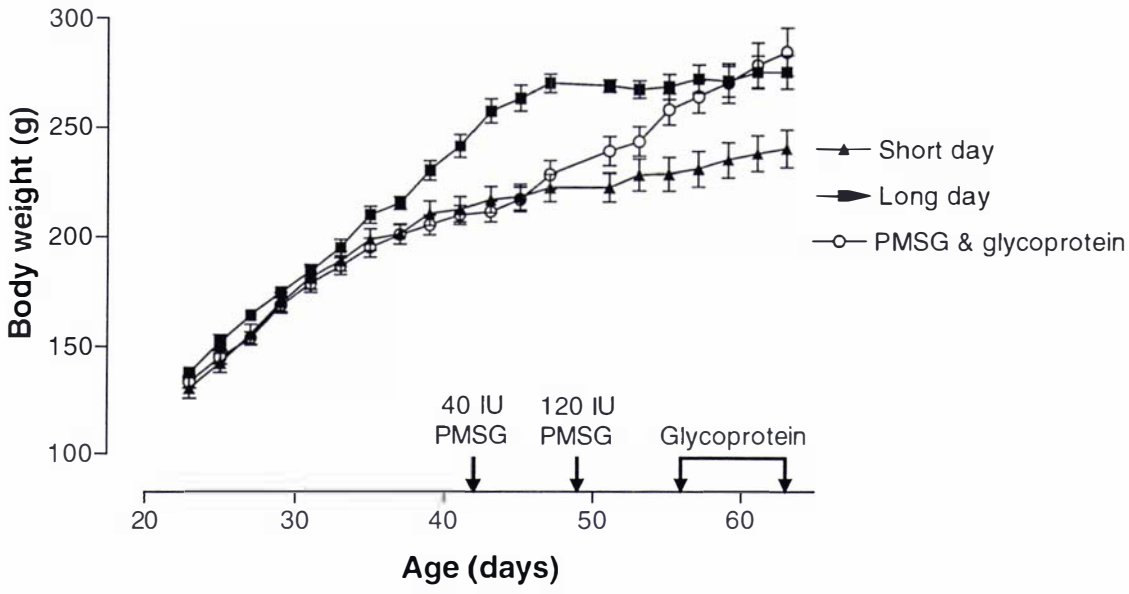

Figure 2.5. Changes in body weight of birds on short days, long days, and short days plus PMSG and glycoprotein treatment (experiment 2). Days of implantation of low dose (40 IU PMSG) and high dose (120 IU PMSG) pumps, and the period of glycoprotein injections are indicated by arrows on the x axis.

Table 2.4. Summary of one-way ANOVA comparing cloacal opening diameter between groups at the start and end of experiment 2, and Bonferroni's posthoc tests comparing cloacal opening between groups at the end of experiment 2.

ANOVA	**F**	**df**	***p***
Cloacal diameter at start	1.398	2, 19	0.271
Cloacal diameter at end	54.061	2, 18	0.000
Bonferroni's posthoc tests (end of experiment 2)			***p***
Short day vs long day			0.000
Short day vs PMSG + glycoprotein			0.000
Long day vs PMSG + glycoprotein			1.000

2.3.1.2 Body weight

Body weight was measured at the end of experiment 1 (Figure 2.4) and every two days in all birds in experiment 2 from three weeks of age (Figure 2.5). Short day controls were significantly lighter than long day controls at the end of both experiments (see Tables 2.5 and 2.6 for statistics). All PMSG and hCG/glycoprotein treated groups were significantly heavier than birds held on short days receiving no treatment, except for one group receiving only PMSG in experiment 1. Birds treated with PMSG only were significantly lighter than the long day birds, but not significantly smaller than the other treated groups. All treated birds, except for the PMSG only group, had similar body weights to the long day controls.

Table 2.5. Summary of Kruskal-Wallis non-parametric ANOVA and Mann-Whitney U tests comparing body weight between groups at the end of experiment 1.

Kruskal-Wallis	**K-W statistic**	**df**	***p***
All groups	11.384	4	0.023
Mann-Whitney U tests			***p***
Short day vs long day			0.004
Short day vs PMSG			0.144
Short day vs PMSG + 3 days hCG			0.023
Short day vs PMSG + 4 days hCG			0.018
Long day vs PMSG			0.045
Long day vs PMSG + 3 days hCG			0.329
Long day vs PMSG + 4 days hCG			0.239
PMSG vs PMSG + 3 days hCG			0.391
PMSG vs PMSG + 4 days hCG			0.317
PMSG + 3 days hCG vs PMSG + 4 days hCG			0.935

Table 2.6. Summary of one-way ANOVA and Bonferroni's posthoc tests comparing body weight between groups at the end of experiment 2.

ANOVA	**F**	**df**	***p***
All groups	5.651	2, 18	0.021
Bonferroni's posthoc tests			***p***
Short day vs long day			0.042
Short day vs PMSG + glycoprotein			0.024
Long day vs PMSG +glycoprotein			1.000

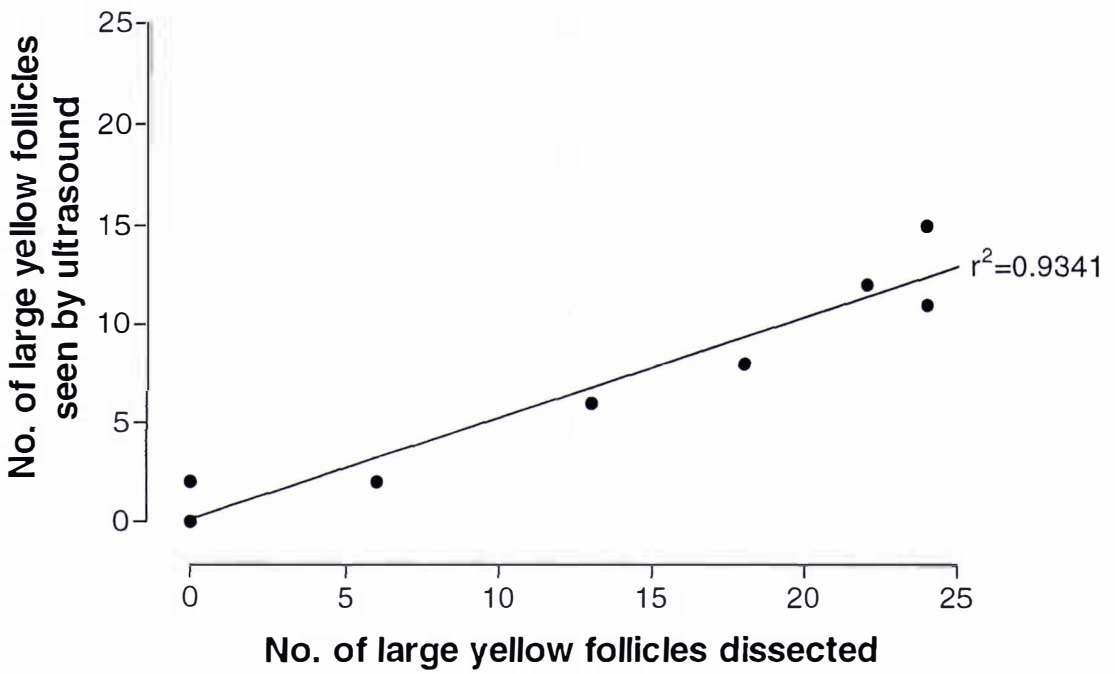

Figure 2.6. Relationship between the numbers of large yellow ovarian follicles in PMSG treated quail seen by ultrasound on day 19 and the number of large yellow follicles dissected on day 21.

2.3.1.3 Ultrasound

The use of ultrasound to detect developing follicles was tested in normal laying females before the start of experiment 2. Follicles greater than 4 mm in diameter could be viewed readily in laying birds. Ultrasound was then used to visualise large yellow follicles in PMSG treated birds (from experiment 2 only) at regular intervals following implantation of the model 2002 osmotic pump. On Day 8, yellow follicles up to 6.5 mm in diameter were observed in six of the treated birds (see Table 2.7). However, none of the PMSG treated birds had developed follicles of an F1 size after one week of treatment, therefore none of the birds were treated with glycoprotein at this time. The ovaries of treated birds were observed again four days later (Day 13), and three of the birds had several follicles greater than 12 mm in size, with at least one follicle being of a suitable size to ovulate. The three birds were subsequently treated with daily injections of glycoprotein. On Day 16, ovaries were viewed again, and a further two birds had sufficient follicular development to attempt induction of ovulation with glycoprotein. The three birds that had been treated with glycoprotein following the previous ultrasound session continued to receive glycoprotein injections. Ovaries of treated birds were observed for the last time 11 days after implantation of the second pump (Day 19). Only one bird, which had not previously received glycoprotein injections, was chosen for treatment following the last ultrasound session. A second bird that had already received treatment continued to receive glycoprotein injections.

There was a very good correlation between the number of large yellow follicles seen by ultrasound on day 19 and the number of large yellow follicles dissected on day 21 ($y = 0.5110x - 0.2888$; Figure 2.6). The number of follicles seen by ultrasound is lower than the actual number of yolky follicles present in the ovary because ultrasound can only detect follicles on one side of the ovary. Ultrasound was a very useful tool for identifying follicles greater than 4 mm in diameter, and providing an indication of the number of follicles present in the ovary.

Table 2.7. Number and size of large yellow follicles present on the ovaries of PMSG treated birds (experiment 2) viewed with ultrasound on four separate occasions.

	Day 8		Day 13		Day 16		Day 19		Dissection	
Bird	No. LYF*	Diam. LYF+	No. LYF	Diam. LYF	No. LYF	Diam. LYF	No. LYF	Diam. LYF	No. LYF	Diam. LYF
Y17	1	5.0	4	8.0	0	-	0	-	0	-
Y53	2	4.4	4	10.4	2	6.7	2	6.9	6	24.1
Y67	0	-	0	-	0	-	0	-	0	-
Y69	3	6.5	3	9.1	4[a]	16.4[a]	6	16.8	13	22.5
Y70	4	6.0	6[a]	15.6[a]	10[a]	20.4[a]	>15	20.4	24	20.1
Y71	0	-	7[a]	17.7[a]	8[a]	25.2[a]	12	23.0	22	19.7
Y72	3	5.5	4	9.4	3	13.9	8[a]	15.5[a]	18	22.1
Y73	0	-	4[a]	14.2[a]	3[a]	20.4[a]	2	15.7	0	-
Y74	3	6.5	3	10.9	5[a]	17.3[a]	11[a]	18.6[a]	24	20.8

* Number of large yellow follicles.
+ Diameter of the largest yellow follicle (mm).
[a] Birds were subsequently treated with glycoprotein.

2.3.1.4 Ovulation and oviposition

All birds in the long day control groups had started laying regularly by the completion of each experiment. Birds usually started laying within two weeks of transfer to a long day photoperiod at 20°C. Human chorionic gonadotropin treatment stimulated ovulation in two of the birds in experiment 1. Single ovulated follicles were found in the body cavity of both the birds upon dissection. Injections of glycoprotein induced egg-laying in one bird (Y71) out of the six that were treated in experiment 2. The bird laid three eggs weighing 9.9 g, 10.2 g and 10.4 g. All eggs were completely shelled and pigmented, but were smaller than eggs laid by long day birds (mean weight = 12.5 ± 0.3 g, n=15). All eggs laid by the PMSG treated bird were fertile (determined by examination of the germinal disc). However, embryos did not develop due to a fault in the turning mechanism of the incubator.

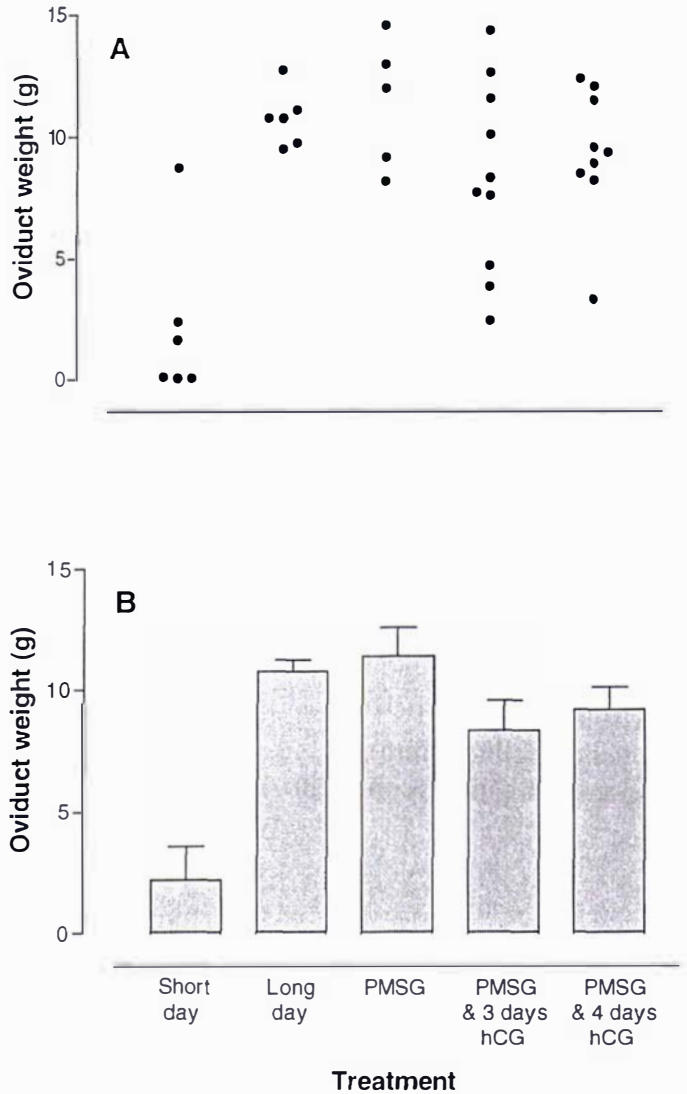

Figure 2.7. Individual oviduct weights (A) and mean oviduct weights (B) for birds on short days, long days, and short days plus treatment with 40 IU PMSG daily for one week followed by 160 IU PMSG daily for two weeks administered by osmotic pump (experiment 1). Two groups also received injections of hCG for three or four days.

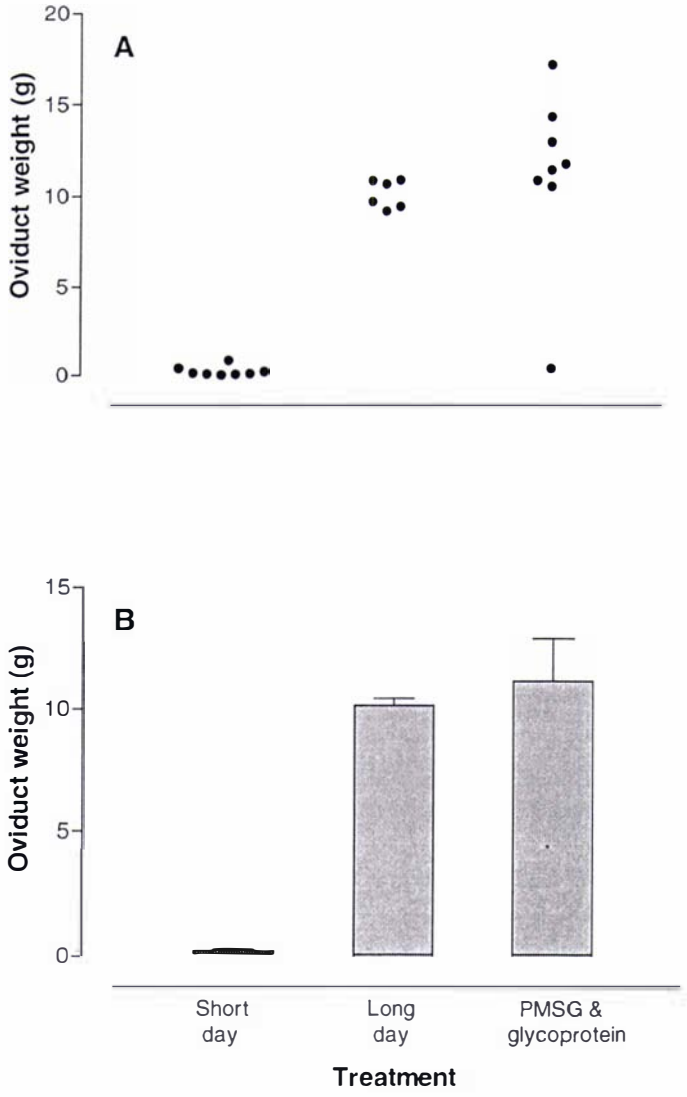

Figure 2.8. Individual oviduct weights (A) and mean oviduct weights (B) for birds on short days, long days, and short days plus treatment with 40 IU PMSG daily for one week followed by 120 IU PMSG daily for two weeks administered by osmotic pump (experiment 2). One group also received glycoprotein injections.

2.3.2 Ovary, oviduct and plasma hormones

2.3.2.1 Oviduct weight

The oviduct remained small in almost all birds held on short days receiving no treatment in both experiments (Figures 2.7 and 2.8). The oviduct developed fully in all birds held on long days, and oviductal development occurred in all but one of the treated birds, although there was considerable variation between birds within a group (Figure 2.7A). The oviduct weights of long day and PMSG treated birds were significantly greater than those of short day birds (see Tables 2.8 and 2.9 for statistics). The mean oviduct weight of PMSG treated birds did not differ significantly from that of long day birds.

Table 2.8. Summary of one-way ANOVA and Bonferroni's posthoc tests comparing oviduct weight between groups at the end of experiment 1.

ANOVA	**F**	**df**	***p***
All groups	8.492	4, 31	0.000
Bonferroni's posthoc tests			***p***
Short day vs long day			0.000
Short day vs PMSG			0.000
Short day vs PMSG + 3 days hCG			0.005
Short day vs PMSG + 4 days hCG			0.001
Long day vs PMSG			1.000
Long day vs PMSG + 3 days hCG			1.000
Long day vs PMSG + 4 days hCG			1.000
PMSG vs PMSG + 3 days hCG			0.774
PMSG vs PMSG + 4 days hCG			1.000
PMSG + 3 days hCG vs PMSG + 4 days hCG			1.000

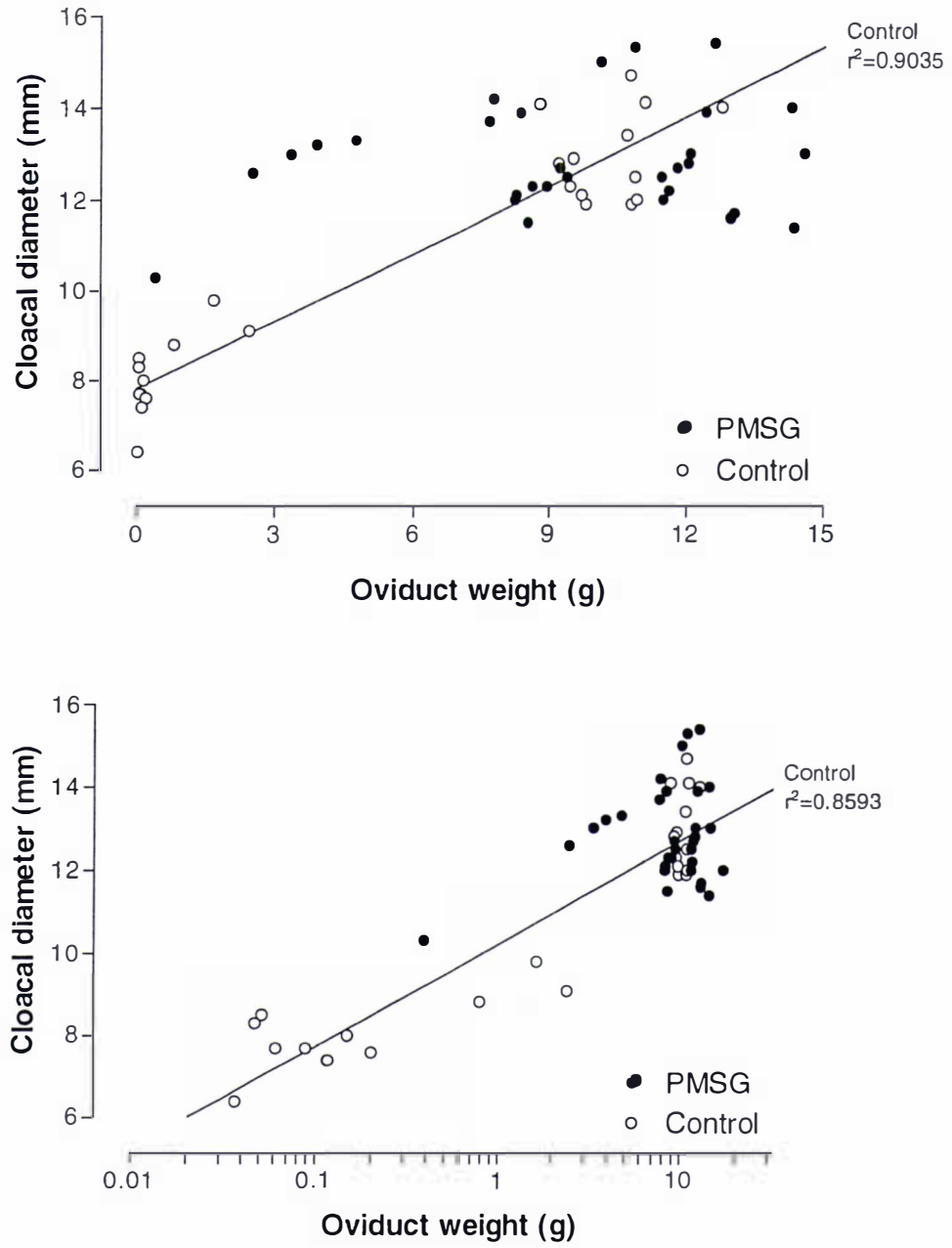

Figure 2.9. Relationship between oviduct weight or log oviduct weight and cloacal diameter of control birds (untreated birds held on long days or short days) and birds held on short days implanted with osmotic pumps that released PMSG continuously.

The relationship between oviduct weight and cloacal diameter for all birds was examined using linear regression (Figure 2.9). There was a clear relationship between oviduct weight and cloacal diameter for untreated birds held on short days or long days (see Table 2.10 for statistics). There was no significant correlation between oviduct weight and cloacal diameter for PMSG treated birds.

Table 2.9. Summary of one-way ANOVA and Bonferroni's posthoc tests comparing oviduct weight between groups at the end of experiment 2.

ANOVA	**F**	**df**	***p***
All groups	32.138	2, 19	0.000
Bonferroni's posthoc tests			***p***
Short day vs long day			0.000
Short day vs PMSG + glycoprotein			0.000
Long day vs PMSG + glycoprotein			1.000

Table 2.10. Summary of linear regression relationship between oviduct weight or log of oviduct weight and cloacal diameter for control and treated birds in experiments 1 and 2.

Group	**●viduct weight**			**Log of oviduct weight**		
	r^2	**Slope**	***p***	**r^2**	**Slope**	***p***
Untreated controls	0.9035	0.49 ± 0.03	<0.0001	0.8593	2.49 ± 0.21	<0.0001
PMSG treated	0.0134	0.04 ± 0.06	0.5347	0.0845	1.09 ± 0.67	0.1127

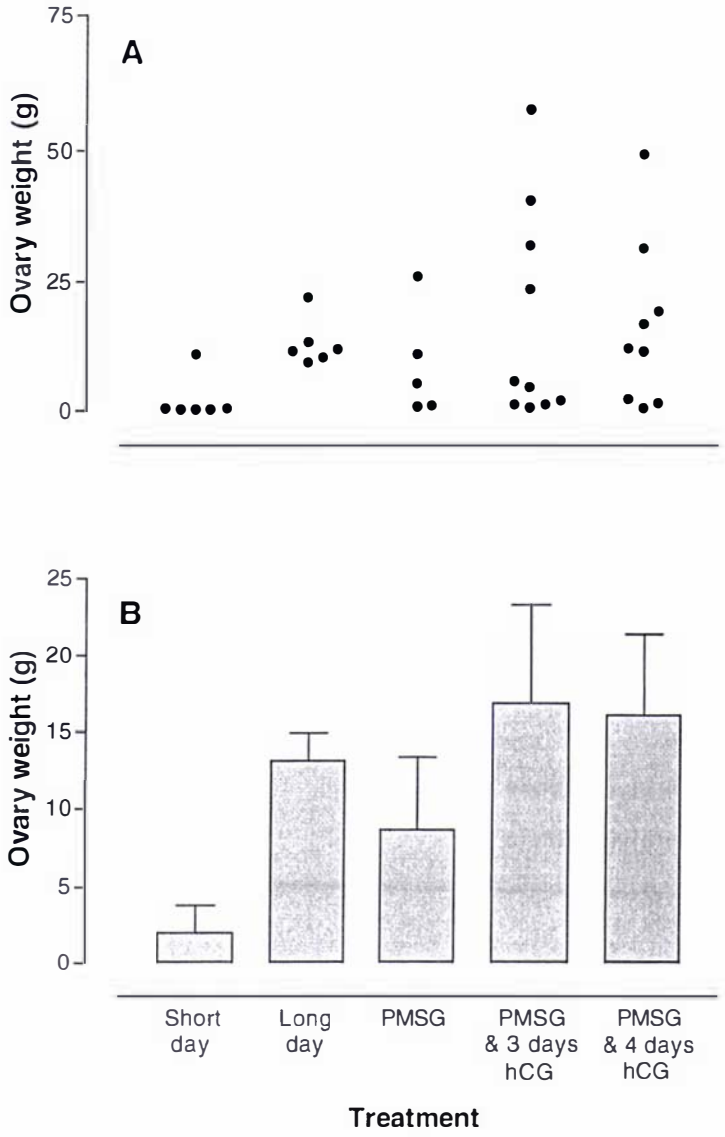

Figure 2.10. Individual ovary weights (A) and mean ovary weights (B) for birds on short days, long days, and short days plus treatment with 40 IU PMSG daily for one week followed by 160 IU PMSG daily for two weeks administered by osmotic pump (experiment 1). Two groups also received injections of hCG for three or four days.

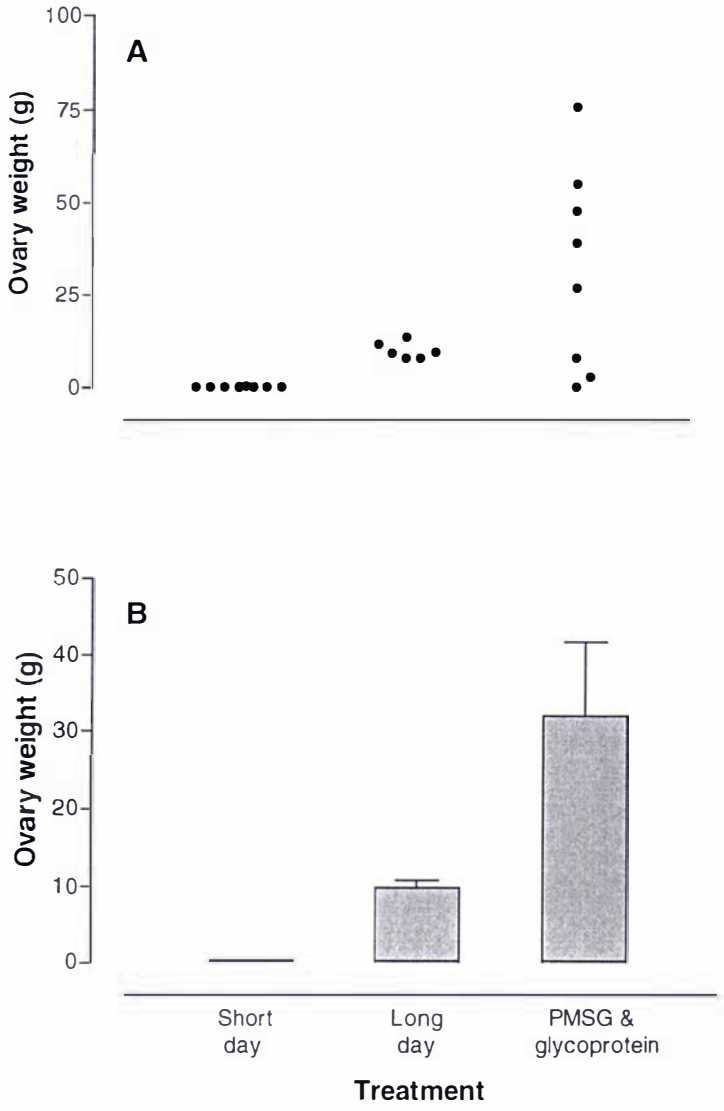

Figure 2.11. Individual ovary weights (A) and mean ovary weights (B) for birds on short days, long days, and short days plus treatment with 40 IU PMSG daily for one week followed by 120 IU PMSG daily for two weeks administered by osmotic pump (experiment 2). One group also received glycoprotein injections.

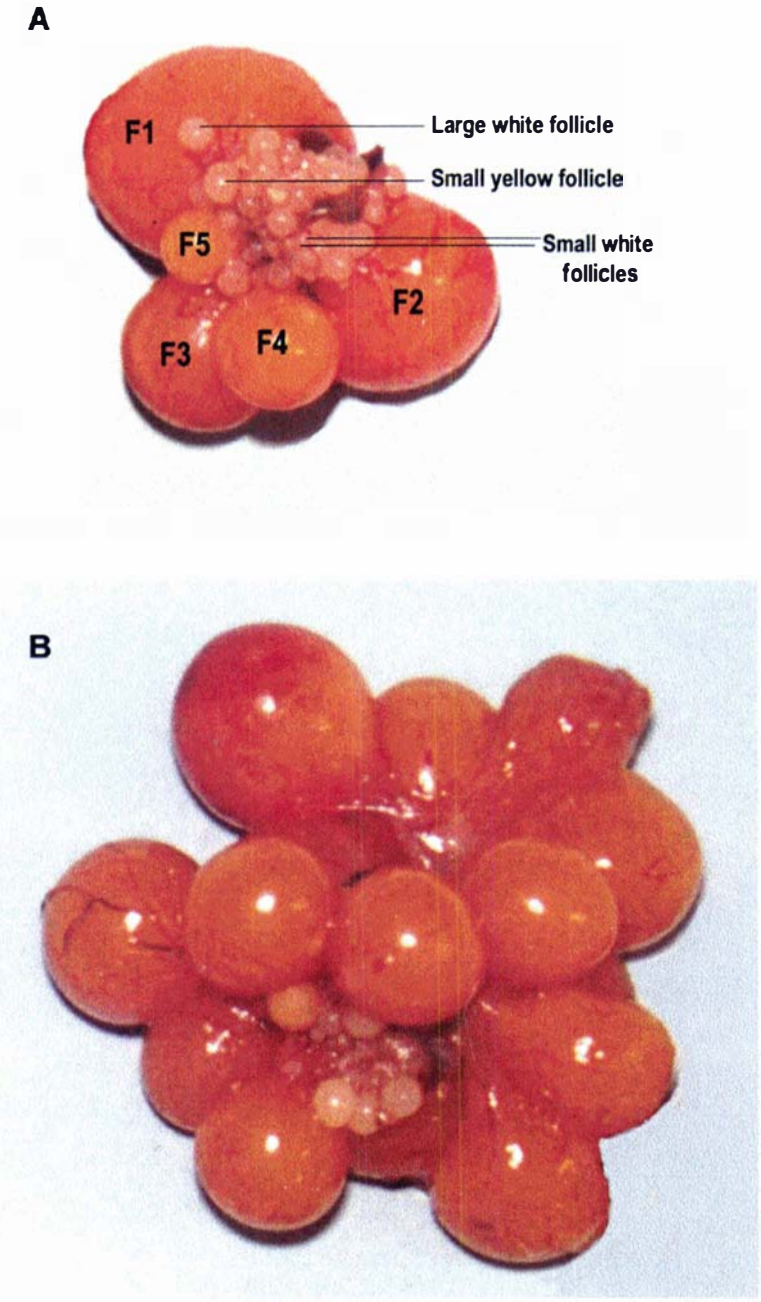

Figure 2.12. The ovary of a normal laying Japanese quail (A) and the ovary of a PMSG treated Japanese quail showing overstimulation of follicular development (B). There are more large yellow follicles present than would be found in a normal laying bird, and the follicles are not arranged in a hierarchy.

2.3.2.2 Ovary weight

With the exception of one bird, the ovaries of all short day control birds remained small (Figures 2.10 and 2.11). The ovaries of all birds held on long days reached full reproductive maturity by the end of the experiment. Treatment with PMSG stimulated ovarian and follicular development but, as with oviduct growth, ovarian weight on dissection was highly variable between birds. The ovaries of several of the PMSG treated birds were up to five times heavier than those of untreated laying birds and showed considerable overstimulation of follicular development (Figure 2.12). In contrast, the ovarian weight of some treated birds was similar to that of untreated short day control birds. The mean ovary weight of all PMSG treated groups did not differ significantly from the ovary weight of birds held on long days (see Tables 2.11 and 2.12 for statistics). Groups of birds on long days or treated with PMSG had significantly larger ovaries than short day birds.

Table 2.11. Summary of Kruskal-Wallis nonparametric ANOVA and Mann-Whitney U tests comparing ovary weight between groups at the end of experiment 1.

Kruskal-Wallis	**K-W statistic**	**df**	***p***
All groups	11.430	4	0.022
Mann-Whitney U tests			***p***
Short day vs long day			0.010
Short day vs PMSG			0.045
Short day vs PMSG + 3 days hCG			0.009
Short day vs PMSG + 4 days hCG			0.005
Long day vs PMSG			0.201
Long day vs PMSG + 3 days hCG			0.515
Long day vs PMSG + 4 days hCG			1.000
PMSG vs PMSG + 3 days hCG			0.462
PMSG vs PMSG + 4 days hCG			0.317
PMSG + 3 days hCG vs PMSG + 4 days hCG			0.807

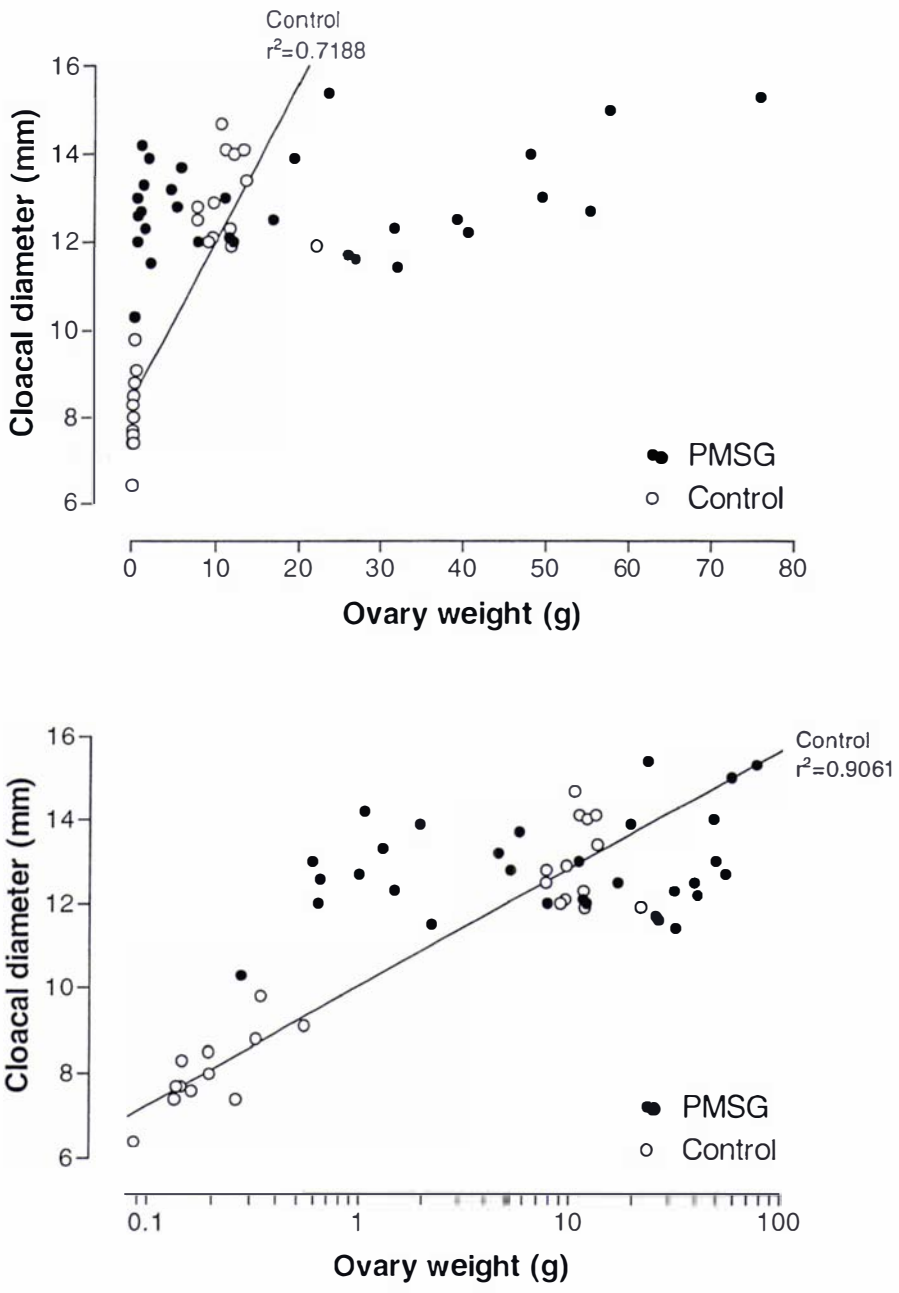

Figure 2.13. Relationship between ovary weight or log ovary weight and cloacal diameter of control birds (untreated birds held on long days or short days) and birds held on short days implanted with osmotic pumps that released PMSG continuously.

Table 2.12. Summary of Kruskal-Wallis nonparametric ANOVA and Mann-Whitney U tests comparing ovary weight between groups at the end of experiment 2.

Kruskal-Wallis	**K-W statistic**	**df**	***p***
All groups	14.488	2	0.001
Mann-Whitney U tests			***p***
Short day vs long day			0.002
Short day vs PMSG + glycoprotein			0.001
Long day vs PMSG + glycoprotein			0.302

The relationship between ovary weight and cloacal diameter for all birds was examined using linear regression (Figure 2.13). There was a clear relationship between ovary weight and cloacal diameter for untreated birds held on short days or long days (see Table 2.13 for statistics). The correlation between ovary weight and cloacal diameter for raw data from PMSG treated birds was almost significant.

Table 2.13. Summary of linear regression relationships between ovary weight or log of ovary weight and cloacal diameter for control and treated birds in experiments 1 and 2.

Group	**Ovary weight**			**Log of ovary weight**		
	r^2	**Slope**	***p***	**r^2**	**Slope**	***p***
Untreated controls	0.8387	0.75 ± 0.07	<0.0001	0.9061	2.79 ± 0.19	<0.0001
PMSG treated	0.1225	0.02 ± 0.01	0.0536	0.0738	0.44 ± 0.29	0.1392

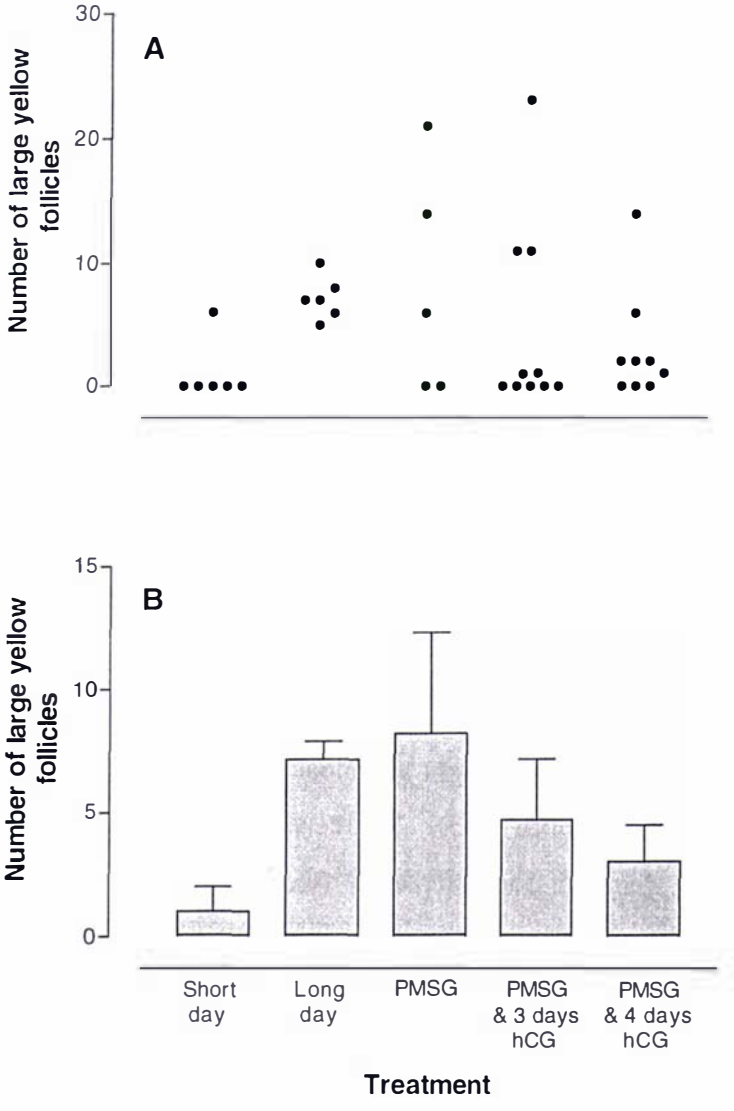

Figure 2.14. Number of large yellow ovarian follicles (A) and mean number of large yellow ovarian follicles (B) for birds on short days, long days, and short days plus treatment with 40 IU PMSG daily for one week followed by 160 IU PMSG daily for two weeks administered by osmotic pump (experiment 1). Two groups also received injections of hCG for three or four days.

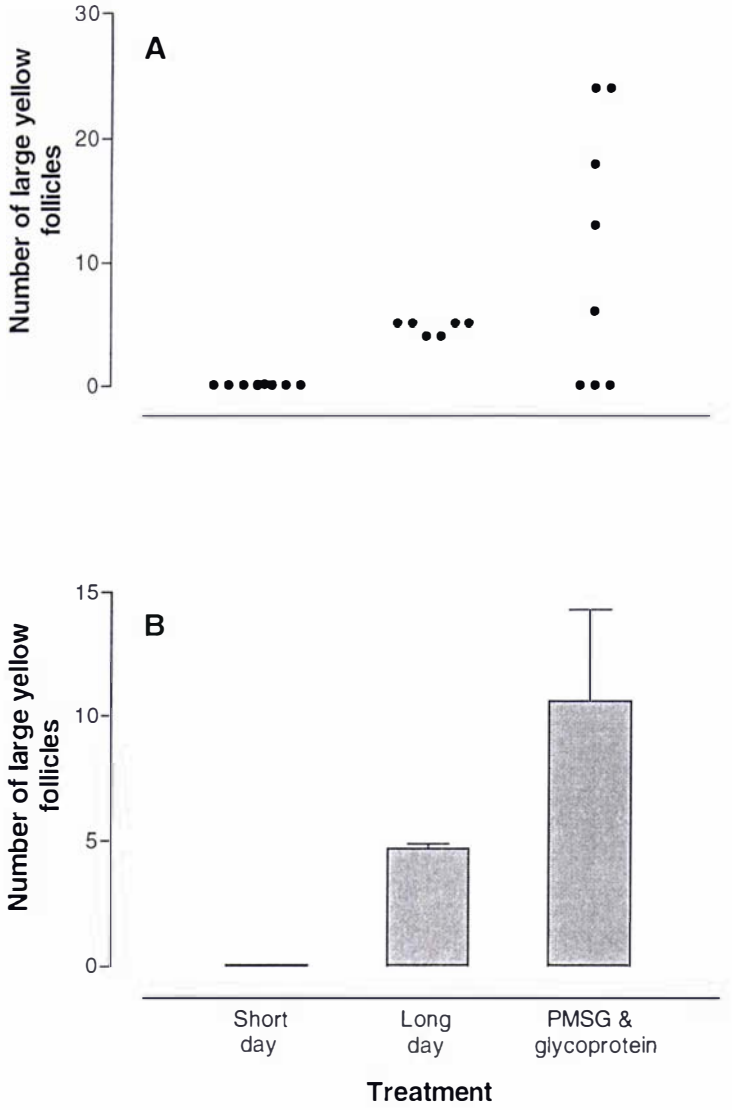

Figure 2.15. Number of large yellow ovarian follicles (A) and mean number of large yellow ovarian follicles (B) for birds on short days, long days, and short days plus treatment with 40 IU PMSG daily for one week followed by 120 IU PMSG daily for two weeks administered by osmotic pump (experiment 2). One group also received glycoprotein injections.

2.3.2.3 Number and diameter of large yellow follicles

All short day birds, except one, did not have yellow ovarian follicles at the time of dissection. The short day control bird whose ovary developed was not included in statistical analyses of these data. The ovaries of birds held on long days contained a normal hierarchy of 5-7 yellow follicles. There was considerable variation in the number and size of yellow follicles in the ovaries of PMSG treated birds (Figures 2.14 and 2.15). Many PMSG treated birds did not develop any yellow follicles after three weeks of treatment. There were significant differences between the numbers of yellow ovarian follicles in short day birds and long day birds for both experiments (see Tables 2.14 and 2.15 for statistics).

Table 2.14. Summary of Kruskal-Wallis nonparametric ANOVA and Mann-Whitney U tests comparing the number of large yellow ovarian follicles between groups in experiment 1.

Kruskal-Wallis	**K-W statistic**	**df**	***p***
All groups	9.847	4	0.043
Mann-Whitney U tests			***p***
Short day vs long day			0.004
Short day vs PMSG			0.054
Short day vs PMSG + 3 days hCG			0.068
Short day vs PMSG + 4 days hCG			0.026
Long day vs PMSG			0.783
Long day vs PMSG + 3 days hCG			0.185
Long day vs PMSG + 4 days hCG			0.020
PMSG vs PMSG + 3 days hCG			0.518
PMSG vs PMSG + 4 days hCG			0.450
PMSG + 3 days hCG vs PMSG + 4 days hCG			0.639

In experiment 1, the group treated with PMSG followed by 4 days of hCG had, on average, significantly more yellow follicles than short day controls, but significantly fewer than birds held under a long day photoperiod. The numbers of large yellow follicles present in the other PMSG treated groups were not significantly different from either control group. Results for experiment 2 were as expected; birds held on long days or treated with PMSG had significantly more yellow follicles than the short day control group. The number of yellow ovarian follicles in treated birds did not differ significantly from that of long day birds.

Table 2.15. Summary of Kruskal-Wallis nonparametric ANOVA and Mann-Whitney U tests comparing the number of large yellow ovarian follicles between groups in experiment 2.

Kruskal-Wallis	**K-W statistic**	**df**	***p***
All groups	10.384	2	0.006
Mann-Whitney U tests			***p***
Short day vs long day			0.000
Short day vs PMSG + glycoprotein			0.001
Long day vs PMSG + glycoprotein			0.430

Several of the large yellow follicles on the ovaries of PMSG treated birds were atretic in appearance. Large yellow atretic follicles were not found in any of the long day control birds.

There were no significant differences in the diameter of the largest yellow ovarian follicle between groups in both experiments ($F_{4, 16}$ =1.874, p=0.169, experiment 1; t=3.007, p=0.117, experiment 2).

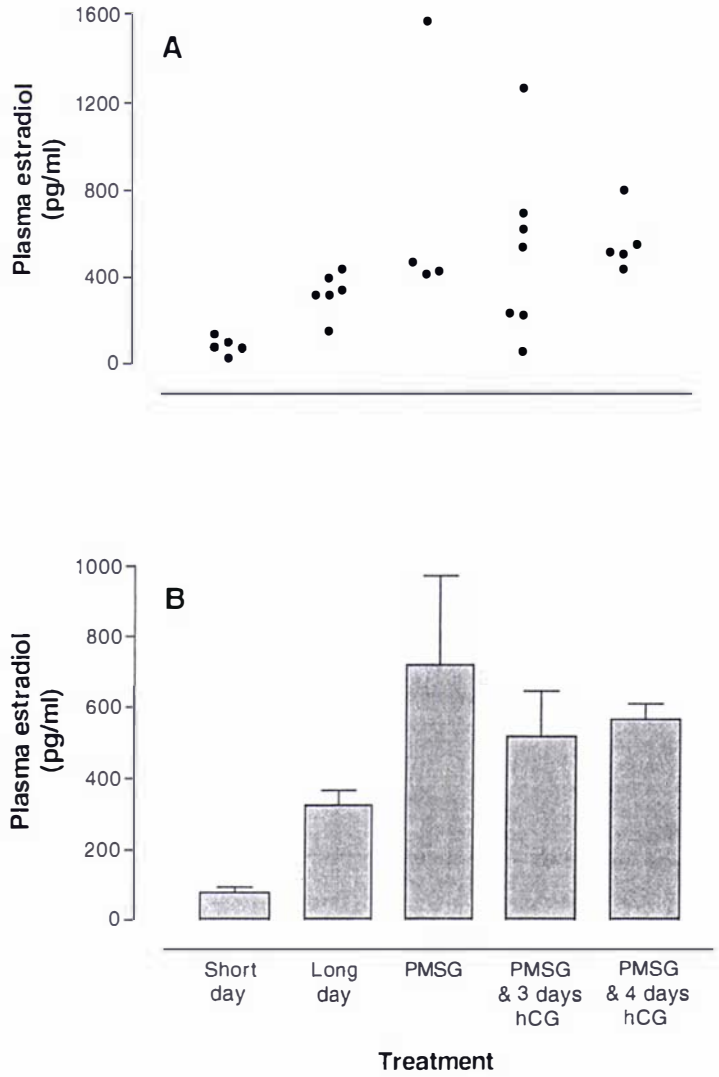

Figure 2.16. Individual plasma estradiol concentrations (A) and mean plasma estradiol concentration (B) for birds on short days, long days, and short days plus treatment with 40 IU PMSG daily for one week followed by 160 IU PMSG daily for two weeks administered by osmotic pump (experiment 1). Two groups also received injections of hCG for three or four days.

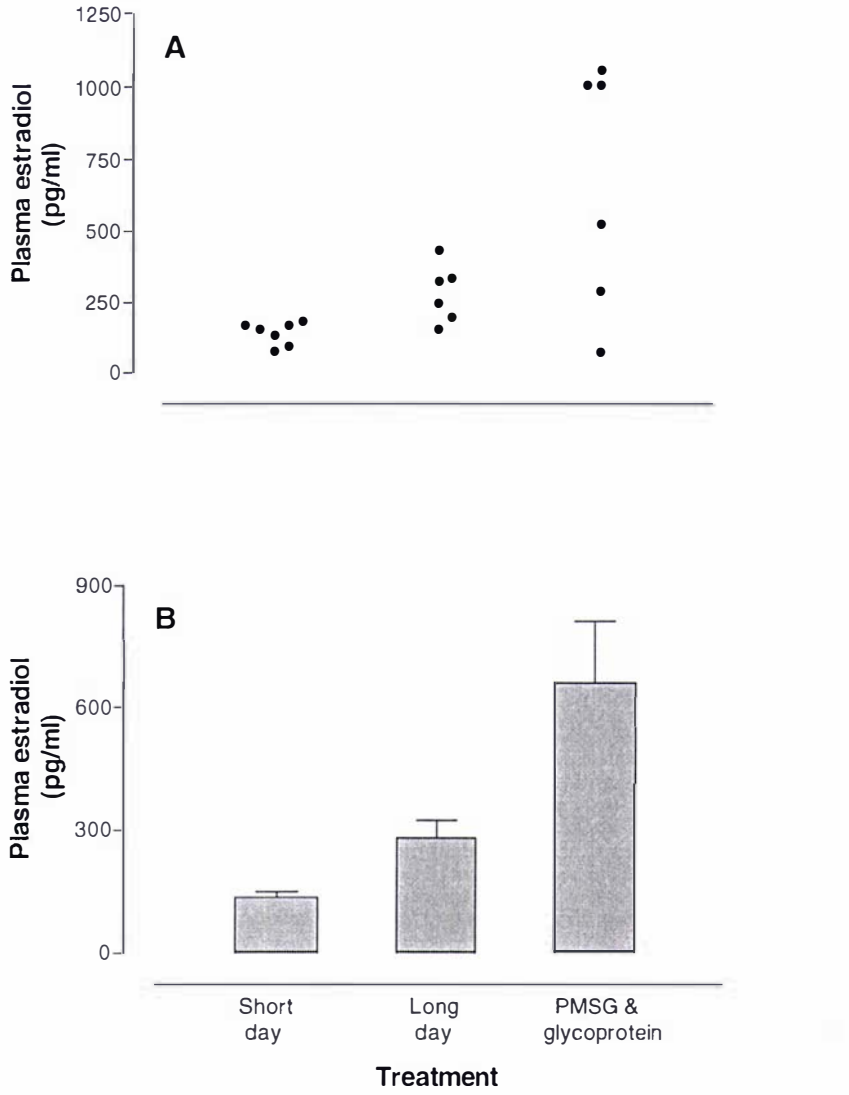

Figure 2.17. Individual plasma estradiol concentrations (A) and mean plasma estradiol concentration (B) for birds on short days, long days, and short days plus treatment with 40 IU PMSG daily for one week followed by 120 IU PMSG daily for two weeks administered by osmotic pump (experiment 2). One group also received glycoprotein injections.

2.3.2.4 Plasma estradiol

Plasma estradiol levels were low in birds held on short days in both experiments, averaging approximately 100 pg/ml (Figures 2.16 and 2.17). Plasma estradiol levels were considerably higher in long day birds (approximately 300 pg/ml), although the difference between short day and long day controls was not significant in experiment 1 (see Table 2.16 for statistics). None of the PMSG treated groups had plasma estradiol levels that were significantly higher than birds held on long days (see Tables 2.16 and 2.17 for statistics), although there was considerable variation in plasma estradiol concentrations between birds in the treated groups (see Figures 2.16A and 2.17A). Estradiol concentrations could not be measured in 20% of the PMSG treated birds because of high lipid levels in those samples. The highest estradiol concentrations in treated birds were two to three times greater than in long day birds.

Table 2.16. Summary of one-way ANOVA and Bonferroni's posthoc tests comparing plasma estradiol concentration between groups in experiment 1.

ANOVA	**F**	**df**	***p***
All groups	5.202	4, 23	0.004
Bonferroni's posthoc tests			***p***
Short day vs long day			0.346
Short day vs PMSG			0.010
Short day vs PMSG + 3 days hCG			0.058
Short day vs PMSG + 4 days hCG			0.009
Long day vs PMSG			0.888
Long day vs PMSG + 3 days hCG			1.000
Long day vs PMSG + 4 days hCG			1.000
PMSG vs PMSG + 3 days hCG			1.000
PMSG vs PMSG + 4 days hCG			1.000
PMSG + 3 days hCG vs PMSG + 4 days hCG			1.000

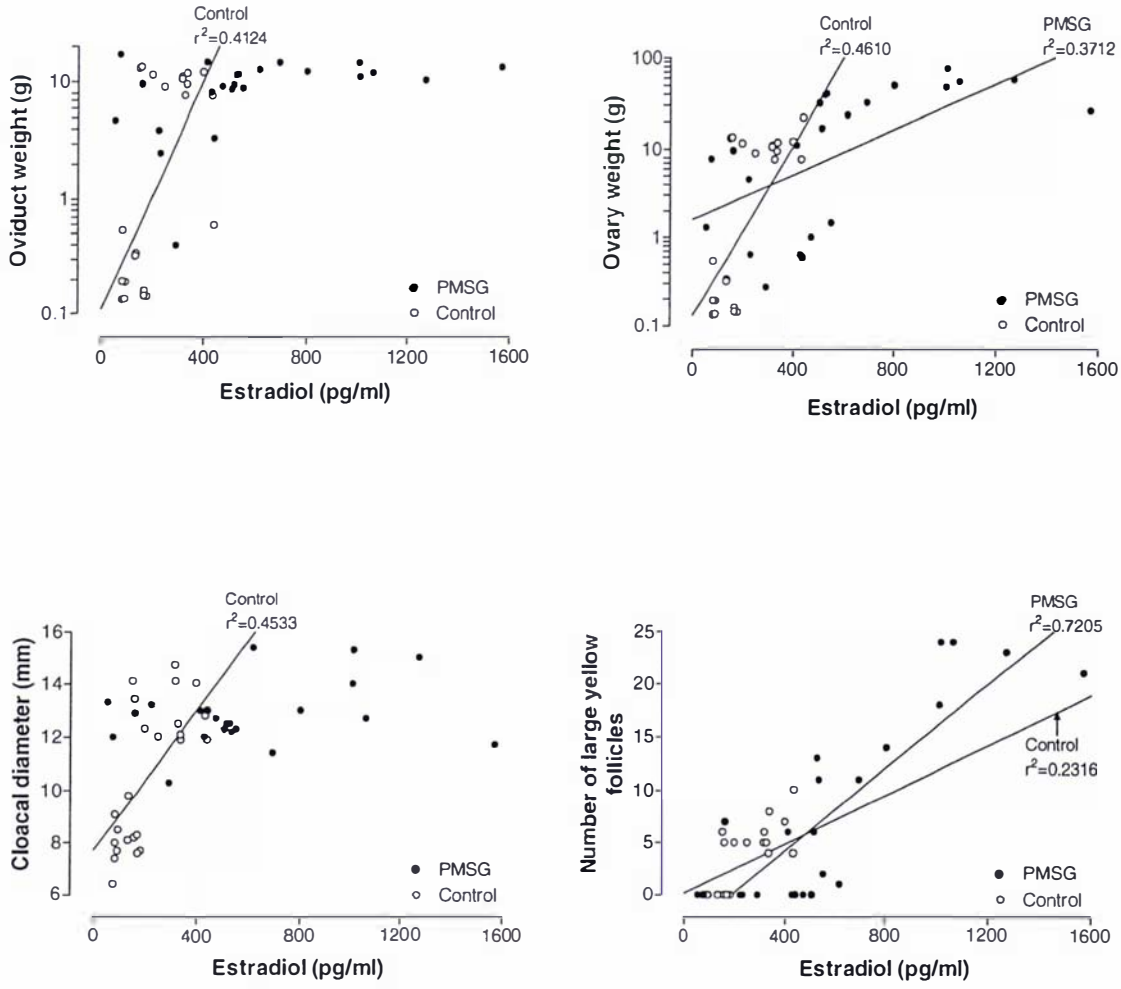

Figure 2.18. Relationships between plasma estradiol concentration and oviduct weight, ovary weight, cloacal diameter and number of large yellow ovarian follicles of control birds (untreated birds held on short days or long days) and birds treated with PMSG by osmotic pumps.

Table 2.17. Summary of Kruskal-Wallis nonparametric ANOVA and Mann-Whitney U tests comparing plasma estradiol concentration between groups in experiment 2.

Kruskal-Wallis	**K-W statistic**	**df**	***p***
All groups	7.830	2	0.020
Mann-Whitney U tests			***p***
Short day vs long day			0.010
Short day vs PMSG + glycoprotein			0.046
Long day vs PMSG + glycoprotein			0.150

The relationships between plasma estradiol concentrations and oviduct weight, ovary weight, cloacal diameter and the number of large yellow follicles were analysed using linear regression (Figure 2.18). There were significant correlations between plasma estradiol concentration and each of the variables listed above in control birds (see Table 2.18 for statistics), with the highest correlation being the relationship between plasma estradiol and ovary weight. There were significant correlations between plasma estradiol concentration and both ovary weight and number of yellow ovarian follicles in PMSG treated birds. There was no significant correlation between plasma estradiol and oviduct weight or cloacal diameter in PMSG treated birds.

Table 2.18. Summary of linear regression relationships between plasma estradiol concentration and log oviduct weight, log ovary weight, cloacal diameter and number of large yellow ovarian follicles (LYF) in experiments 1 and 2.

	Control		**PMSG treated**	
Linear regression	r^2	***p***	r^2	***p***
Oviduct weight	0.4124	0.0003	0.1492	0.0687
Ovary weight	0.4610	<0.0001	0.3712	0.0020
Cloacal diameter	0.4533	0.0001	0.0793	0.2044
Number of LYF	0.2316	0.0110	0.7205	<0.0001

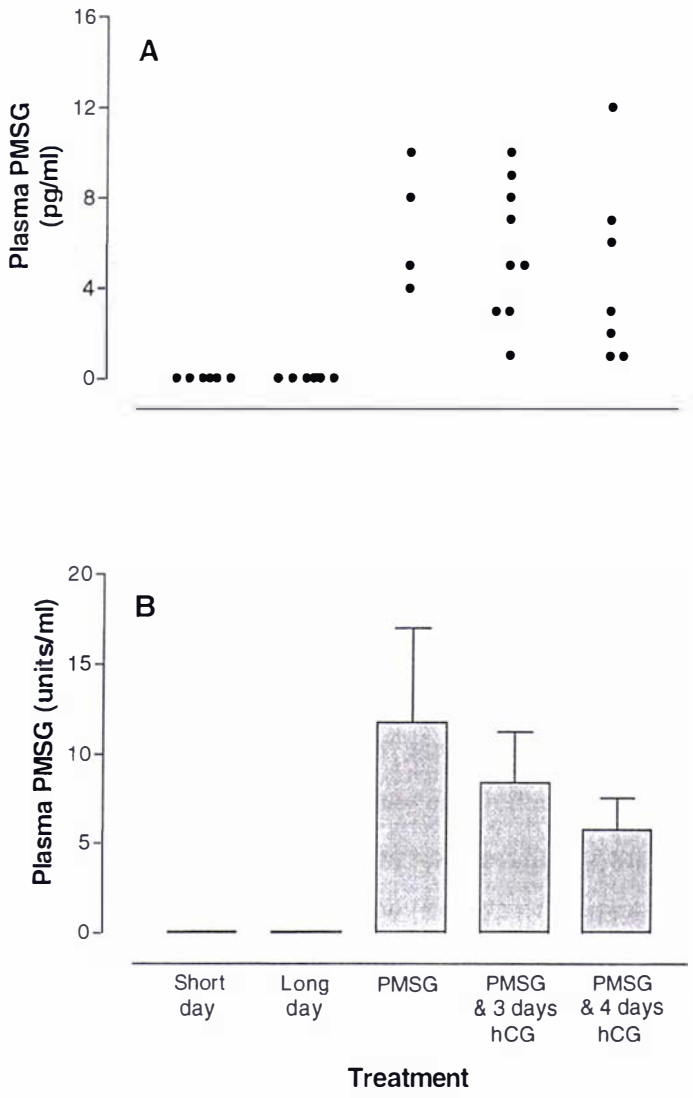

Figure 2.19. Individual plasma PMSG concentrations (A) and mean plasma PMSG concentration (B) for birds on short days, long days, and short days plus treatment with 40 IU PMSG daily for one week followed by 160 IU PMSG daily for two weeks administered by osmotic pump (experiment 1). Two groups also received injections of hCG for three or four days.

2.3.2.5 Plasma PMSG

Short day and long day birds did not have any PMSG present in the plasma. All of the treated birds had measurable amounts of PMSG in the plasma, although the concentrations of plasma PMSG varied considerably between birds (Figure 2.19 shows data from experiment 1 only). Two birds with exceptionally high levels of PMSG (>30 units/ml) have been excluded from the graphs and the analyses. There were no significant differences in plasma PMSG levels between PMSG treated groups in experiment 1 (see Tables 2.19 for statistics).

Table 2.19. Summary of Kruskal-Wallis nonparametric ANOVA and Mann-Whitney U tests comparing plasma PMSG concentration between groups in experiment 1.

Kruskal-Wallis	**K-W statistic**	**df**	***p***
All groups	24.925	4	0.000

Mann-Whitney U tests	***p***
Short day vs long day	1.000
Short day vs PMSG	0.003
Short day vs PMSG + 3 days hCG	0.001
Short day vs PMSG + 4 days hCG	0.002
Long day vs PMSG	0.003
Long day vs PMSG + 3 days hCG	0.001
Long day vs PMSG + 4 days hCG	0.002
PMSG vs PMSG + 3 days hCG	0.460
PMSG vs PMSG + 4 days hCG	0.122
PMSG + 3 days hCG vs PMSG + 4 days hCG	0.259

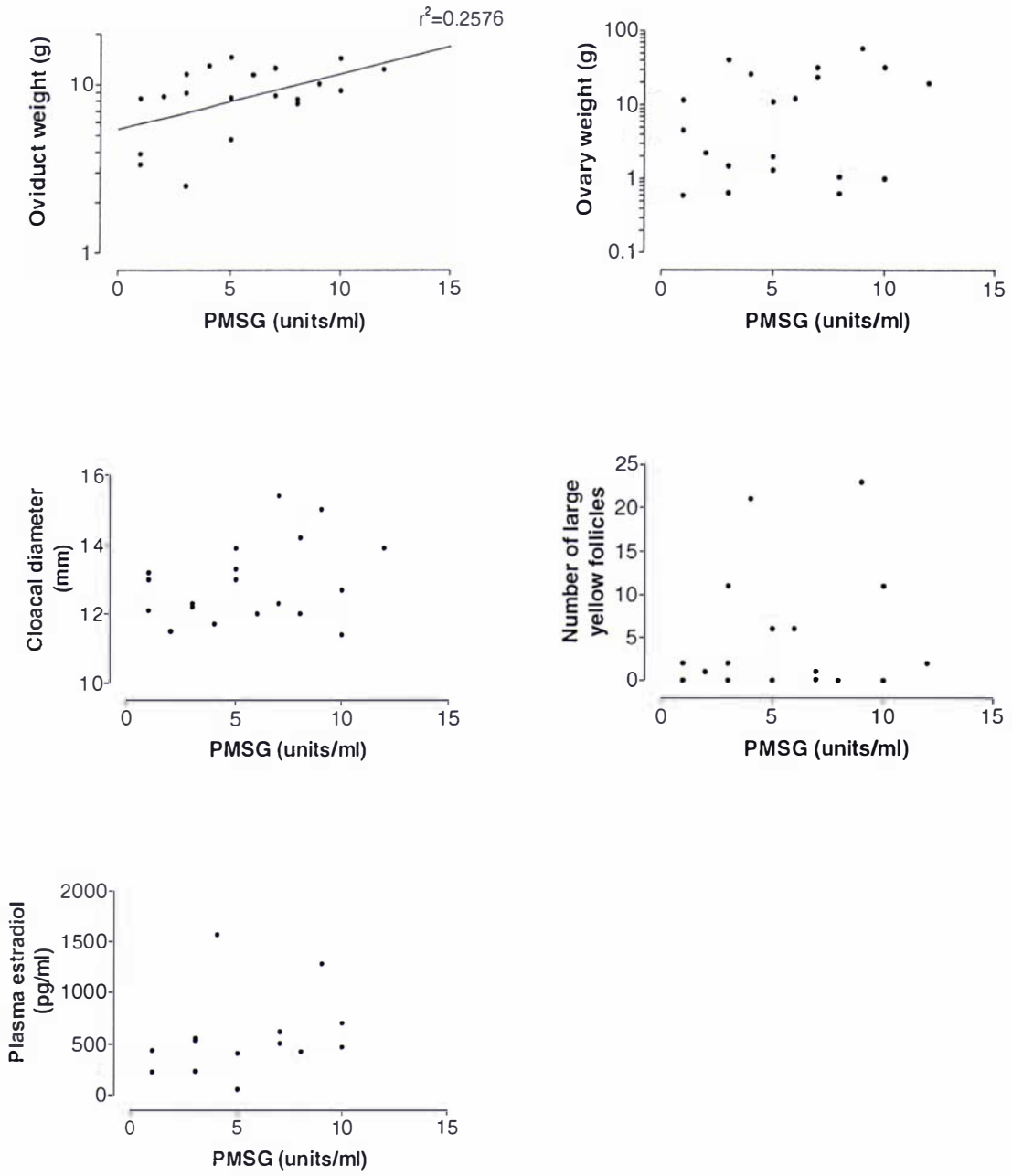

Figure 2.20. Relationships between plasma PMSG concentration and oviduct weight, ovary weight, cloacal diameter, number of large yellow ovarian follicles and plasma estradiol concentration of control birds (untreated birds held on short days or long days) and birds treated with PMSG by osmotic pumps.

The relationships between plasma PMSG concentration and oviduct weight, ovary weight, cloacal diameter, number of large yellow ovarian follicles, and plasma estradiol concentration were analysed using linear regression (Figure 2.20). There was a significant relationship between plasma PMSG and oviduct weight, although the r^2 value was low (see Table 2.20 for statistics). There was no relationship between plasma PMSG and any of the other variables listed above.

Table 2.20. Summary of linear regression relationship between plasma PMSG concentration and log oviduct weight, log ovary weight, cloacal diameter, number of large yellow ovarian follicles (LYF) and plasma estradiol concentration in experiments 1 and 2.

Linear regression	r^2	*p*
Oviduct weight	0.2576	0.0224
Ovary weight	0.0631	0.2854
Cloacal diameter	0.0890	0.2147
Number of LYF	0.0241	0.5137
Plasma estradiol	0.0661	0.3748

2.4 Discussion

Treatment with PMSG stimulated oviductal and ovarian development in Japanese quail and induced egg laying in one bird. This has been achieved previously in Japanese quail (Wakabayashi *et al.*, 1996), but PMSG had not been used to stimulate ovarian development in the New Zealand strain of Japanese quail. Although ovarian and follicular growth did occur, there was considerable variation between birds receiving the same dose of PMSG. In many of the treated birds ovarian growth was excessive, with

several ovaries growing to a weight five times greater than would be expected in a normal laying bird. Individual follicles did not grow any larger than would be expected in an untreated laying bird, indicating normal functioning of the factors controlling maximal follicular growth. One bird did produce three eggs, and all three eggs were fertile.

In the birds in which follicular development occurred in response to PMSG, many ovaries contained numerous large yellow follicles of a similar size and maturity. PMSG treatment often appeared to lead to a breakdown of the mechanisms controlling the entry of follicles into the rapid growth phase and of maintenance of the follicular hierarchy. Several authors have reported a breakdown in the follicular hierarchy in response to PMSG in chickens (Imai *et al.*, 1972; Palmer and Bahr, 1992; Hocking and McCormack, 1995), turkeys (Zadworny and Etches, 1988) and Japanese quail (Wakabayashi *et al.*, 1996). Treatment with pituitary extracts of avian or mammalian origin has also led to overstimulation of follicular development, to varying degrees, in chickens (Das and Nalbandov, 1955; Opel and Nalbandov, 1961a; Mitchell, 1967a; 1967b; Imai, 1972; Wakabayashi *et al.*, 1992). A hierarchy of follicles has developed and been maintained in response to exogenous gonadotropins on some occasions in a few birds, but the result was not consistent among all birds receiving the same treatment (Opel and Nalbandov, 1961a; Mitchell, 1967a; 1967b, Petitte and Etches, 1989). Some authors have attributed breakdown of the follicular hierarchy to high doses of PMSG (Wakabayashi *et al.*, 1996) or pituitary extracts (Mitchell, 1967a), and this may have caused overstimulation of follicular development in the current experiments. Wakabayashi *et al.* (1996) implanted osmotic pumps into Japanese quail that delivered 40 to 160 IU PMSG per day for 3 weeks. Although overstimulation of follicular growth was reported by Wakabayashi and colleagues, similar doses of PMSG where chosen for the experiments described in this chapter because the body weight of the New Zealand strain of Japanese quail is almost twice that of the quail bred in Japan.

Taber *et al.* (1958) and Mitchell (1967a; 1967b) reported ovulation in chickens treated with chicken pituitary extract to stimulate ovarian development, but there have been no reports of egg laying or ovulation in chickens treated with PMSG. Zadworny and Etches (1988) induced ovulation in out-of-lay turkeys treated with PMSG. Induction of egg laying in Japanese quail treated with PMSG to stimulate ovarian development has

been achieved with hCG, chicken pituitary glycoprotein or PMSG (Wakabayashi *et al.*, 1996). Approximately 6% of birds induced to ovulate with PMSG laid eggs, 14% of birds treated with PMSG and hCG laid eggs, and 38% of birds laid eggs in response to treatment with pituitary glycoprotein. All of the eggs hatched and were raised through to maturity. The results from the current experiment are similar. Although only one bird laid eggs in response to glycoprotein injections, this was 12% of the total number of birds treated. Two birds ovulated in response to hCG treatment, approximately 10% of the total number treated with hCG.

Very few birds ovulated in response to hCG or a glycoprotein extract, although ovarian follicles of F1 size had developed in several birds. Ovulation has been induced previously in untreated laying birds using exogenous hormones such as progesterone (Sekiguchi and Imai, 1987; Onagbesan and Peddie, 1988; Nakada *et al.*, 1994), ovine and porcine LH (Fraps and Riley, 1942; Fraps *et al.*, 1942a; 1942b; Hosoda *et al.*, 1956; Kamiyoshi and Tanaka, 1972; Sekiguchi and Imai, 1987; Onagbesan and Peddie, 1988), ovine and porcine LHRH (van Tienhoven and Schally, 1972; Reeves *et al.*, 1973; Johnson *et al.*, 1984b; Sekiguchi and Imai, 1987; Onagbesan and Peddie, 1988), porcine ACTH (Etches *et al.*, 1982; Etches and Croze, 1983), corticosterone (Etches and Croze, 1983) and even PMSG (Fraps *et al.*, 1942a). Successful ovulation in birds in which ovarian growth was stimulated with exogenous hormones has been achieved rarely (Wakabayashi *et al.*, 1992; 1996). Birds treated with PMSG or glycoprotein extracts to stimulate ovarian development have laid spontaneously without treatment with another hormone to induce ovulation (Wakabayashi *et al.*, 1996). The birds that ovulated and oviposited in the experiments described in this chapter may have ovulated regardless of whether or not they were treated with hCG or glycoprotein. PMSG stimulates ovarian and follicular development and presumably initiates some of the normal steroid pathways that influence ovulation and oviposition in untreated laying birds. Treatment with PMSG alone might be as effective as PMSG plus other hormones for stimulating ovarian growth and inducing ovulation in Japanese quail.

The oviducts of PMSG treated birds appeared morphologically similar to those of untreated laying birds. The eggs that were laid were completely shelled and normally pigmented. Histological examination of oviductal tissue showed that there were no differences in cell appearance, size or numbers between treated and untreated birds (J.

Girling, unpublished data). This is consistent with the results from other experiments (Imai *et al.*, 1972; Zadworny and Etches, 1988; Wakabayashi *et al.*, 1996). However, approximately half of the follicles that ovulated were found in the body cavity on dissection. This phenomenon was also noted by Fraps and Riley (1942) in chickens treated with PMSG, but occurs rarely in untreated laying quail (unpublished observations). The fact that such a large proportion of the ovulated follicles failed to enter the oviduct suggests complete or partial failure of the infundibulum to receive the newly ovulated ovum. This could be to large number of yolky ovarian follicles present in some PMSG treated birds. The infundibulum may be unable to "catch" all of the ovulated follicles from such a large ovary.

The ratio of LH to FSH activity present in pituitary extracts or PMSG may not be sufficiently similar to the ratio that occurs naturally in the bird during reproductive activity. The concentration of FSH in the plasma of domestic chickens is 13 to 50 times greater than that of LH during the ovulatory cycle (Scanes *et al.*, 1977). However, the production of, and responses to LH and FSH change during the reproductive cycle and are therefore difficult to determine and mimic accurately. Imai (1972) treated chickens receiving a pituitary inhibitor with various combinations of ovine LH and FSH, and found the most successful to be FSH:LH ratios of 2:1 or 1:1. Lower ratios of FSH:LH (e.g. 1:2) stimulated some oviductal growth, but ovary weights and follicular development were similar to that of saline treated birds. The ratio of FSH:LH in the plasma and the biological activity of the gonadotropins are not often measured when PMSG or pituitary extracts are used to stimulate reproduction. Stewart *et al.* (1976) used a rat radioreceptor assay to measure the ratio of FSH:LH activity in numerous preparations of PMSG and found the ratio to vary between 0.87 and 1.30. The biological activities of LH and FSH in the PMSG preparation used in the experiments described in this chapter were not known.

The FSH binding sites in the testis of Japanese quail are similar to those in mammalian testis in that they show high affinity, low capacity, are saturable, and have a low affinity for LH-like hormones (Licht and Midgley, 1976a; Ishii and Adachi, 1977; Gordon *et al.*, 1989). Radioiodinated human FSH and rat FSH are able to bind to the testis of the Japanese quail (Ishii and Adachi, 1977; Tsutsui and Ishii, 1978), chicken (Licht and Midgley, 1976a) and mallard duck (Licht and Midgley, 1976a). These results indicate

that the biological characteristics of the avian FSH receptor, and presumably the LH receptor, are similar to those of the mammalian gonadotropin receptors. The binding affinity of PMSG to avian FSH and LH receptors is unknown. However, equine FSH exhibits a binding affinity to chicken and rat FSH receptors at least one order of magnitude greater than human or porcine FSH (Gordon *et al.*, 1989). The degree of specific binding to chicken FSH receptors in the absence of any competing unlabelled FSH was greater for eFSH (14%) than for porcine FSH (9%).

Many of the gonadotropin preparations that have been used to stimulate ovarian development in birds, particularly crude pituitary extracts, were not pure preparations of LH and FSH. Thyroid stimulating hormone (TSH) has been identified in numerous pituitary extracts (Taber, 1958; Opel and Nalbandov, 1961a; Mitchell, 1970; Wakabayashi et al., 1992). ACTH was also found in partially purified extracts used by Taber (1958) and Opel and Nalbandov (1961a). These hormones may hinder normal ovarian development, but it is more likely that additional hormones are required in conjunction with LH and FSH to induce normal follicular growth. The left adrenal gland is embedded in the ovary, suggesting some influence on ovarian function, and adrenal hormones such as corticosterone can induce ovulation in hens (Etches *et al.*, 1982; Etches and Croze, 1983). ACTH and TSH may be involved in controlling yolk deposition into developing follicles (Opel and Nalbandov, 1961a). This might explain why normal hierarchies developed in some birds treated with crude extracts of avian and mammalian gonadotropins. It is possible that other hormones, such as estradiol, progesterone, or growth factors, are required in addition to PMSG for normal development and maintenance of follicular hierarchies.

Although excessive follicular development occurred in many of the PMSG treated birds, several birds showed little or no response to PMSG, and did not develop any yellow follicles. PMSG acts on the ovary in a similar way to LH and FSH, but the high doses of PMSG used in this experiment may be interfering with the regular stimulatory and inhibitory feedback loops that normally occur in response to endogenous plasma LH concentrations. However, plasma estradiol concentrations are high in birds with excessive follicular development, indicating relatively normal production of estradiol by large yellow follicles. There was also no relationship between plasma PMSG and ovary weight, as would be expected if high doses of PMSG were affecting normal feedback

control mechanisms. Petitte and Etches (1989) showed that large yellow follicles did not develop on the ovaries of birds treated with corticosterone and PMSG. Birds that showed little or no sign of ovarian development in response to PMSG may have had increased plasma corticosterone levels. An increase in corticosterone production may have occurred in response to regular handling or cold temperatures. This, in turn, could have resulted in the failure of the reproductive system to respond to exogenous gonadotropins.

Plasma estradiol concentrations in all short day and long day birds were within the range normally expected in female Japanese quail (Doi *et al.*, 1980; Gulati *et al.*, 1981). Plasma estradiol has not previously been measured in PMSG treated Japanese quail, but has been measured in PMSG treated chickens (Petitte and Etches, 1989) and turkeys (Zadworny and Etches, 1988). Zadworny and Etches (1988) treated incubating and out-of-lay turkeys every two days with injections of PMSG, and found that plasma estradiol concentrations increased significantly in response to high doses. In normal laying birds, it is the F2-F5 follicles that produce estradiol, so it is not surprising that there is a positive linear relationship between plasma estradiol and the numbers of large yellow follicles present on the ovary. In PMSG treated birds, there are often many more large yellow follicles than would be found in the follicular hierarchy of an untreated laying bird, leading to increased production of estradiol. There were also positive relationships between plasma estradiol and oviduct weight, ovary weight and cloacal diameter.

In previous experiments, PMSG was usually administered to birds via daily injections. Osmotic pumps have been used rarely to deliver PMSG, with the only published example being Wakabayashi *et al.* (1996). The ovaries of many birds did develop large yellow follicles, indicating that the pumps were releasing PMSG. There was, however, considerable variation in ovarian weight and the number of yellow ovarian follicles between birds receiving the same dose of PMSG. Although the delivery rate of the osmotic pump is fixed, and pumps were empty at the time of dissection, it was difficult to determine whether or not all pumps delivered at the same rate for the duration of the experiment. The site of implantation and the positioning of the pump were chosen to minimize discomfort to the bird, and had been successful in previous experiments (Wakabayashi *et al.*, 1996). However, implantation beside the inner thigh may not have been the most ideal position for regular functioning of osmotic pumps. The fate of

PMSG once it leaves the pump is unknown, but presumably it is transported into the blood stream and eventually carried to the ovary. However, plasma PMSG concentrations were highly variable between birds, suggesting potential variation in delivery rate between pumps, or failure of some or all of the PMSG to enter the circulatory system. There may have also been a change in the metabolism of PMSG, or a change in the production of antibodies against PMSG. Witschi and Johnson (1960) found antibodies for PMSG present in Weaver finches after several injections, but this did not affect the physiological responsiveness to PMSG. The use of osmotic pumps for delivery of gonadotropins is practical and convenient, but may not be the best option.

Several external characteristics were measured throughout the experiments as an indication of the stage of development of the reproductive tract. Cloacal opening diameter proved to be a good external indicator of oviductal growth, and to a lesser extent, ovarian development. Measuring the cloacal opening diameter is quick and simple, and provides useful information about the development of the reproductive tract, but is not useful for determining the number and size of yellow ovarian follicles present. An increase in the size of the oviduct and ovary would lead to an increase in body weight, and therefore body weight was recorded regularly. However, birds were still maturing, and some of the increase in weight was due to increase in body size and deposition of fat stores. There was also considerable variation in body weight between birds within the same treatment group, so body weight was not a particularly useful indicator of reproductive tract development.

The use of ultrasound to examine the avian ovary has been used rarely as a diagnostic tool as the extensive air sac system found in bird's limits ultrasound penetration (Hildebrandt *et al.*, 1998). Subcutaneous fat and feather follicles can also interfere with ultrasound waves. Hildebrandt *et al.*, (1994; 1995; 1996) used transintestinal and transcloacal ultrasound methods involving high-resolution, miniaturised probes to examine the avian reproductive tract in chickens, penguins and several species of raptors. Although this work concentrated on visualization of the oviduct for sex identification, ovarian follicles could also be seen. However, there are no published data in which numbers or diameters of avian ovarian follicles have been measured using ultrasound. Ultrasound was a very useful tool for examining follicle sizes and numbers in PMSG treated Japanese quail in the current experiments. Identification of large

yellow follicles greater than 15 mm in diameter enabled selection of birds suitable for induction of ovulation. Other papers have reported instances where birds were treated with hormones to induce ovulation, but on dissection it was noted that the ovary contained no yellow follicles of F1 size. Measuring follicles using ultrasound eliminates unnecessary wastage of hormones on birds that are not ready to ovulate.

Although PMSG was able to stimulate development of the reproductive tract in Japanese quail, and a few birds could be induced to ovulate, treatment with PMSG needs extensive refinement before being used in endangered or threatened avian species. Attempting to minimise overstimulation of follicular development will be the main focus of future experiments, and may be achieved by using lower doses of PMSG. Alternative methods for delivering known concentrations of PMSG to Japanese quail should also be investigated and compared.

3 Effect of one, two or three weeks of PMSG treatment on ovarian development, and comparison of delivery by osmotic pumps and daily injections

3.1 Introduction

Exogenous gonadotropins have been used previously to stimulate development of the reproductive system in Japanese quail. However, considerable over-stimulation of follicular development occurred when large doses of PMSG were used to stimulate ovarian growth (Wakabayashi *et al.*, 1996; Bennett *et al.*, unpublished observations, see previous chapter). Numerous large yellow follicles developed on the ovaries of many of the PMSG treated birds, and these were not arranged in a hierarchy. In previous experiments, birds were treated with either hCG or a glycoprotein extract from chicken pituitaries to induce ovulation. Very few of the treated birds ovulated, despite the ovaries of several birds containing follicles of F_1 size. The current set of experiments focused on stimulating normal ovarian growth using various doses of PMSG (5 to 80 IU) over a one, two or three week period, without attempting to induce ovulation.

Recently, PMSG has been administered to birds using small osmotic pumps for the continuous delivery of hormones (Wakabayashi *et al.*, 1992; 1996). Osmotic pumps involve initial minor surgery for implantation, and continue to deliver a specific dose for a controlled period of time. Birds have also been treated with PMSG using injections (Opel and Nalbandov, 1961a; Imai *et al.*, 1972; Zadworny and Etches, 1988; Petitte and Etches, 1989). Administration of PMSG using daily injections is a considerably less invasive procedure than the implantation of osmotic pumps, although regular capture and handling of the birds is required. Unlike osmotic pumps, injections allow the dose of hormone and timing of treatment to be easily varied from day to day if necessary.

The first experiment in a set of three compared the ovarian responses of birds given PMSG via daily injections or via osmotic pumps. The effectiveness of daily injections compared with osmotic pumps has not previously been examined in a single experiment. Two further experiments examined the ovarian response to daily PMSG treatment over a two or three week period. Data from previous experiments were used to choose PMSG doses with the aim of stimulating a pattern of ovarian growth similar to that which occurs in response to normal stimulatory photoperiod conditions.

3.2 Materials and Methods

3.2.1 Animals

Quail (n=261) were purchased at three weeks of age from our normal supplier, Rangitikei Game Farm. Before the start of experiments, birds were housed under a short day photoperiod at 10°C as described in Chapter 2.

3.2.2 Hormone Preparation and Administration

Pregnant mare serum gonadotropin (PMSG; Teikoku Zoki, Tokyo, Japan) was kindly donated by Professor Susumu Ishii, Waseda University, Tokyo.

PMSG was dissolved in saline and administered using either Alzet mini-osmotic pumps, model 2001, or by daily injections. The model 2001 pump has a delivery rate of 1.0 µl/hour, and delivers hormone continuously for one week. The pumps were loaded with solutions of PMSG so that 0, 5, 10, 20, 40 or 80 IU of PMSG would be delivered daily to the bird. The pumps were implanted subcutaneously on the underside of the body beside the thigh as described in Chapter 2. Daily injections were administered subcutaneously in

the abdomen using a 0.5 ml insulin syringe and a 27 gauge, ½ inch needle. Each bird received a volume of 200 µl of solution per injection, containing 0, 5, 10, 20, 40 or 80 IU PMSG.

3.2.3 Experimental Design

3.2.3.1 Experiment 1

At six weeks of age, 105 female Japanese quail were divided into 15 groups (n=7 birds per group) and treated for seven days as shown in Table 3.1.

Table 3.1. Experimental groups for experiment 1.

Group number	Photoperiod	Dose of PMSG/day	Delivery method
1	Euthanased at start of experiment	-	-
2	Short day	None	None
3	Long day	None	None
4	Short day	0 IU	Injection
5	Short day	0 IU	Pump
6	Short day	5 IU	Pump
7	Short day	10 IU	Pump
8	Short day	20 IU	Pump
9	Short day	40 IU	Pump
10	Short day	80 IU	Pump
11	Short day	5 IU	Injection
12	Short day	10 IU	Injection
13	Short day	20 IU	Injection
14	Short day	40 IU	Injection
15	Short day	80 IU	Injection

3.2.3.2 Experiment 2

Seventy-eight, six week old female Japanese quail were divided into 12 groups (n=6 birds per control group, n=7 birds per treatment group) and treated for 14 days as shown in Table 3.2.

Table 3.2. Experimental groups for experiment 2.

Group number	Photoperiod	Dose of PMSG/day (week 1)	Dose of PMSG/day (week 2)
1	Euthanased at start of experiment	-	-
2	Short day	None	None
3	Long day	None	-
4	Long day	None	None
5	Long day (7 days) then short day (7 days)	None	None
6	Short day	0 IU	0 IU
7	Short day	10 IU	-
8	Short day	10 IU	0 IU
9	Short day	10 IU	5 IU
10	Short day	10 IU	10 IU
11	Short day	10 IU	20 IU
12	Short day	10 IU	40 IU

3.2.3.3 Experiment 3

At six weeks of age, seventy-eight female quail were divided into 13 groups (n=6 birds per group) and treated for 21 days as shown in Table 3.3.

Table 3.3. Experimental groups for experiment 3.

Group number	Photoperiod	Dose of PMSG/day (week 1)	Dose of PMSG/day (week 2)	Dose of PMSG/day (week 3)
1	Euthanased at start of experiment	-	-	-
2	Short day	None	None	None
3	Long day	None	-	-
4	Long day	None	None	-
5	Long day	None	None	None
6	Short day	0 IU	0 IU	0 IU
7	Short day	10 IU	-	-
8	Short day	10 IU	20 IU	-
9	Short day	10 IU	20 IU	0 IU
10	Short day	10 IU	20 IU	5 IU
11	Short day	10 IU	20 IU	10 IU
12	Short day	10 IU	20 IU	20 IU
13	Short day	10 IU	20 IU	40 IU

For each experiment, group one was euthanased at the start of the treatment period to determine the size of the ovary and oviduct prior to treatment, and group two remained on short days, received no treatment, and was euthanased at the end of the experiment. Long day control groups were transferred to a 16L:8D photoperiod at 20°C on the same day as the other treatments began. In each experiment, the amount of oviductal and ovarian development stimulated by a long day photoperiod provided a target for the PMSG treatment to achieve. Sham treated groups were also included in each experiment.

In experiment 1, treated groups were held on short days and birds were given daily injections for one week of either 0, 5, 10, 20, 40 or 80 IU PMSG per injection, or implanted subcutaneously with an osmotic pump delivering 0, 5, 10, 20, 40 or 80 IU PMSG per day. The results from this experiment were used to determine PMSG doses and the delivery method that would be used for subsequent experiments.

Experiments 2 and 3 examined the ovarian and oviductal response to daily injections of PMSG over two- and three-week periods respectively. In experiment 2, all treated groups were held on short days and given 10 IU PMSG daily for the first week, and were then given 0, 5, 10, 20 or 40 IU PMSG for the second week. Groups 3 and 7 were euthanased on day 8 of the experiment to determine the amount of ovarian and oviductal development after one week of a long day photoperiod or daily PMSG treatment. Group 5 was transferred back to short days (8L:16D) after one week under a stimulatory long day photoperiod. This group was compared with group 8 in which birds were treated with 10 IU PMSG for the first week, and then received saline injections for the second week.

The experimental design for experiment 3 was similar to that of experiment 2, but covered a three-week treatment period. Control groups were similar to those in experiment 2 (Tables 3.2 and 3.3). Groups treated with PMSG received daily injections containing 10 IU PMSG for the first week, 20 IU PMSG for the second week, and varying doses in the third week.

Regular measurements (daily or every two days) of cloacal opening diameter were made during each experiment as an indicator of development of the oviduct. Body weight was also measured regularly to ensure birds maintained a healthy weight.

At the end of each experiment, birds were euthanased, and ovary and oviduct excised and weighed, and body weight and cloacal opening diameter recorded. Ovaries were divided into five size classes (0.00-0.15 g, 0.16-0.50 g, 0.51–1.00 g, 1.01–5.00 g, and 5.01–9.00 g) and the numbers and sizes of yellow follicles present in each ovary were recorded. The follicular distribution within each ovarian weight class was compared between control and

treated groups. Blood samples were taken and stored for radioimmunoassay of plasma estradiol and plasma LH.

All experimental procedures were approved by the Massey University Animal Ethics Committee.

3.2.4 Radioimmunoassay of plasma estradiol

Plasma samples were assayed for estradiol using the method described in Chapter 2.

Plasma samples from experiment 1 were assayed by Dr Jane Girling. The percentage recovery for estradiol in quail plasma for this set of assays ranged from 91.4% to 99.0%. The intra-assay coefficients of variation for estradiol assays were 14.5%, 8.5% and 11.8% for high, medium and low quality controls respectively (n=20). The inter-assay coefficients of variation for the assays were 8.7%, 12.0% and 17.9% for high, medium and low quality controls respectively (n=12).

The least detectable dose of the assay was equivalent to an estradiol concentration in quail plasma of 73.1 pg/ml (n=3 assays).

3.2.5 Radioimmunoassay of luteinising hormone

Plasma LH was assayed in the Department of Biology, Waseda University, Tokyo, Japan by Dr Motoshi Kikuchi using their validated radioimmunoassay for LH in Japanese quail (Hattori and Wakabayashi, 1979; Kikuchi and Ishii, 1989).

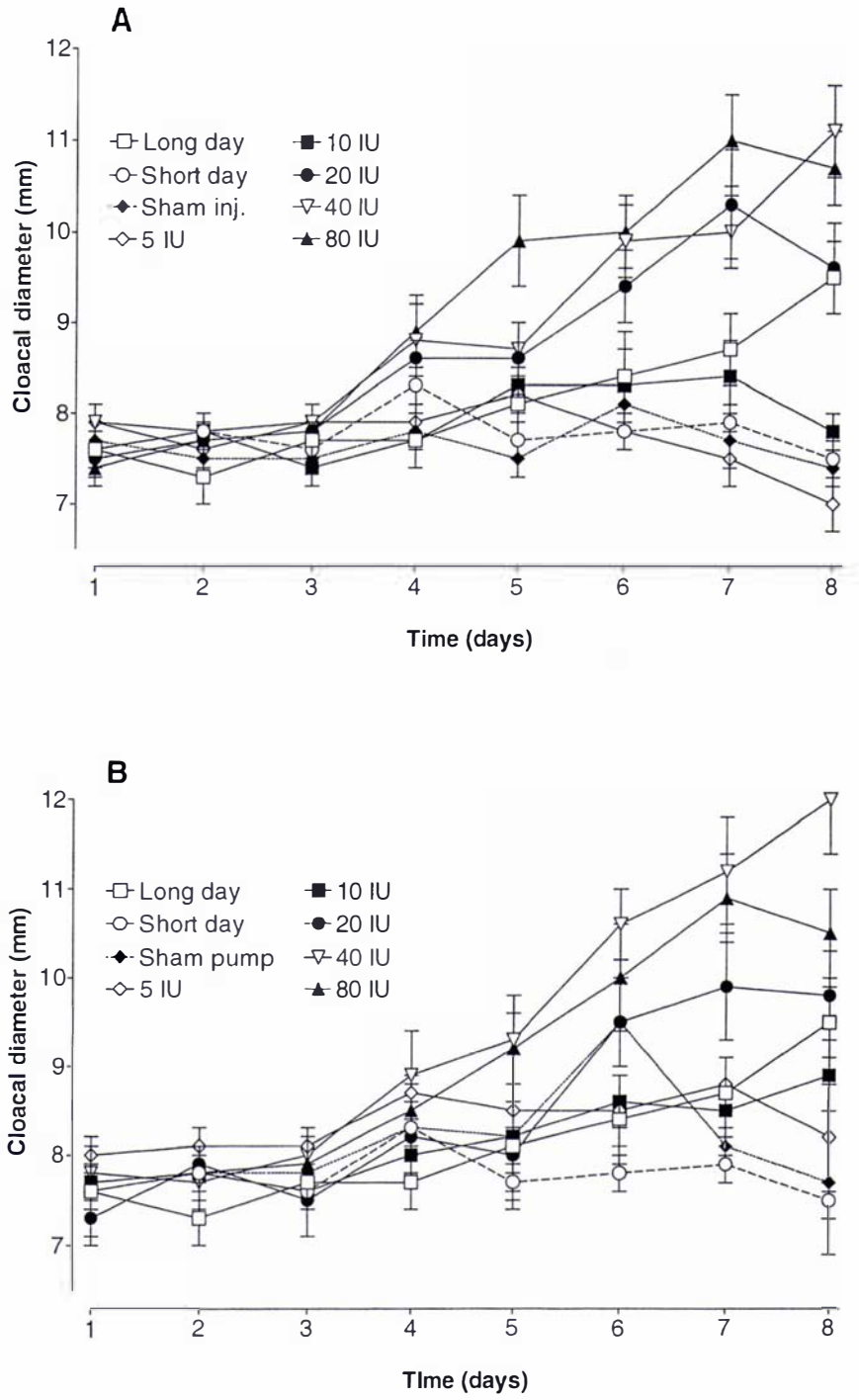

Figure 3.1. Changes in mean cloacal diameter of female Japanese quail held on short days, long days, and short days plus treatment with 0, 5, 10, 20, 40 or 80 IU PMSG for seven days by daily injection (A) or osmotic pump (B).

3.2.6 Statistics

All variables were tested for homogeneity of variance using Levene's test. When variances were homogeneous across groups for a single variable, ANOVA followed by linear contrasts were used to analyse data. Linear contrasts were used rather than Bonferroni's posthoc tests due to the large number of comparisons. When variances were not homogeneous across groups, Kruskal-Wallis non-parametric ANOVA followed by Mann-Whitney U tests were performed to determine differences between groups. One-way ANOVA or Student's t-tests were used to analyse follicular size frequency distribution data. Repeated measures ANOVA was used to analyse changes in cloacal diameter over time. Relationships between variables were investigated using linear regression to calculate r^2 values.

ANOVAs and non-parametric equivalents were carried out using Systat Version 8.0 (SPSS Inc., 1988), and relationships between variables (linear regression) were analysed using GraphPad Prism Version 3.0 (GraphPad Software Inc., 1999). Data were transformed to logarithms where necessary. Data are presented as individual points or as means ± S.E.

3.3 Results

3.3.1 Experiment 1

3.3.1.1 Cloacal opening

Cloacal opening diameter was not significantly different between groups at the start of the treatment period ($F_{13, 72}$=0.737, p=0.721). Cloacal diameter increased in female quail after seven days under a long day photoperiod at 20°C (Figures 3.1, see Table 3.4 for statistics). There were also increases in the diameter of the cloacal opening in birds treated by daily

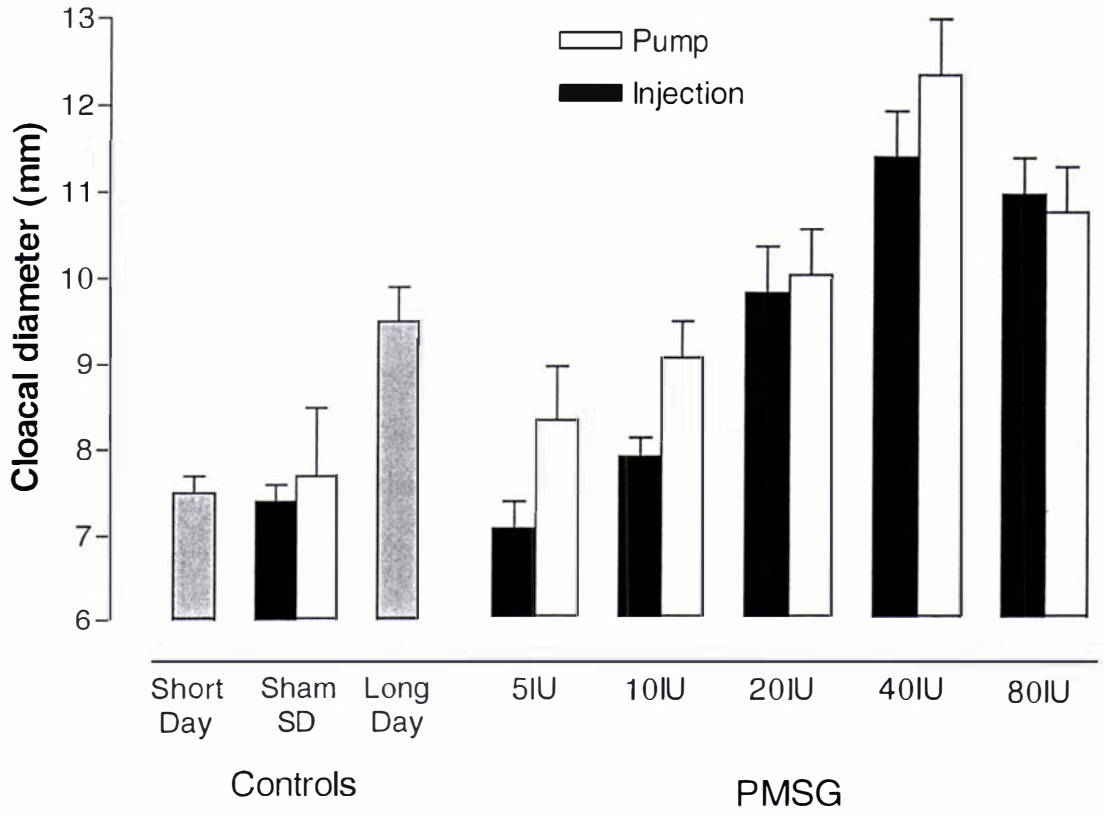

Figure 3.2. Mean cloacal diameter of female Japanese quail after seven days on short days, long days, or short days plus treatment with 0, 5, 10, 20, 40 or 80 IU PMSG by osmotic pump or daily injection.

injection with 10, 20, 40 or 80 IU PMSG, and in birds implanted with osmotic pumps delivering 10, 20, 40 or 80 IU PMSG per day. There were no changes in the cloacal opening diameters of birds that received no treatment and remained under short days at 10°C, birds that were treated with sham injections or a sham pump, or birds receiving 5 IU PMSG by daily injections or osmotic pumps.

Table 3.4. Summary of results of repeated measures ANOVA for changes in cloacal diameter across days for each group in experiment 1.

Group	F	df	*p*
Long day	5.172	6, 36	0.001
Short day	0.604	6, 30	0.725
Sham injection	0.761	6, 36	0.605
Sham pump	1.739	6, 12	0.195
5 IU pump	1.725	6, 24	0.158
10 IU pump	2.652	6, 30	0.035
20 IU pump	9.552	6, 24	0.000
40 IU pump	21.024	6, 18	0.000
80 U pump	20.761	6, 24	0.000
5 IU injection	1.081	6, 36	0.392
10 IU injection	3.272	6, 36	0.011
20 IU injection	9.707	6, 36	0.000
40 IU injection	19.408	6, 36	0.000
80 IU injection	12.358	6, 36	0.000

At the end of the experiment, there were significant differences in cloacal opening diameter between groups (Figure 3.2, see Table 3.5 for statistics). Delivery method had no effect on cloacal diameter. The cloacal opening of untreated birds held on short days was not significantly different from sham treated birds, but was significantly smaller than birds

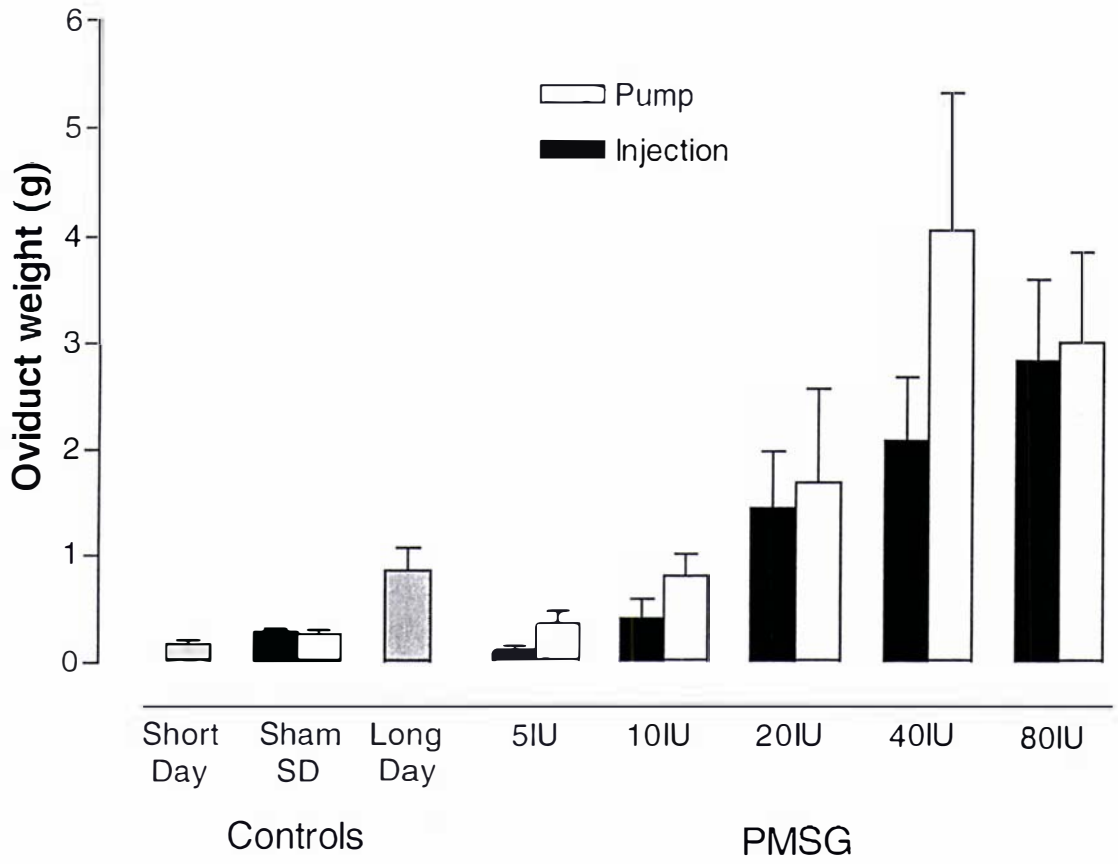

Figure 3.3. Mean oviduct weight of female Japanese quail after seven days on short days, long days, or short days plus treatment with 0, 5, 10, 20, 40 or 80 IU PMSG by osmotic pump or daily injection.

transferred to long days. Birds treated with 5 or 10 IU PMSG also had significantly smaller cloacal diameters than birds held on long days. Cloacal diameters of birds treated with the two highest doses, 40 and 80 IU PMSG, were significantly larger than birds kept on a long day photoperiod. Daily treatment with 20 IU PMSG caused cloacal diameter to increase to a similar size to that found in long day birds.

Table 3.5. Summary of one-way ANOVA and linear contrasts comparing cloacal opening diameter and ovary weight between groups at the end of experiment 1.

	Cloacal diameter			Ovary weight		
ANOVA	**F**	**df**	***p***	**F**	**df**	***p***
All groups	11.808	14, 75	0.000	15.724	14, 78	0.000
Linear contrasts	**F**	**df**	***p***	**F**	**df**	***p***
All pumps vs All injections	2.643	1, 75	0.108	2.352	1, 78	0.129
Long day vs Short day	12.141	1, 75	0.001	27.510	1, 78	0.000
Short day vs Sham injection	0.260	1, 75	0.612	2.185	1, 78	0.143
Short day vs Sham pump	0.281	1, 75	0.598	0.000	1, 78	0.996
Long day vs 5 IU	11.968	1, 75	0.001	8.976	1, 78	0.004
Long day vs 10 IU	7.295	1, 75	0.009	2.155	1, 78	0.146
Long day vs 20 IU	1.343	1, 75	0.250	0.491	1, 78	0.486
Long day vs 40 IU	38.200	1, 75	0.000	16.345	1, 78	0.000
Long day vs 80 IU	13.730	1, 75	0.000	28.484	1, 78	0.000

3.3.1.2 Oviduct weight

There were significant differences in oviduct weight between groups at the time of dissection (Figure 3.3, see Table 3.6 for statistics). There was no significant difference in oviduct weight between pump and injection treated groups. The oviduct weight of

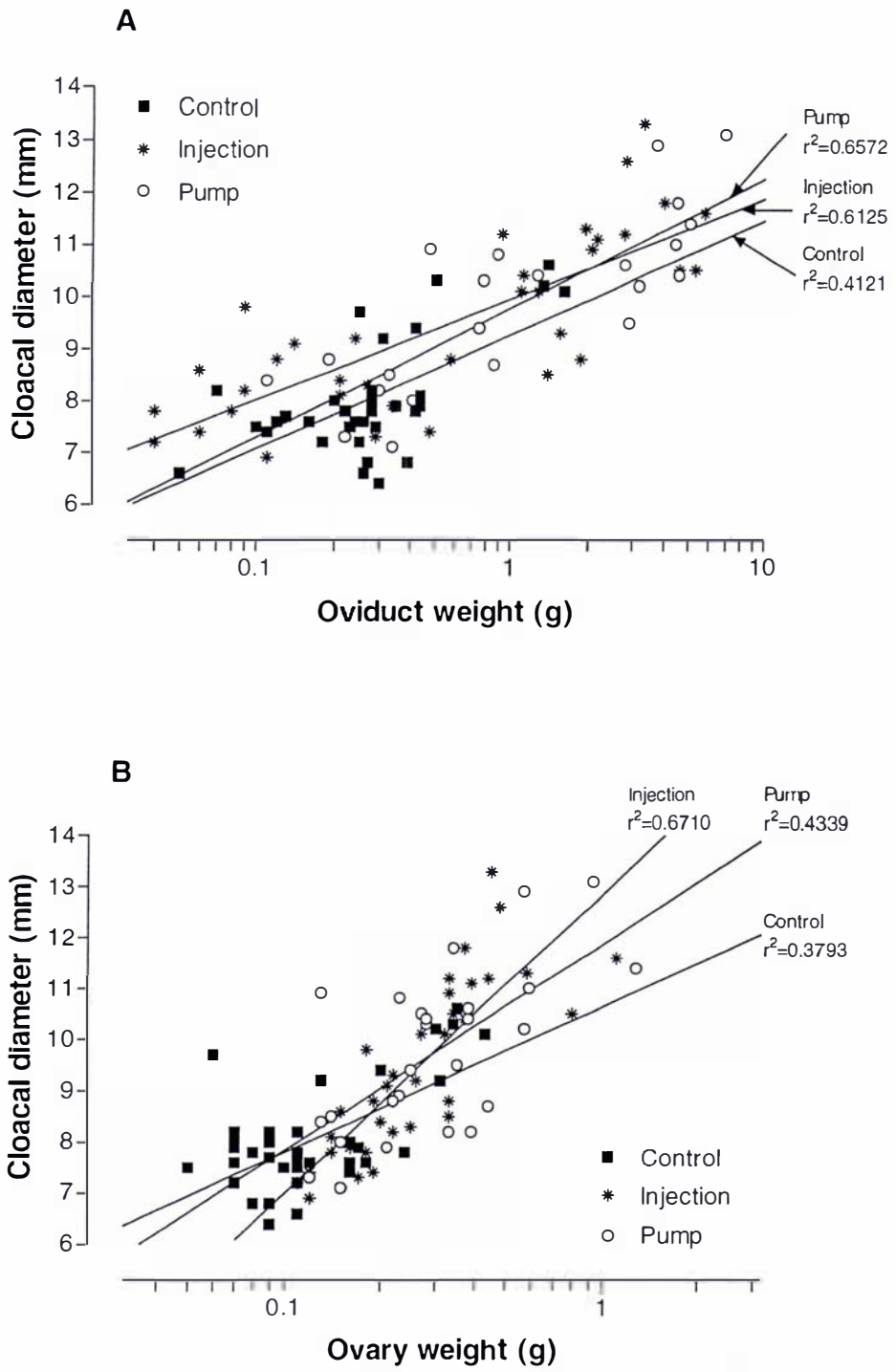

Figure 3.4. Relationships between oviduct (A) or ovary weight (B) and cloacal diameter of control birds (untreated birds held on short days or long days) and birds held on short days receiving daily treatment with 0, 5, 10, 20, 40 or 80 IU PMSG by osmotic pump or daily injection.

untreated birds held on short days was not significantly different from sham pump treated birds, but was significantly smaller than long day birds. Although the mean oviduct weight of the sham injection group was significantly different to the short day control group, little or no oviductal growth had occurred in all birds in the sham treated group. Birds treated with 5 IU PMSG had significantly smaller oviducts than birds transferred to long days. Mean oviduct weight of birds treated with 80 IU PMSG was significantly greater than the oviduct weight of birds held on long days. Birds treated daily with 10, 20 or 40 IU PMSG had oviducts of a similar weight to those of birds held on long days. Oviduct weight showed a positive correlation with cloacal diameter for both control and treated groups (Figure 3.4A, see Table 3.7 for statistics).

Table 3.6. Summary of Kruskal-Wallis non-parametric ANOVA and Mann-Whitney U tests comparing oviduct weight and plasma estradiol concentration between groups at the end of the experiment.

	Oviduct weight			**Plasma estradiol**		
Kruskal-Wallis	**K-W**	**df**	***p***	**K-W**	**df**	***p***
All groups	56.722	14	0.000	22.263	14	0.073
Mann-Whitney U tests	**M-W**	**df**	***p***	**M-W**	**df**	***p***
All pumps vs All injections	529.5	1	0.168	497.0	1	0.171
Long day vs Short day	60.0	1	0.003	41.5	1	0.107
Short day vs Sham injection	12.0	1	0.039	33.5	1	0.449
Short day vs Sham pump	5.0	1	0.116	13.0	1	0.813
Long day vs 5 IU	79.5	1	0.001	66.5	1	0.031
Long day vs 10 IU	62.5	1	0.178	63.5	1	0.140
Long day vs 20 IU	40.0	1	0.866	44.5	1	0.586
Long day vs 40 IU	18.0	1	0.063	46.5	1	0.259
Long day vs 80 IU	12.0	1	0.011	39.0	1	0.798

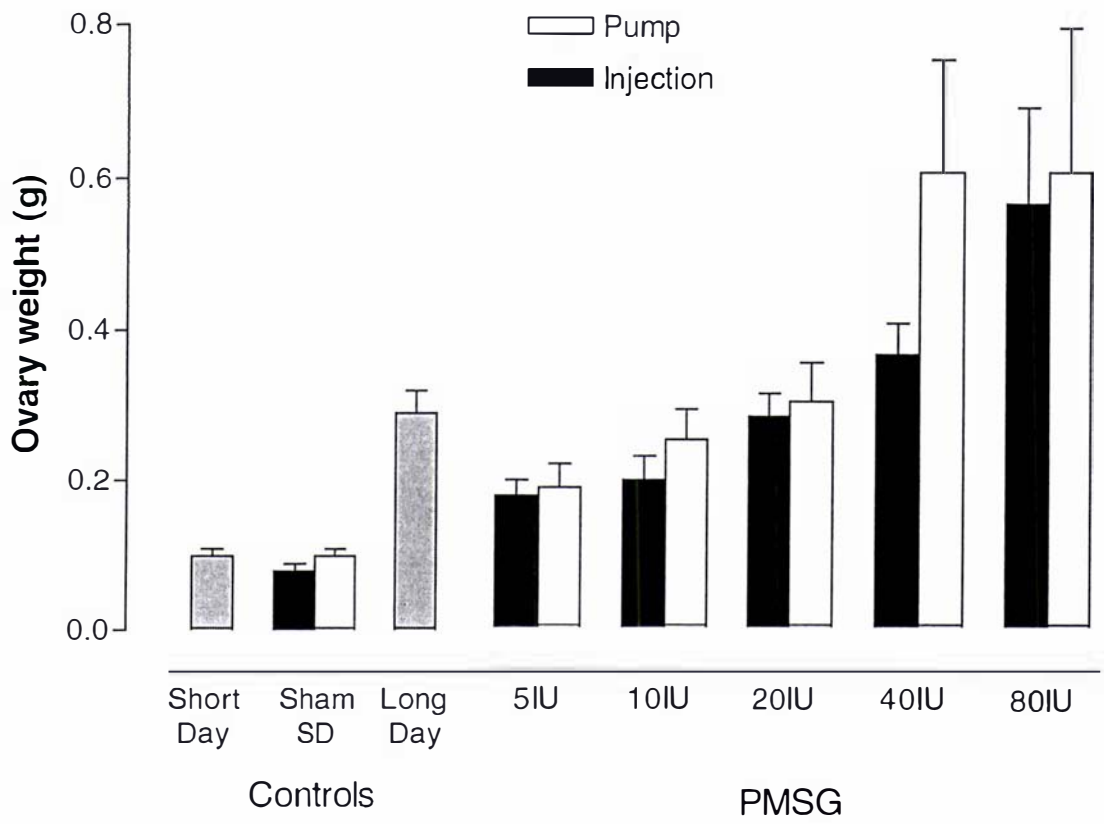

Figure 3.5. Mean ovary weight of female Japanese quail after seven days on short days, long days, or short days plus treatment with 0, 5, 10, 20, 40 or 80 IU PMSG by osmotic pump or daily injection.

Table 3.7. Summary of linear regression relationship between log ovary weight and cloacal diameter, and between log oviduct weight and cloacal diameter for all control birds, and for each delivery method in experiment 1.

Group	Oviduct weight			Ovary weight		
	r^2	Slope	*p*	r^2	Slope	*p*
Untreated controls	0.4121	2.20 ± 0.47	<0.0001	0.3793	2.85 ± 0.62	<0.0001
Pump treated	0.6572	2.48 ± 0.39	<0.0001	0.4339	4.64 ± 0.94	0.0003
Injection treated	0.6125	1.93 ± 0.27	<0.0001	0.6710	5.87 ± 0.72	<0.0001

3.3.1.3 Ovary weight

Some ovarian growth was stimulated by all doses of PMSG, although seven days of treatment was insufficient for yellow follicles to develop. There were significant differences in mean ovary weight between groups (Figure 3.5, see Table 3.5 for statistics). Delivery method had no effect on ovary weight. The ovary weight of untreated birds kept under a short day photoperiod was not significantly different from sham treated birds, but was significantly smaller than long day birds. Birds treated with 5 IU PMSG also had significantly smaller ovaries than birds held on long days. Mean ovary weight of birds treated with 40 or 80 IU PMSG was significantly greater than the ovary weight of birds transferred to long days. Daily treatment with 10 or 20 IU PMSG led to an increase in ovarian weight similar to that found in birds held on long days. Ovarian weight showed a positive correlation with cloacal diameter for both control and treated groups (Figure 3.4B, see Table 3.7 for statistics).

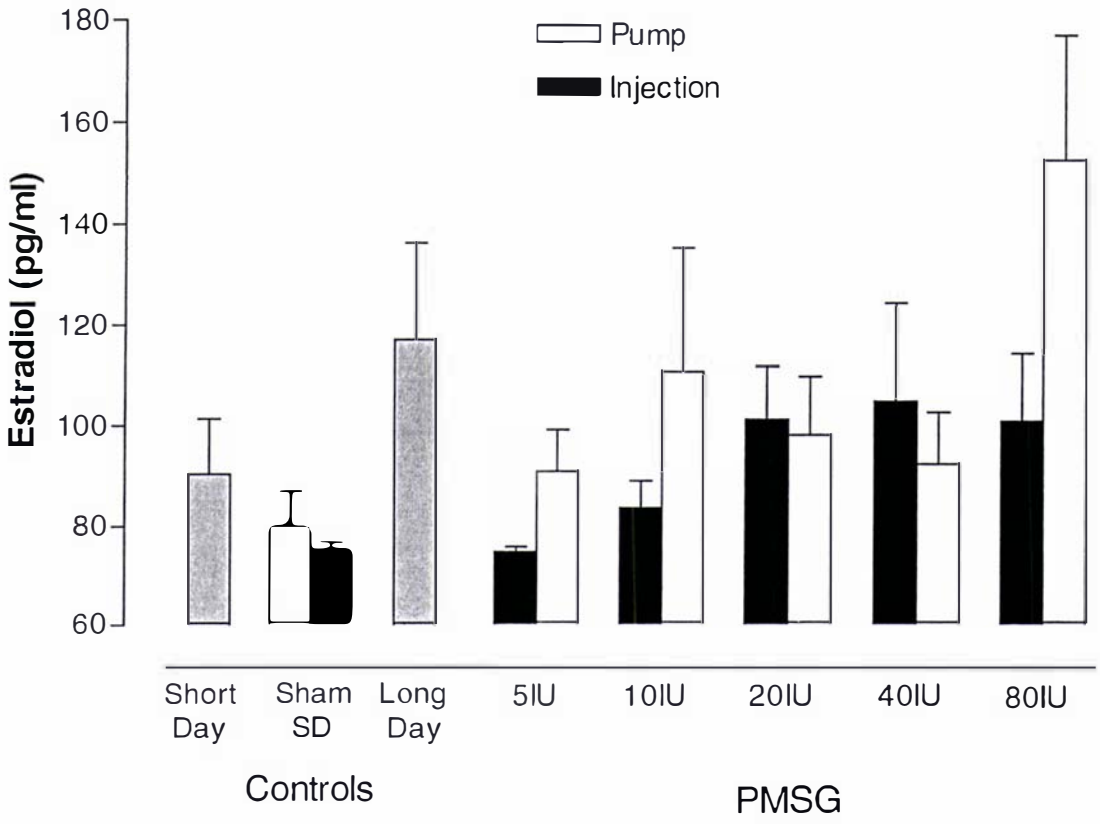

Figure 3.6. Mean plasma estradiol concentration of female Japanese quail after seven days on short days, long days, or short days plus treatment with 0, 5, 10, 20, 40 or 80 IU PMSG by osmotic pump or daily injection.

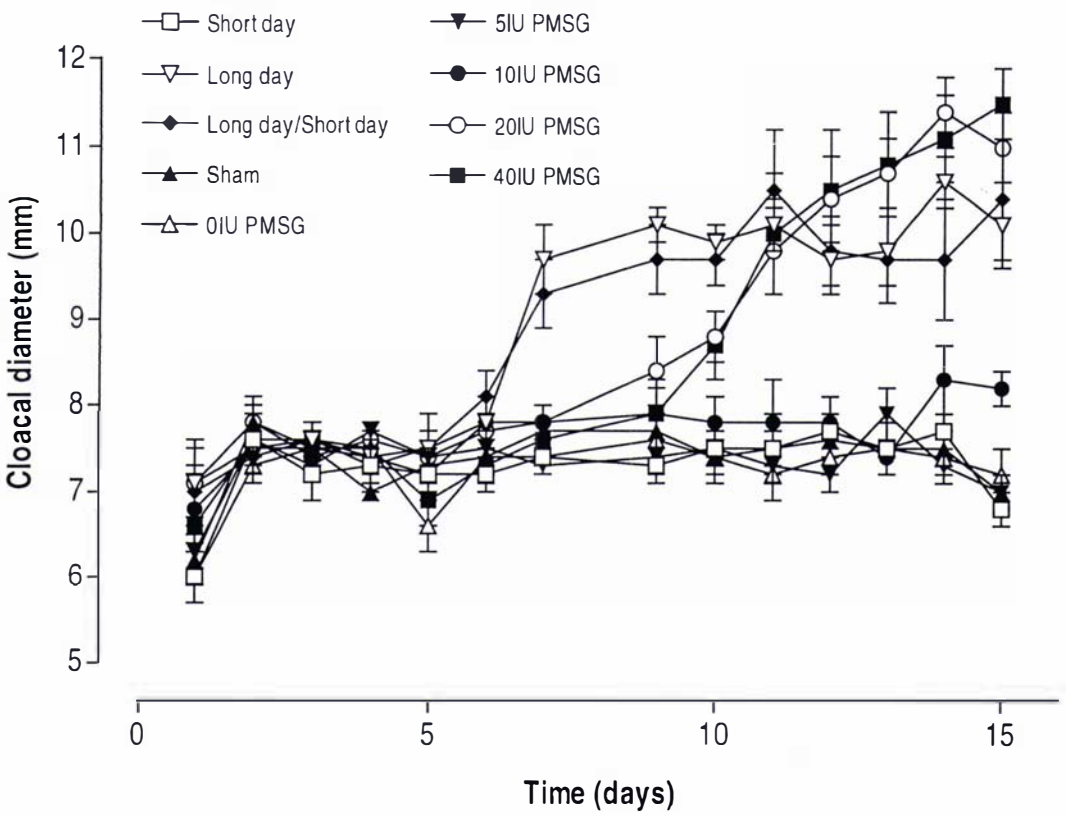

Figure 3.7. Changes in mean cloacal diameter of female Japanese quail held on short days, long days, and short days plus treatment with daily injections of 10 IU PMSG for one week followed by treatment with 0, 5, 10, 20 or 40 IU PMSG for the second week.

3.3.1.4 Plasma estradiol

There were no significant differences in plasma estradiol concentrations between groups at the end of the experiment (Figure 3.6, see Table 3.6 for statistics). Neither delivery method or dose affected plasma estradiol concentration, with only the 5 IU PMSG group having significantly lower levels of plasma estradiol than long day birds. There was no significant correlation between plasma estradiol concentration and ovary or oviduct weight (data not shown). These results may be have been influenced by the relatively high least detectable dose for the estradiol assays.

3.3.2 Experiment 2

3.3.2.1 Cloacal opening

At the start of the treatment period, cloacal opening diameter did not differ significantly between groups ($F_{10, 61}$=0.826, p=0.606, Figure 3.7). The cloacal opening of birds that remained on short days and received no treatment did not change over two weeks (see Table 3.8 for statistics). Birds receiving saline injections for two weeks or daily injections of 10 IU PMSG for one week also showed no significant increase in cloacal diameter. There was a significant increase in cloacal diameter of birds that were transferred to long days for one week or two weeks, and in birds held under long days for one week and then transferred back to short days for one week. Birds that received daily injections of 10 IU PMSG for one week, followed by saline injections for one week showed a significant change in cloacal opening diameter, although the change was relatively small compared to other groups. All groups treated daily with PMSG for two weeks showed a significant change in mean cloacal diameter during the treatment period. The change in cloacal opening diameter was most marked in groups treated with 20 IU or 40 IU PMSG in the second week of treatment. The cloacal opening in both of these groups had increased to a similar diameter to the cloacal opening of birds held on long days.

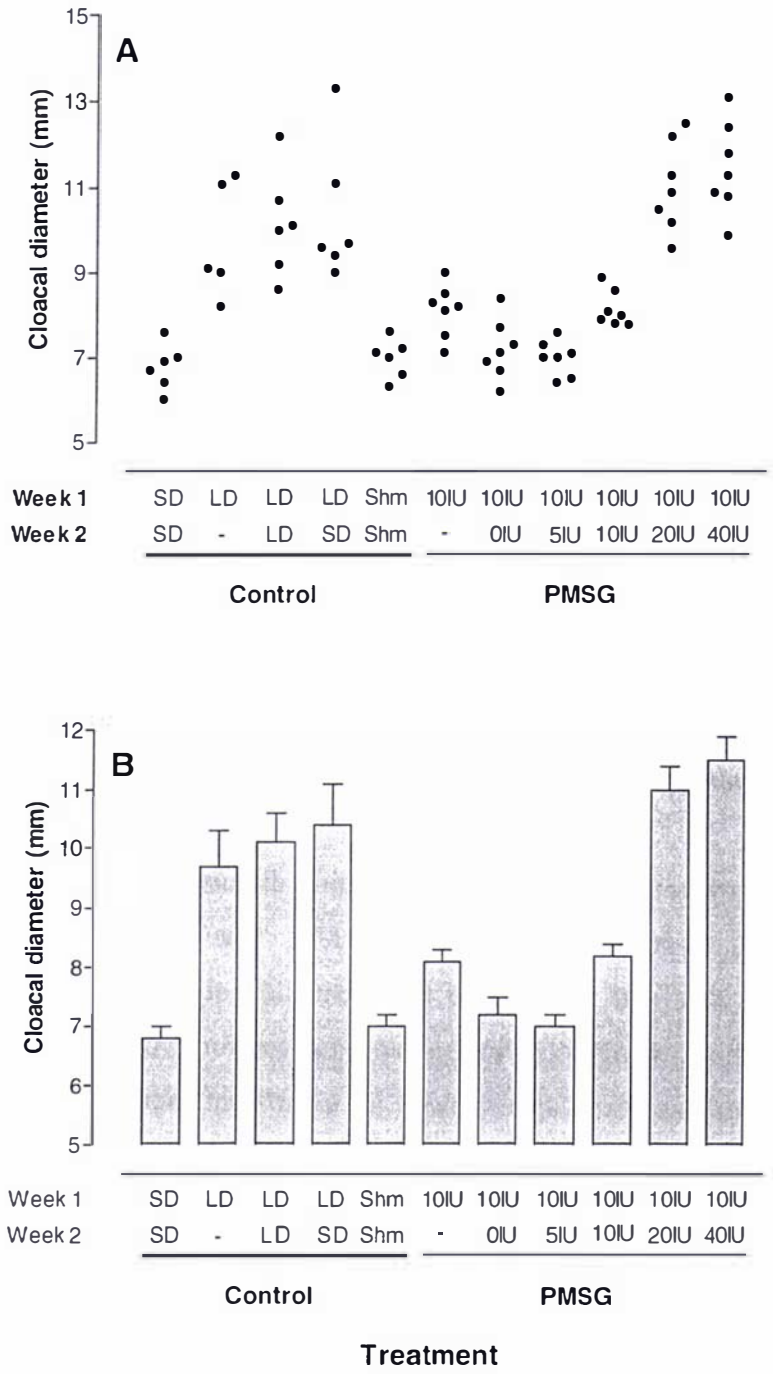

Figure 3.8. Raw data (A) and mean (B) cloacal diameter of female Japanese quail after two weeks on short days, long days, or short days plus treatment with daily injections of 10 IU PMSG for one week, followed by treatment with 0, 5, 10, 20 or 40 IU PMSG for the second week.

Table 3.8. Summary of results of repeated measures ANOVA for changes in cloacal diameter across days for each group in experiment 2.

Group	F	df	*p*
Short day	1.766	12, 60	0.075
Long day (1 week)	16.713	6, 24	0.000
Long day (2 weeks)	24.957	12, 60	0.000
Long day to short day	8.216	12, 60	0.000
Sham injection	1.519	12, 60	0.142
10 IU (1 week)	1.936	6, 36	0.101
10 IU/0 IU PMSG	1.931	12, 72	0.044
10 IU/5 IU PMSG	2.299	12, 72	0.015
10 IU/10 IU PMSG	2.046	12, 72	0.032
10 IU/20 IU PMSG	30.453	12, 72	0.000
10 IU/40 IU PMSG	33.300	12, 72	0.000

At the end of the treatment period, there were significant differences in cloacal opening diameter between groups (Figure 3.8, see Table 3.9 for statistics). The cloacal opening of birds held on short days for two weeks was significantly smaller than that of birds held on long days for the same period. Birds that received daily saline injections had a mean cloacal opening diameter that was not significantly different from short day controls. One week of daily treatment with 10 IU PMSG led to a significant increase in cloacal diameter compared to short day controls. However, the mean cloacal diameter of birds treated for one week was significantly smaller than that of birds held on long days for one week. The mean cloacal opening of birds that received 10 IU PMSG for one week followed by saline injections for one week was not significantly different from short day controls, but was significantly smaller than that of birds held on long days for one week and then transferred to short days for one week. Treatment with 10 IU PMSG for one week followed by 5 IU for one week was insufficient to increase cloacal diameter, as the mean was not significantly different from that of short day controls. The cloacal opening of birds treated

with 10 IU PMSG daily for two weeks was significantly larger that short day controls, and significantly smaller than the cloacal opening of birds held under long day conditions. Birds that received daily injections of 20 IU PMSG during the second week of treatment had a mean cloacal opening diameter that was significantly different to short day controls, but not significantly different from the mean cloacal diameter of birds held under long days. Daily treatment with 40 IU PMSG during the second week of treatment led to an increase in cloacal diameter that was significantly different from both short day and long day control groups.

Table 3.9. Summary of one-way ANOVA and linear contrasts comparing cloacal opening diameter between groups at the end of experiment 2.

ANOVA	**F**	**df**	***p***
All groups	26.177	11, 63	0.000
Linear contrasts	**F**	**df**	***p***
Long day vs short day	50.504	1, 63	0.000
Short day vs Sham injection	0.284	1, 63	0.596
Short day vs 10 IU (1 week)	10.987	1, 63	0.002
Long day (1 week) vs 10 IU (1 week)	16.486	1, 63	0.000
Short day vs 10 IU/0 IU PMSG	1.167	1, 63	0.284
Long days vs 10 IU/0 IU PMSG	39.619	1, 63	0.000
Long day/short day vs 10 IU/0 IU PMSG	43.935	1, 63	0.000
Short day vs 10 IU/5 IU PMSG	0.370	1, 63	0.545
Long days vs 10 IU/5 IU PMSG	45.791	1, 63	0.000
Short day vs 10 IU/10 IU PMSG	12.052	1, 63	0.001
Long days vs 10 IU/10 IU PMSG	15.235	1, 63	0.000
Short day vs 10 IU/20 IU PMSG	80.590	1, 63	0.000
Long days vs 10 IU/20 IU PMSG	2.567	1, 63	0.114
Short day vs 10 IU/40 IU PMSG	93.740	1, 63	0.000
Long days vs 10 IU/40 IU PMSG	5.323	1, 63	0.024

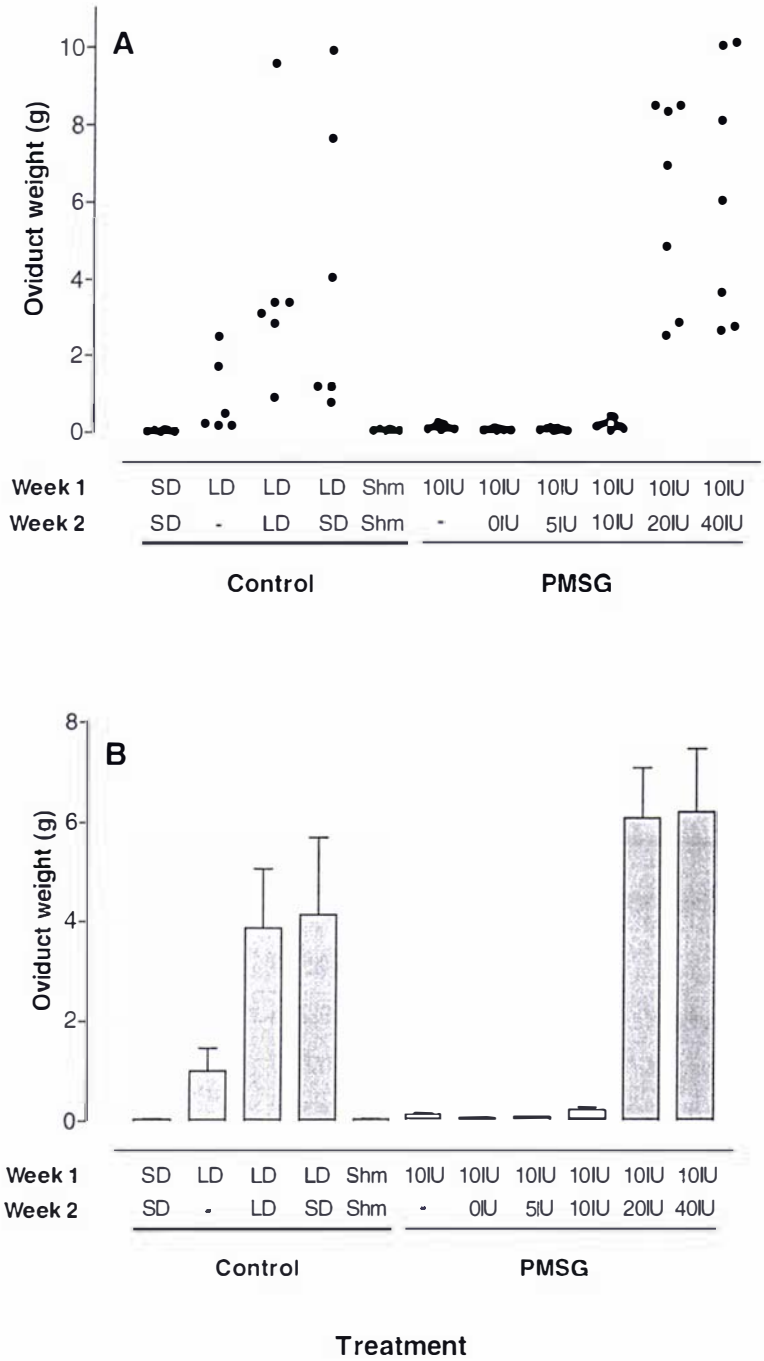

Figure 3.9. Raw data (A) and mean (B) oviduct weight of female Japanese quail after two weeks on short days, long days, or short days plus treatment with daily injections of 10 IU PMSG for one week, followed by treatment with 0, 5, 10, 20 or 40 IU PMSG for the second week.

3.3.2.2 Oviduct weight

There were significant differences in oviduct weight between groups at the end of the treatment period (Figure 3.9, see Table 3.10 for statistics). There was a significant difference in mean oviduct weight between short day and long day control groups.

Table 3.10. Summary of Kruskal-Wallis non-parametric ANOVA and Mann-Whitney U tests comparing ovary weight between groups and oviduct weight between groups at the end of experiment 2.

	Ovary weight			Oviduct weight		
Kruskal-Wallis	**K-W**	**df**	***p***	**K-W**	**df**	***p***
All groups	63.782	11	0.000	65.265	11	0.000
Mann-Whitney U tests			***p***			***p***
Long day vs short day	0.0	1	0.004	0.0	1	0.004
Short day vs Sham injection	13.0	1	0.421	16.0	1	0.739
Short day vs 10 IU (1 week)	3.0	1	0.009	4.0	1	0.015
Long day (1 wk) vs 10 IU (1 wk)	42.0	1	0.003	42.0	1	0.003
Short day vs 10 IU/0 IU PMSG	14.5	1	0.345	11.5	1	0.170
Long days vs 10 IU/0 IU PMSG	42.0	1	0.003	42.0	1	0.003
Long/short day vs 10 IU/0 IU PMSG	42.0	1	0.003	42.0	1	0.003
Short day vs 10 IU/5 IU PMSG	15.5	1	0.428	9.0	1	0.083
Long days vs 10 IU/5 IU PMSG	42.0	1	0.003	42.0	1	0.003
Short day vs 10 IU/10 IU PMSG	3.0	1	0.010	2.5	1	0.080
Long days vs 10 IU/10 IU PMSG	42.0	1	0.003	42.0	1	0.003
Short day vs 10 IU/20 IU PMSG	0.0	1	0.003	0.0	1	0.003
Long days vs 10 IU/20 IU PMSG	25.2	1	0.520	14.5	1	0.352
Short day vs 10 IU/40 IU PMSG	0.0	1	0.003	0.0	1	0.003
Long days vs 10 IU/40 IU PMSG	20.5	1	0.943	13.0	1	0.253

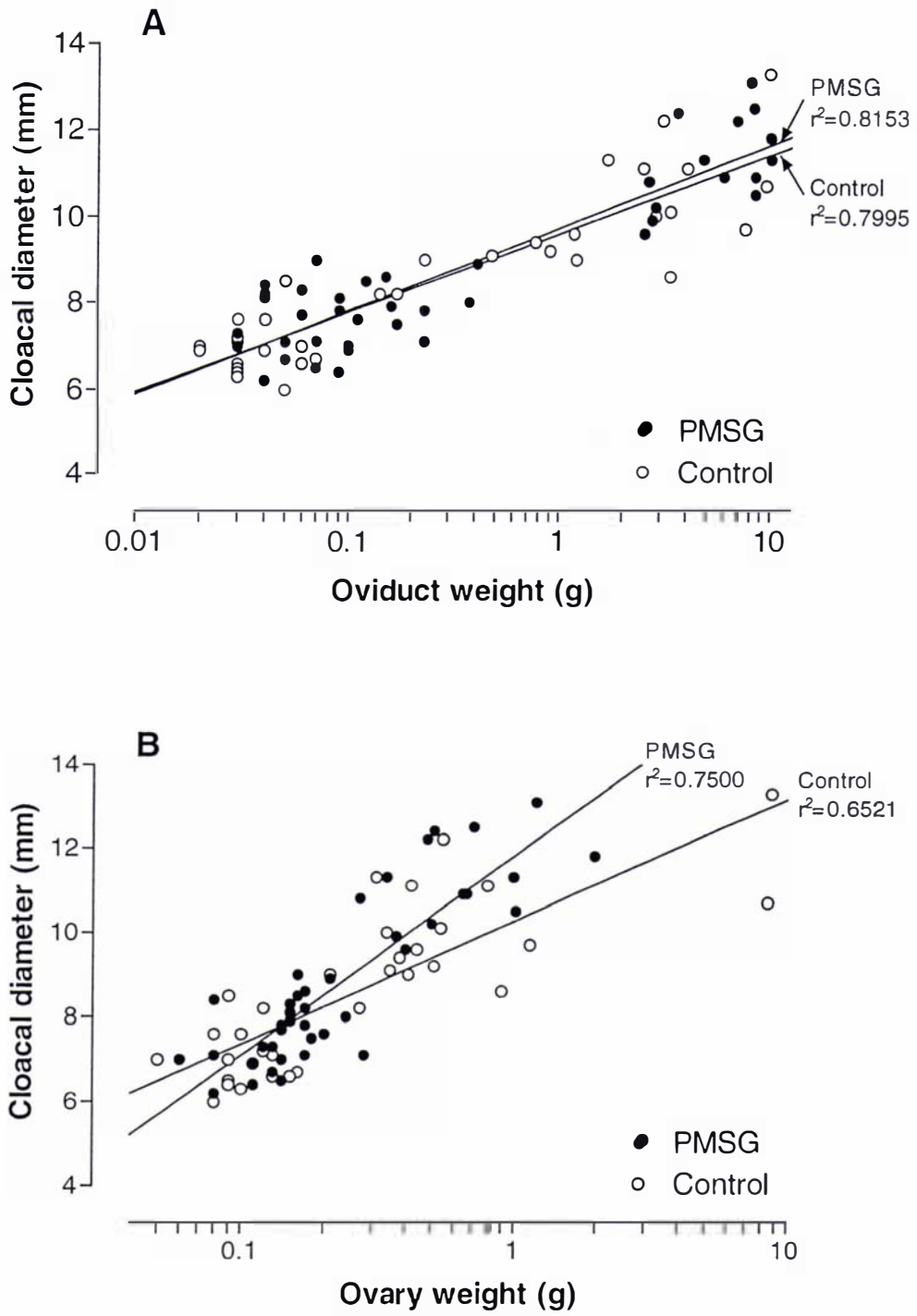

Figure 3.10. Relationships between oviduct (A) or ovary (B) weight and cloacal diameter of control birds (untreated birds held on short days or long days) and birds held on short days receiving daily treatment for one week with 10 IU PMSG, followed by one week of treatment with 0, 5, 10, 20 or 40 IU PMSG.

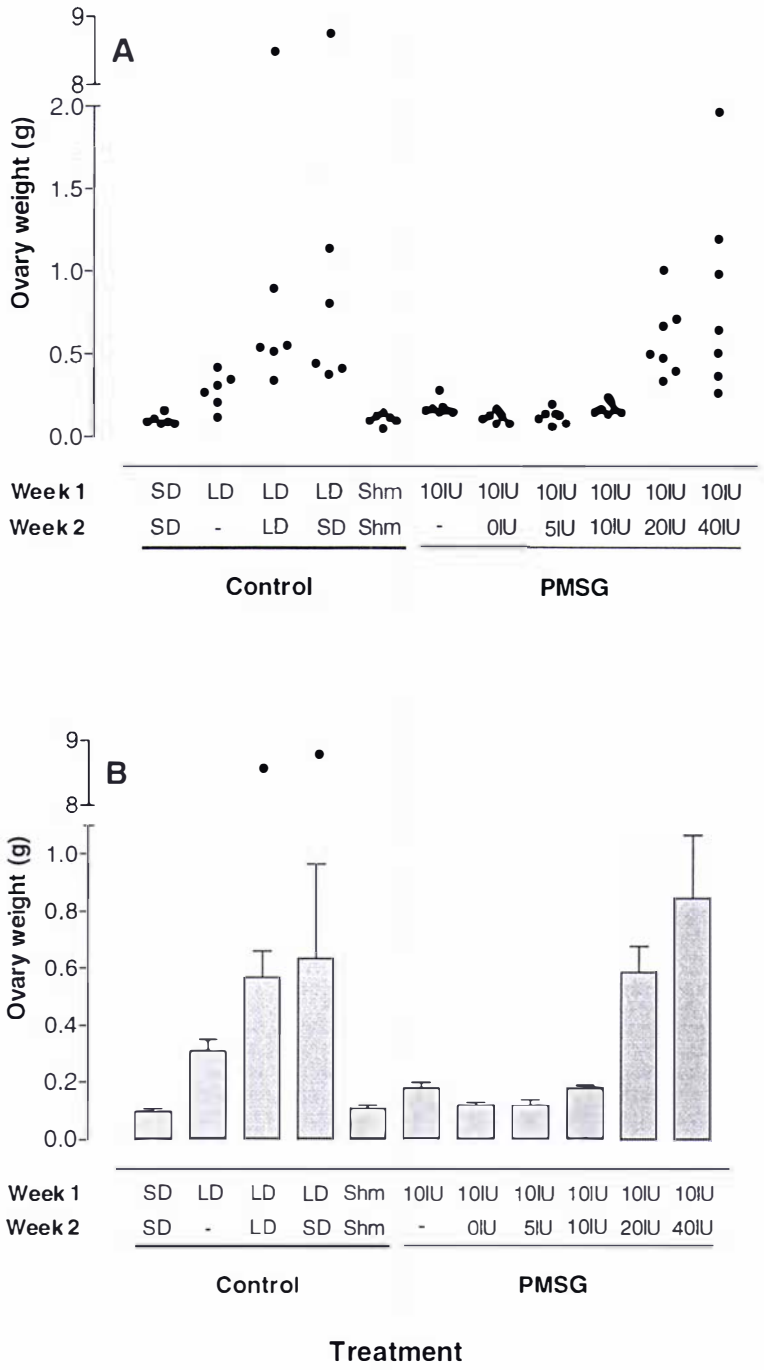

Figure 3.11. Raw data (A) and mean (B) ovary weight of female Japanese quail after two weeks on short days (n=6), long days (n=5), or short days plus treatment with daily injections of 10 IU PMSG for one week, followed by treatment with 0, 5, 10, 20 or 40 IU PMSG for the second week (n=6).

The mean oviduct weights of birds treated with saline injections or 10 IU PMSG for one week followed by 0, 5 or 10 IU PMSG for the second week were not significantly different to that of short day controls. Birds treated with 10 IU PMSG for one week had significantly larger oviducts than birds held on short days, but mean oviduct weight was significantly smaller than that of birds held on long days for one week. The mean oviduct weight of birds treated with 10 IU PMSG for one week followed by saline injections for one week was significantly smaller than the oviduct weight of birds held on long days for one week, and then transferred to short days for one week. The oviduct weight of birds transferred from long days to short days was not significantly different from that of birds maintained on long days for two weeks. Treatment with 10 IU PMSG daily for one week followed by treatment with 20 IU or 40 IU PMSG led to a significant increase in oviduct weight compared to short day controls, with oviduct weights as great as long day controls. Oviduct weight showed a positive correlation with cloacal diameter for both control and treated groups (Figure 3.10A and Table 3.11).

3.3.2.3 Ovary weight

Some ovarian growth was stimulated by doses of 10 IU PMSG or higher when compared to short day controls (Figure 3.11, see Table 3.10 for statistics). However, yellow follicles developed in the ovary of only one treated bird which received 10 IU PMSG for one week followed by daily treatment with 40 IU PMSG for the second week. Similarly, birds transferred to long days for two weeks showed a significant increase in ovarian weight compared to short day controls, but large yellow follicles were present in the ovary of only a single bird. The mean ovarian weights of birds treated with saline injections or 10 IU PMSG for one week followed by 0 IU or 5 IU PMSG for the second week were not significantly different to that of short day controls. Birds treated with 10 IU PMSG for one week or two weeks had significantly larger ovaries than short day controls, but mean ovarian weights were significantly smaller than those of birds held on long days for one or two weeks. The mean ovarian weight of birds held on long days for one week, and then transferred to short days for one week was significantly larger than the ovarian weight of

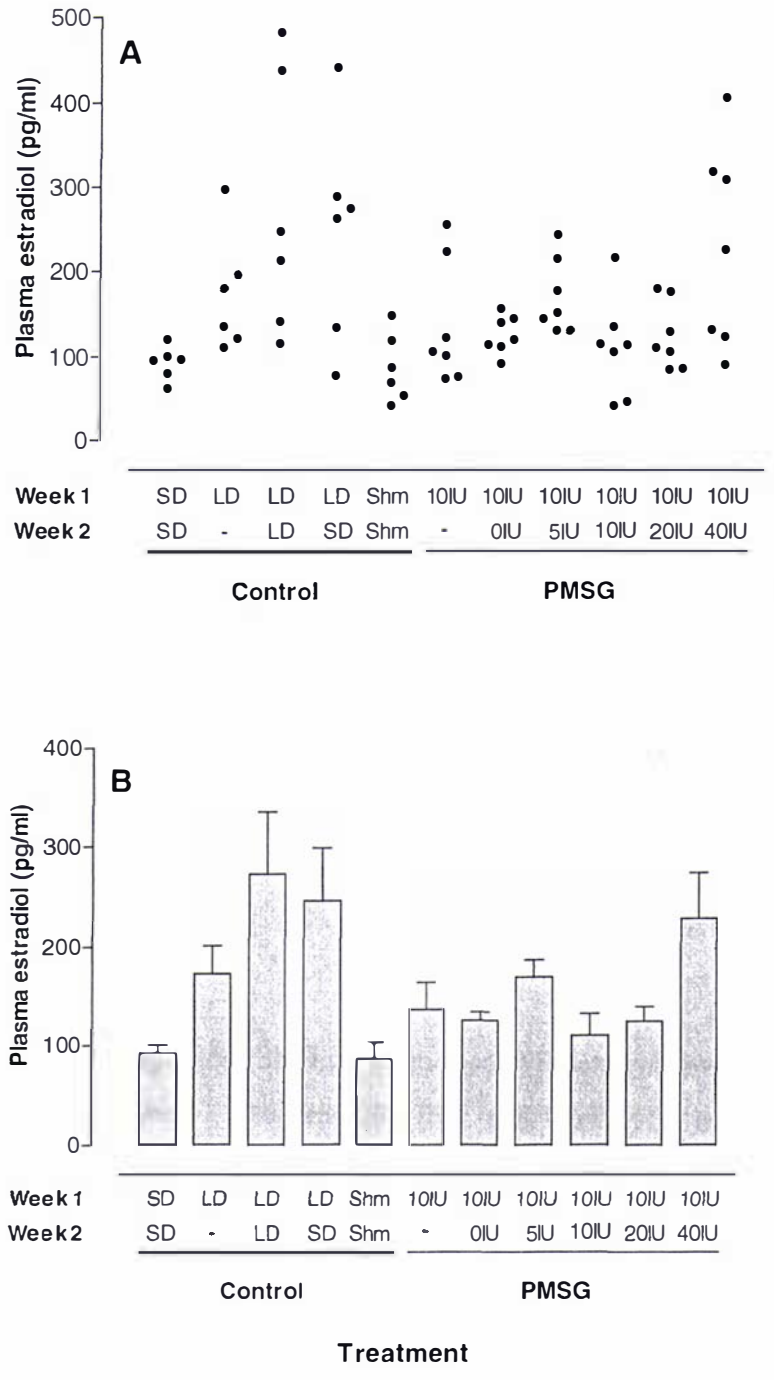

Figure 3.12. Raw data (A) and mean (B) plasma estradiol concentration of female Japanese quail after two weeks on short days, long days, or short days plus treatment with daily injections of 10 IU PMSG for one week, followed by treatment with 0, 5, 10, 20 or 40 IU PMSG for the second week.

birds that received PMSG treatment for one week followed by saline injections for one week. The ovary weight of birds transferred from long days to short days was not significantly different from that of birds maintained on long days for two weeks. Treatment with 10 IU PMSG daily for one week followed by treatment with 20 IU or 40 IU PMSG led to an increase in ovarian weight significantly greater than short day controls, and not significantly different to long day controls. Ovarian weight showed a positive correlation with cloacal diameter for both control and treated groups (Figure 3.10B and Table 3.11).

Table 3.11. Summary of linear regression relationship between ovary weight and cloacal diameter, and between oviduct weight and cloacal diameter for all control and treated groups in experiment 2.

Group	Ovary weight			Oviduct weight		
	r^2	Slope	*p*	r^2	Slope	*p*
Untreated controls	0.6521	2.91 ± 0.38	<0.0001	0.7995	1.81 ± 0.16	<0.0001
PMSG treated	0.7500	4.71 ± 0.43	<0.0001	0.8153	1.91 ± 0.14	<0.0001

3.3.2.4 Plasma estradiol

There was a significant difference in plasma estradiol concentrations between groups at the end of the experiment (Figure 3.12, see Table 3.12 for statistics). Birds maintained on long days for two weeks had significantly higher plasma estradiol concentrations than short day controls. Birds treated daily with saline injections or 10 IU PMSG for one week had similar plasma estradiol concentrations to short day controls. Daily treatment with 10 IU PMSG for one week followed by 10 or 20 IU PMSG did not increase plasma estradiol concentrations, as mean values were not significantly different to those of birds held on short days. Birds treated with 10 IU PMSG for one week followed by 5 or 40 IU for one

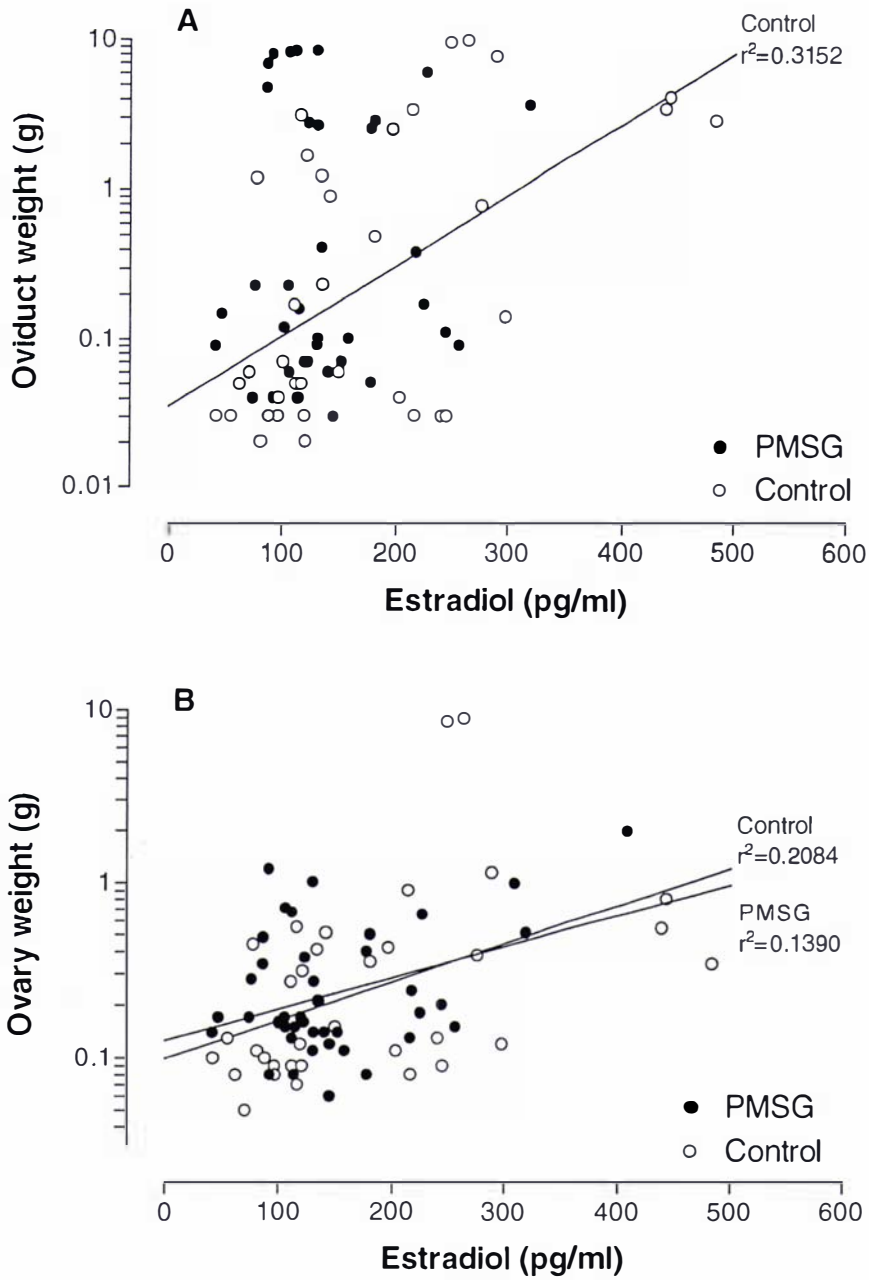

Figure 3.13. Relationships between plasma estradiol concentration and oviduct (A) or ovary weight (B) of control birds (untreated birds held on short days or long days) and birds held on short days receiving daily treatment for one week with 10 IU PMSG, followed by one week of treatment with 0, 5, 10, 20 or 40 IU PMSG.

week had plasma estradiol concentrations significantly greater than short day controls, and not significantly different to birds held on long days.

Table 3.12. Summary of Kruskal-Wallis non-parametric ANOVA and Mann-Whitney U tests comparing plasma estradiol concentrations between groups at the end of experiment 2.

ANOVA	**K-W**	**df**	***p***
All groups	30.786	11	0.001
Mann-Whitney U tests	**K-W**	**df**	***p***
Long day vs short day	1.0	1	0.006
Short day vs Sham injection	22.0	1	0.522
Short day vs 10 IU (1 week)	12.0	1	0.199
Long day (1 week) vs 10 IU (1 week)	34.0	1	0.063
Short day vs 10 IU/0 IU PMSG	7.0	1	0.046
Long days vs 10 IU/0 IU PMSG	36.0	1	0.032
Long day/short day vs 10 IU/0 IU PMSG	32.0	1	0.116
Short day vs 10 IU/5 IU PMSG	0.0	1	0.003
Long days vs 10 IU/5 IU PMSG	28.0	1	0.317
Short day vs 10 IU/10 IU PMSG	15.0	1	0.391
Long days vs 10 IU/10 IU PMSG	38.0	1	0.015
Short day vs 10 IU/20 IU PMSG	10.0	1	0.116
Long days vs 10 IU/20 IU PMSG	37.0	1	0.022
Short day vs 10 IU/40 IU PMSG	4.0	1	0.015
Long days vs 10 IU/40 IU PMSG	25.0	1	0.568

There were significant positive correlations between plasma estradiol concentration and ovary weight for treated groups and untreated controls (Figure 3.13, see Table 3.13 for statistics). A linear relationship between oviduct weight and plasma estradiol concentration was significant for control groups, but not for groups treated with PMSG.

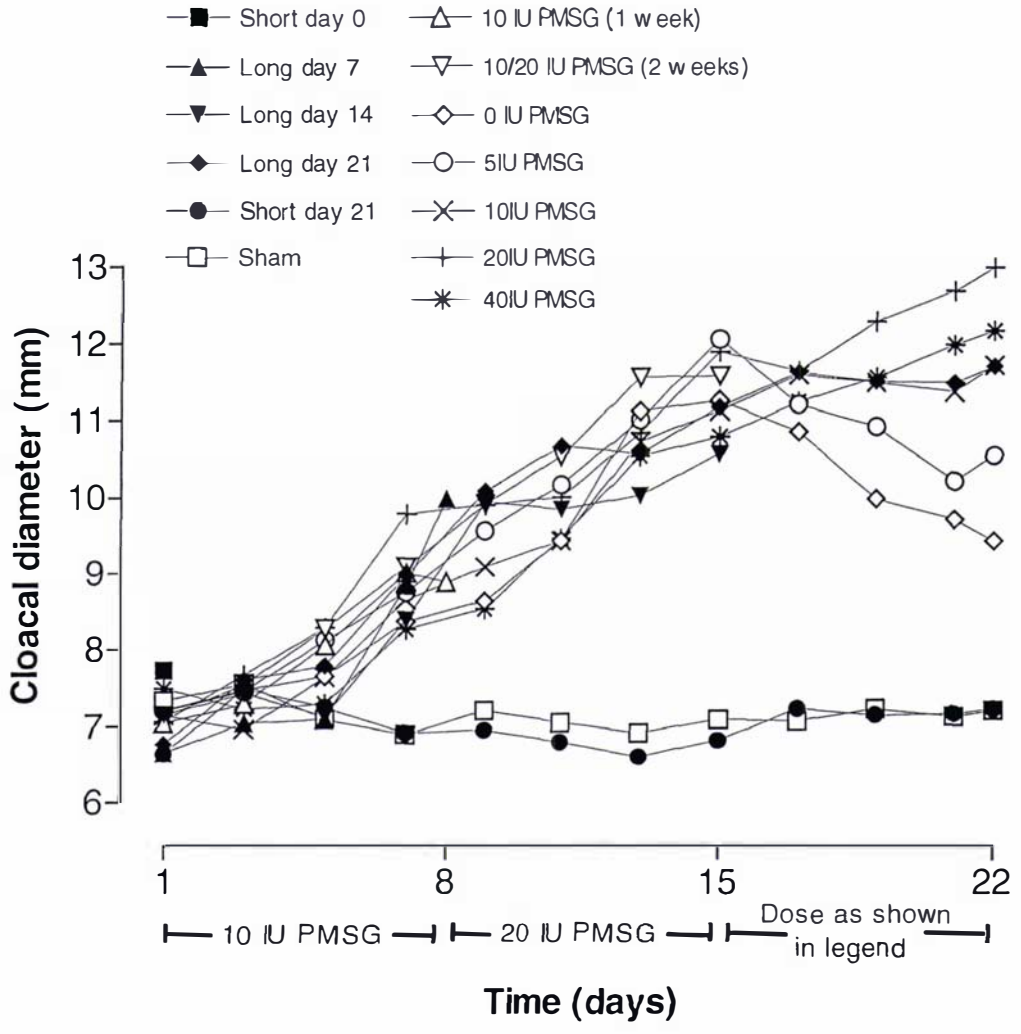

Figure 3.14. Changes in mean cloacal diameter of female Japanese quail held on short days, long days, and short days plus treatment with daily injections of 10 IU PMSG for one week, 20 IU for the second week, and 0, 5, 10, 20 or 40 IU PMSG for the third week.

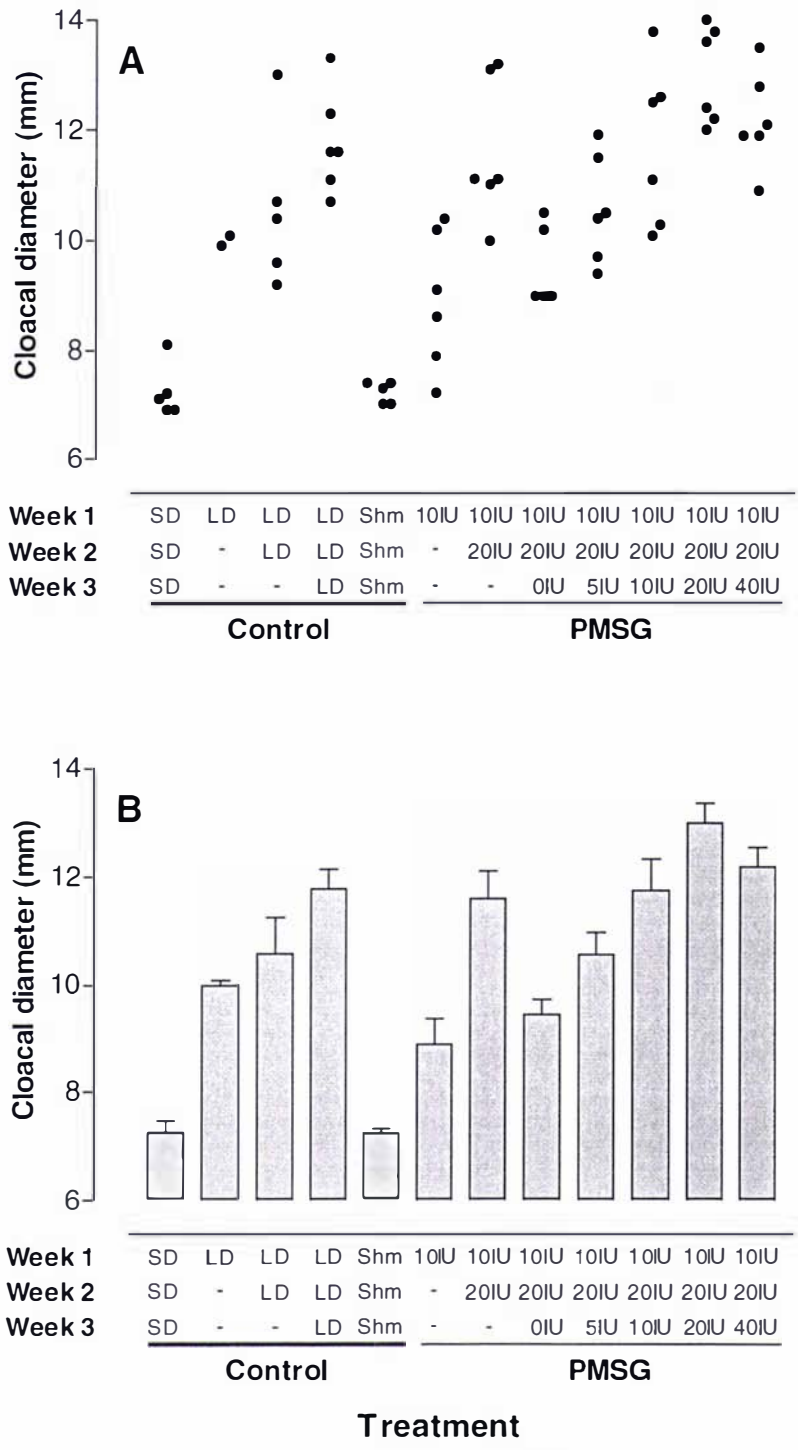

Figure 3.15. Raw data (A) and mean (B) cloacal diameter of female Japanese quail after three weeks on short days, long days, or short days plus treatment with daily injections of 10 IU PMSG for one week, 20 IU for the second week, and 0, 5, 10, 20 or 40 IU PMSG for the third week.

Table 3.13. Summary of linear regression relationship between ovary weight and plasma estradiol concentration, and between oviduct weight and plasma estradiol concentration for all control and treated groups in experiment 2.

Group	Ovary weight			Oviduct weight		
	r^2	Slope	*p*	r^2	Slope	*p*
Untreated controls	0.2084	0.002 ± 0.001	0.0051	0.3152	0.004 ± 0.001	0.0004
PMSG treated	0.1390	0.002 ± 0.001	0.0150	0.0761	0.003 ± 0.002	0.0770

3.3.3 Experiment 3

3.3.3.1 Cloacal opening

At the start of the treatment period, cloacal opening diameter did not differ significantly between groups ($F_{12, 56}$=1.162, *p*=0.332; Figure 3.14). The cloacal openings of birds that remained on short days and received no treatment, and birds that received daily saline injections for three weeks did not change during the treatment period (see Table 3.14 for statistics). All PMSG treated groups, and groups transferred to long day conditions, showed an increase in cloacal opening diameter during the treatment period.

At the end of the treatment period there were significant differences in cloacal opening diameter between groups (Figure 3.15, see Table 3.15 for statistics). Birds held under a long day photoperiod for three weeks had significantly larger cloacal openings than birds held on short days for the same period. The mean cloacal opening of birds that received daily saline injections was not significantly different from that of short day controls. One week of daily treatment with 10 IU PMSG only, or one week of treatment with 10 IU

PMSG followed by one week of treatment with 20 IU PMSG led to a significant increase in cloacal opening diameter compared to short day controls. Furthermore, mean cloacal diameters in both these groups were not significantly different from the mean cloacal diameter of birds held on long days for one or two weeks. The mean cloacal opening of birds that received 10 IU PMSG for one week, 20 IU for the second week, followed by saline injections for the third week was significantly greater than that of short day controls, but significantly smaller than birds held on long days for three weeks. Birds that received daily injections of 5 or 10 IU PMSG during the third week of treatment had mean cloacal opening diameters that were significantly different to short day controls, and not significantly different from the mean cloacal diameter of birds held under long days. The mean cloacal opening of groups that received 20 or 40 IU PMSG during the third week of treatment were significantly larger than the mean cloacal opening of the short day control group and long day control group.

Table 3.14. Summary of results of repeated measures ANOVA for changes in cloacal diameter across days for each group in experiment 3.

Group	F	df	*p*
Short day	1.770	11, 44	0.089
Long day (1 week)	7.127	4, 16	0.002
Long day (2 weeks)	11.656	7, 35	0.000
Long day (3 weeks)	30.783	11, 55	0.000
Sham injection	0.653	11, 44	0.773
10 IU (1 week)	5.539	4, 20	0.004
10 IU/20 IU PMSG	33.589	7, 35	0.000
10 IU/20 IU/0 IU PMSG	26.010	11, 55	0.000
10 IU/20 IU/5 IU PMSG	38.776	11, 55	0.000
10 IU/20 IU/10 IU PMSG	21.356	11, 55	0.000
10 IU/20 IU/20 IU PMSG	28.413	11, 55	0.000
10 IU/20 IU/40 IU PMSG	35.448	11, 55	0.000

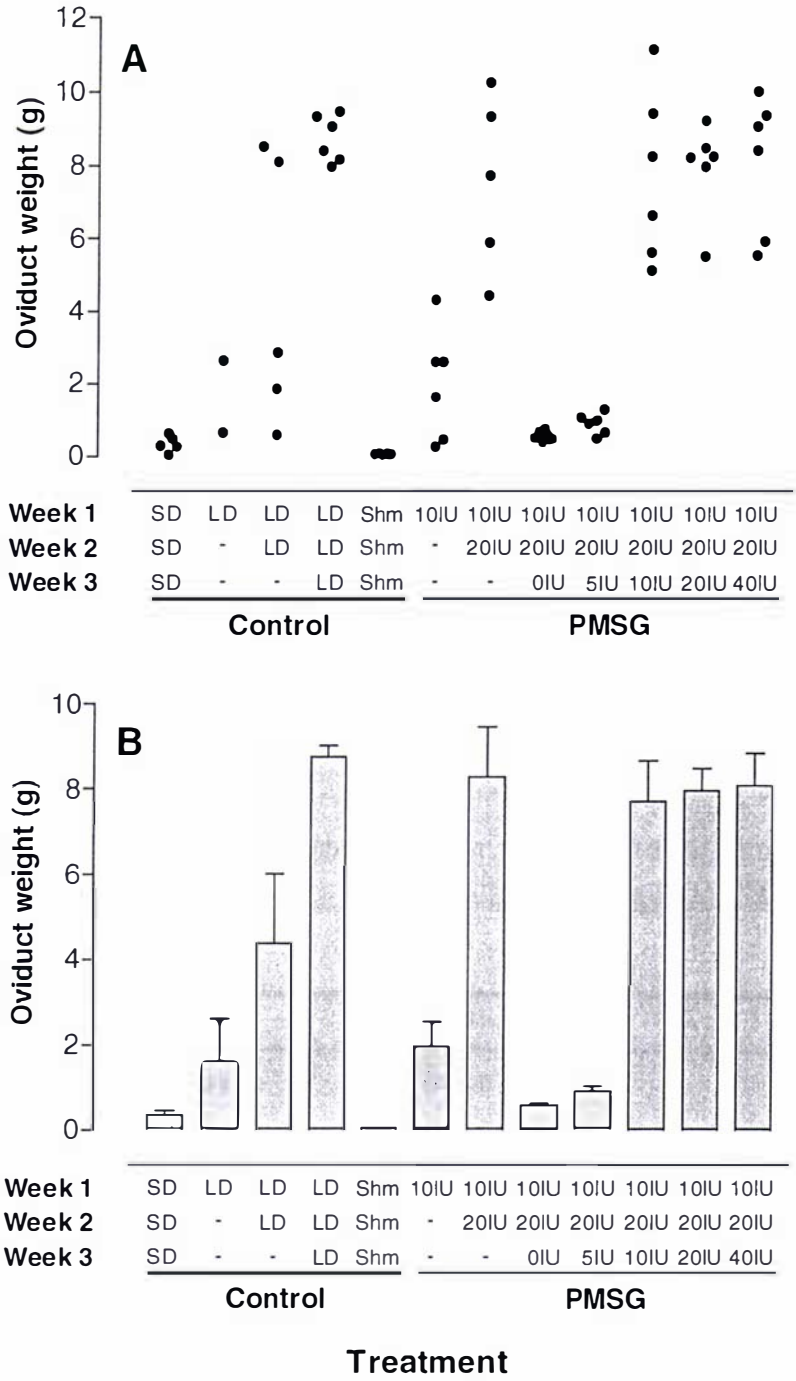

Figure 3.16. Raw data (A) and mean (B) oviduct weight of female Japanese quail after three weeks on short days, long days, or short days plus treatment with daily injections of 10 IU PMSG for one week, 20 IU for the second week, and 0, 5, 10, 20 or 40 IU PMSG for the third week.

Table 3.15. Summary of Kruskal-Wallis non-parametric ANOVA and Mann-Whitney U tests comparing cloacal opening diameter between groups and oviduct weights between groups at the end of experiment 3.

	Cloacal diameter			Oviduct weight		
Kruskal-Wallis	**K-W**	**df**	***p***	**K-W**	**df**	***p***
All groups	55.26	12	0.000	58.22	12	0.000
Mann-Whitney U tests	**M-W**	**df**	***p***	**M-W**	**df**	***p***
Long day vs short day	0.0	1	0.006	0.0	1	0.006
Short day vs Sham injection	9.0	1	0.461	21.0	1	0.076
Short day vs 10 IU (1 week)	2.5	1	0.022	1.0	1	0.011
Long day (1 week) vs 10 IU (1 week)	8.0	1	0.505	7.0	1	0.739
Short day vs 10 IU/20 IU (2 weeks)	0.0	1	0.006	0.0	1	0.006
Long day (1 wk) vs 10 IU/20 IU (2 wks)	6.0	1	0.100	6.0	1	0.100
Short day vs 10 IU/20 IU/0 IU PMSG	0.0	1	0.005	6.0	1	0.100
Long days vs 10 IU/20 IU/0 IU PMSG	36.0	1	0.003	36.0	1	0.004
Short day vs 10 IU/20 IU /5 IU PMSG	0.0	1	0.006	1.0	1	0.011
Long days vs 10 IU/20 IU /5 IU PMSG	30.0	1	0.054	36.0	1	0.004
Short day vs 10 IU/20 IU /10 IU PMSG	0.0	1	0.006	0.0	1	0.006
Long days vs 10 IU/20 IU /10 IU PMSG	18.5	1	0.936	23.0	1	0.423
Short day vs 10 IU/20 IU /20 IU PMSG	0.0	1	0.006	0.0	1	0.006
Long days vs 10 IU/20 IU /20 IU PMSG	5.0	1	0.037	24.0	1	0.337
Short day vs 10 IU/20 IU /40 IU PMSG	0.0	1	0.006	0.0	1	0.006
Long days vs 10 IU/20 IU /40 IU PMSG	5.0	1	0.037	24.0	1	0.337

3.3.3.2 Oviduct weight

There were significant differences in oviduct weight between groups at the end of the treatment period (Figure 3.16, see Table 3.15 for statistics). There was a significant

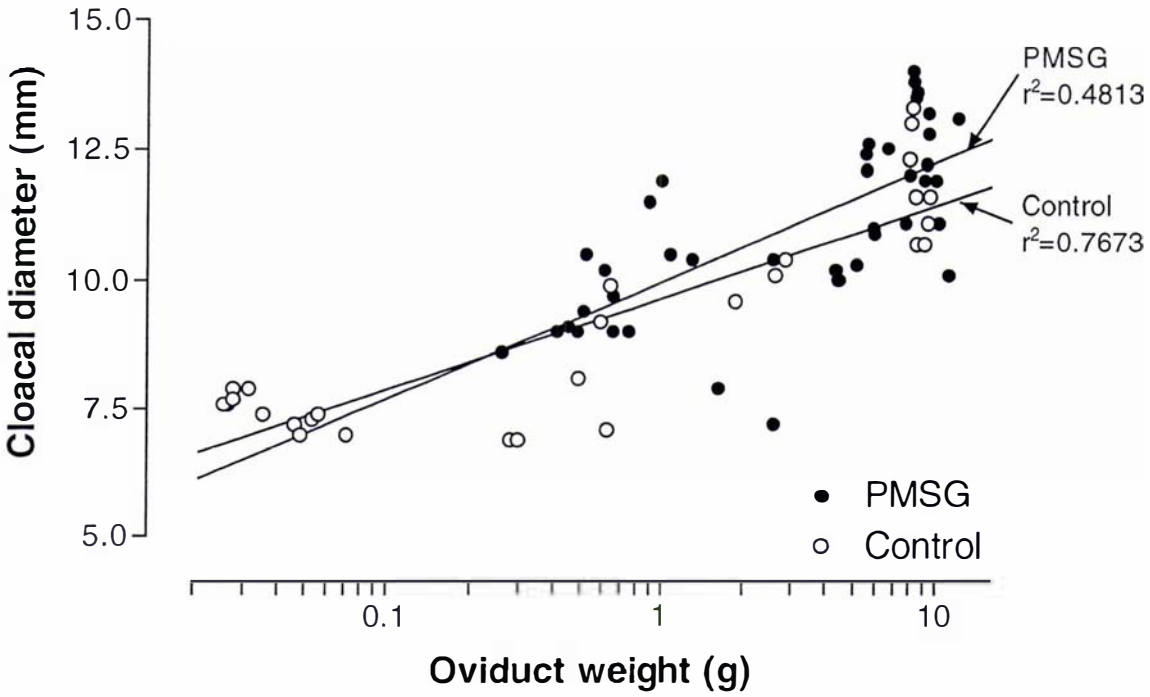

Figure 3.17. Relationships between oviduct weight and cloacal diameter of control birds (untreated birds held on short days or long days) and birds held on short days plus treatment with daily injections of 10 IU PMSG for one week, 20 IU for the second week, and 0, 5, 10, 20 or 40 IU PMSG for the third week.

difference in mean oviduct weight between short day and long day control groups. The mean oviduct weights of birds treated with saline injections for three weeks, or birds given saline injections for the final week of treatment only, were not significantly different to that of short day controls. Birds treated with 10 IU PMSG for one week only, or treated with 10 IU PMSG for one week followed by 20 IU PMSG for one week, had significantly larger oviducts than birds held on short days. Furthermore, mean oviduct weights in both groups were not significantly different to those of birds held on long days for one or two weeks. Birds treated with 10 IU PMSG for one week, 20 IU PMSG for the second week, and 5 IU for the final week had mean oviduct weights that were significantly larger than short day controls, and significantly smaller than long day controls. Treatment with 10, 20 or 40 IU in the third week of treatment led to an increase in mean oviduct weight compared to short day controls. The mean oviduct weights in these three treatment groups were not significantly different to the mean oviduct weight of long day controls. Oviduct weight showed a positive correlation with cloacal diameter for both control and treated groups (Figure 3.17 and Table 3.16).

Table 3.16. Summary of linear regression relationship between ovary weight and cloacal diameter, and between oviduct weight and cloacal diameter for all control and treated groups in experiment 3.

	Oviduct weight			**Ovary weight**		
Group	r^2	**Slope**	*p*	r^2	**Slope**	*p*
Untreated controls	0.7673	1.78 ± 0.19	<0.0001	0.8109	2.25 ± 0.21	<0.0001
PMSG treated	0.4813	2.27 ± 0.37	<0.0001	0.2451	1.49 ± 0.41	0.0009

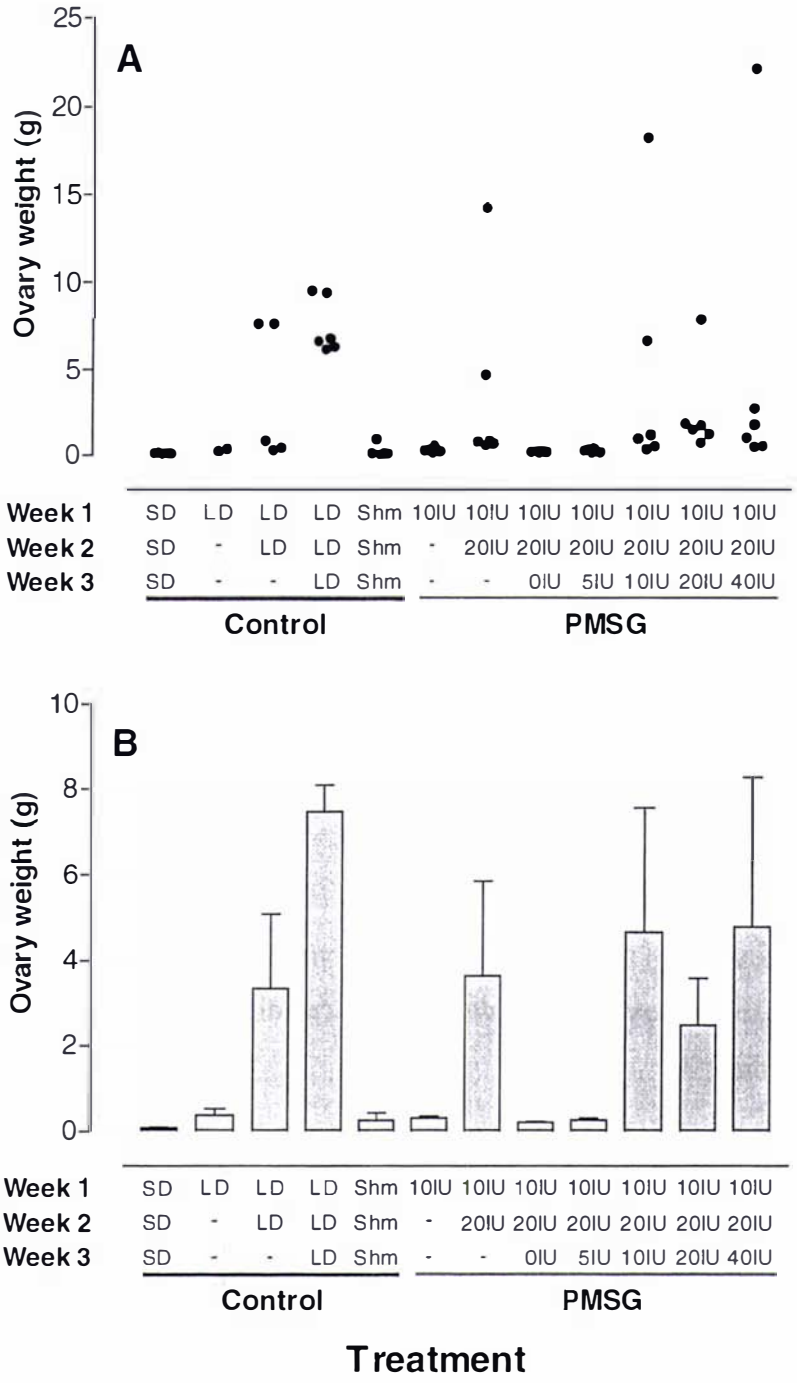

Figure 3.18. Raw data (A) and mean (B) ovary weight of female Japanese quail after three weeks on short days, long days, or short days plus treatment with daily injections of 10 IU PMSG for one week, 20 IU for the second week, and 0, 5, 10, 20 or 40 IU PMSG for the third week.

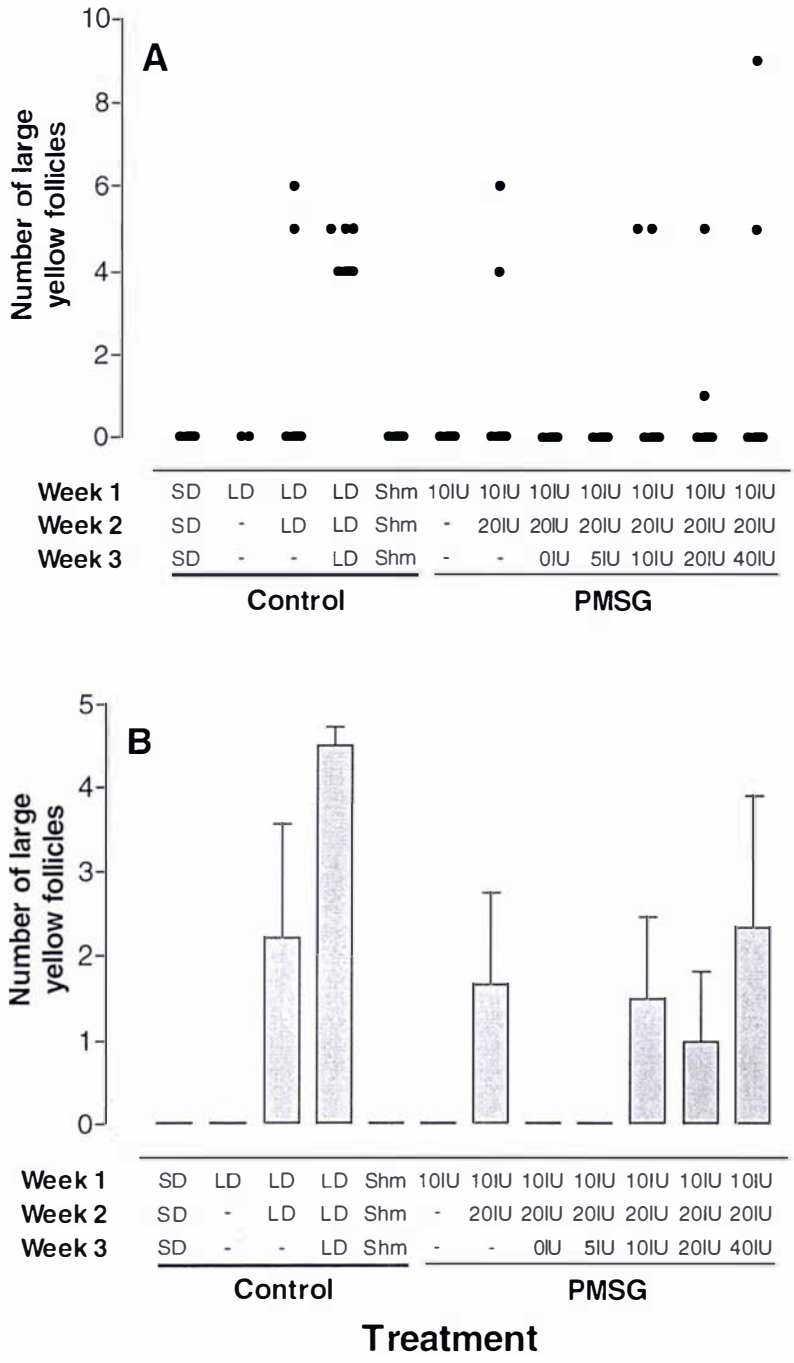

Figure 3.19. Raw data (A) and mean (B) number of large yellow follicles in the ovaries of female Japanese quail after three weeks on short days, long days, or short days plus treatment with daily injections of 10 IU PMSG for one week, 20 IU for the second week, and 0, 5, 10, 20 or 40 IU PMSG for the third week.

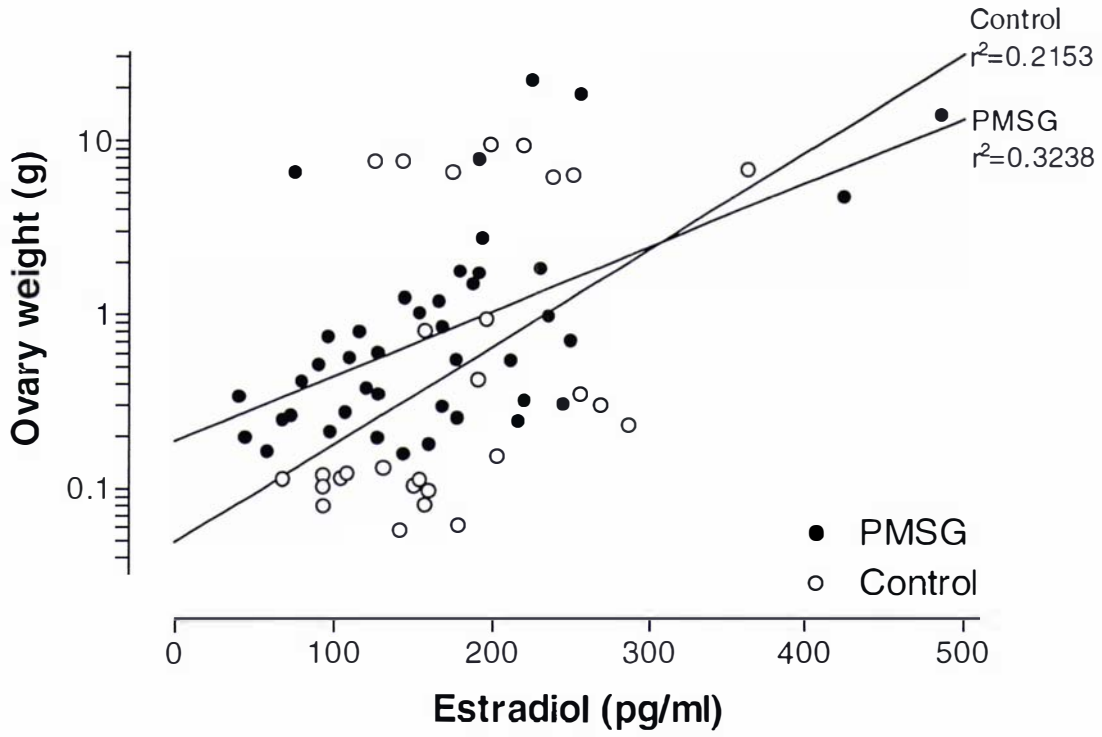

Figure 3.20. Relationships between ovary weight and cloacal diameter of control birds (untreated birds held on short days or long days) and birds held on short days plus treatment with daily injections of 10 IU PMSG for one week, 20 IU for the second week, and 0, 5, 10, 20 or 40 IU PMSG for the third week.

3.3.3.3 Ovary weight and number of large yellow follicles

Ovarian growth was stimulated by all combinations of PMSG treatment when compared to short day controls (Figure 3.18, see Table 3.17 for statistics). However, large yellow follicles had developed in the ovaries of relatively few PMSG treated birds (Figure 3.19, see Table 3.17 for statistics). Birds held under a long day photoperiod for three weeks had significantly larger ovaries and significantly more yellow follicles than birds held on short days for the same period, and all birds in the long day group had large yellow follicles in the ovary. The mean ovary weight and mean number of yellow follicles present in the ovary of birds that received daily saline injections were not significantly different from those of short day controls. One week of daily treatment with 10 IU PMSG only, or one week of treatment with 10 IU PMSG followed by one week of treatment with 20 IU PMSG led to a significant increase in mean ovary weight compared to short day controls. Furthermore, mean ovary weight in each group was not significantly different to the mean ovary weight of birds held on long days for one or two weeks. Yellow follicles did not develop in the ovaries of birds held on long days for one week or in birds treated with 10 IU PMSG for one week. The ovaries of birds that received 0 or 5 IU PMSG in the final week of treatment contained no yellow follicles, but were significantly larger than short day controls. Birds treated with 20 or 40 IU PMSG in the third week of treatment also had mean ovary weights that were significantly larger than short day controls and significantly smaller than long day controls. However, only two birds in each treatment group had yellow follicles present in the ovary. Ovarian weight showed a positive correlation with cloacal diameter for both control and treated groups (Figure 3.20 and Table 3.16). PMSG treated birds did not lay any eggs, whereas 50% of birds held on long days started laying within three weeks.

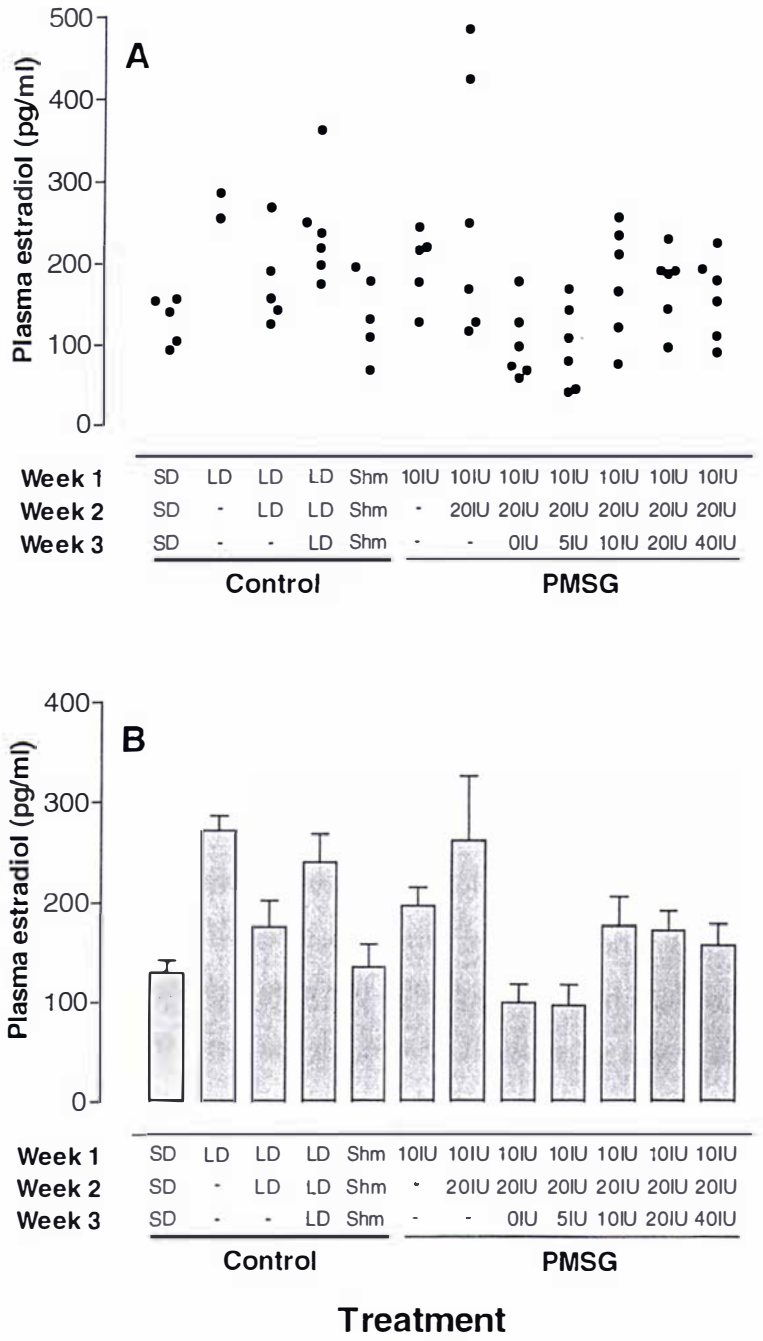

Figure 3.21. Raw data (A) and mean (B) plasma estradiol concentration of female Japanese quail after three weeks on short days, long days, or short days plus treatment with daily injections of 10 IU PMSG for one week, 20 IU for the second week, and 0, 5, 10, 20 or 40 IU PMSG for the third week.

Table 3.17. Summary of Kruskal-Wallis nonparametric ANOVA and Mann-Whitney U tests comparing ovary weight and number of large yellow follicles (LYF) between groups at the end of experiment 3.

	Ovary weight			Number of LYF		
Kruskal-Wallis	**K-W**	**df**	***p***	**K-W**	**df**	***p***
All groups	55.50	12	0.000	29.74	12	0.003
Mann-Whitney U tests	**M-W**	**df**	***p***	**M-W**	**df**	***p***
Long day vs short day	0.0	1	0.006	0.0	1	0.003
Short day vs Sham injection	5.0	1	0.117	12.5	1	1.000
Short day vs 10 IU (1 week)	0.0	1	0.006	15.0	1	1.000
Long day (1 week) vs 10 IU (1 week)	5.0	1	0.739	6.0	1	1.000
Short day vs 10 IU/20 IU (2 weeks)	0.0	1	0.006	10.0	1	0.176
Long day (1 wk) vs 10 IU/20 IU (2 wks)	13.0	1	0.715	16.5	1	0.750
Short day vs 10 IU/20 IU/0 IU PMSG	0.0	1	0.006	15.0	1	1.000
Long days vs 10 IU/20 IU/0 IU PMSG	36.0	1	0.004	36.0	1	0.002
Short day vs 10 IU/20 IU/5 IU PMSG	0.0	1	0.006	15.0	1	1.000
Long days vs 10 IU/20 IU/5 IU PMSG	36.0	1	0.004	36.0	1	0.002
Short day vs 10 IU/20 IU/10 IU PMSG	0.0	1	0.006	10.0	1	0.174
Long days vs 10 IU/20 IU/10 IU PMSG	27.0	1	0.150	27.0	1	0.125
Short day vs 10 IU/20 IU/20 IU PMSG	0.0	1	0.006	10.0	1	0.176
Long days vs 10 IU/20 IU/20 IU PMSG	32.0	1	0.025	31.5	1	0.024
Short day vs 10 IU/20 IU/40 IU PMSG	0.0	1	0.006	10.0	1	0.176
Long days vs 10 IU/20 IU/40 IU PMSG	32.0	1	0.025	31.5	1	0.024

3.3.3.4 Plasma estradiol

There were significant differences in plasma estradiol concentrations between groups at the end of the experiment (Figure 3.21, see Table 3.18 for statistics). Birds maintained on long days for three weeks had significantly higher plasma estradiol concentrations than short day

controls. Mean plasma estradiol concentration in birds treated with saline injections for three weeks, or receiving 0 or 5 IU PMSG during the final week of treatment were not significantly different to the mean plasma estradiol concentration in birds maintained on short days. Birds treated with daily 10, 20 or 40 IU PMSG injections in the third week had plasma estradiol concentrations that were not significantly different from short day or long day control birds.

Table 3.18. Summary of one-way ANOVA and linear contrasts comparing plasma estradiol concentrations between groups at the end of experiment 3.

ANOVA	**F**	**df**	***p***
All groups	3.630	12, 56	0.000
Linear contrasts	**F**	**df**	***p***
Long day vs short day	6.251	1, 56	0.015
Short day vs Sham injection	0.000	1, 56	0.994
Short day vs 10 IU (1 week)	2.622	1, 56	0.111
Long day (1 week) vs 10 IU (1 week)	1.032	1, 56	0.314
Short day vs 10 IU/20 IU (2 weeks)	5.409	1, 56	0.024
Long day (1 wk) vs 10 IU/20 IU (2 wks)	1.260	1, 56	0.266
Short day vs 10 IU/20 IU/0 IU PMSG	1.705	1, 56	0.197
Long days vs 10 IU/20 IU/0 IU PMSG	15.935	1, 56	0.000
Short day vs 10 IU/20 IU/5 IU PMSG	2.814	1, 56	0.099
Long days vs 10 IU/20 IU/5 IU PMSG	19.199	1, 56	0.000
Short day vs 10 IU/20 IU/10 IU PMSG	1.063	1, 56	0.307
Long days vs 10 IU/20 IU/10 IU PMSG	2.375	1, 56	0.129
Short day vs 10 IU/20 IU/20 IU PMSG	1.259	1, 56	0.267
Long days vs 10 IU/20 IU/20 IU PMSG	2.089	1, 56	0.154
Short day vs 10 IU/20 IU/40 IU PMSG	0.498	1, 56	0.483
Long days vs 10 IU/20 IU/40 IU PMSG	3.542	1, 56	0.065

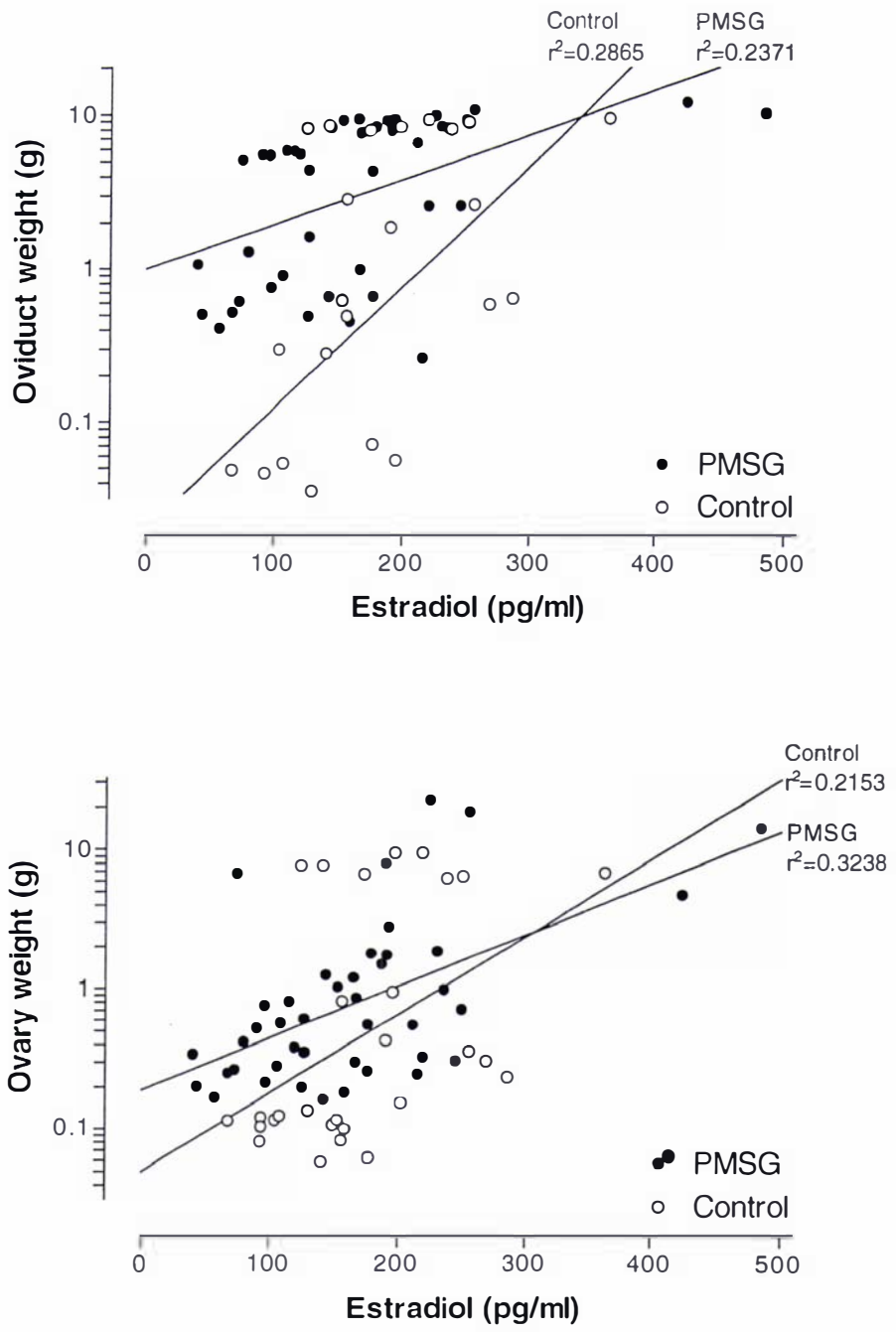

Figure 3.22. Relationships between plasma estradiol concentration and oviduct or ovary weight of control birds (untreated birds held on short days or long days) and birds held on short days plus treatment with daily injections of 10 IU PMSG for one week, 20 IU for the second week, and 0, 5, 10, 20 or 40 IU PMSG for the third week.

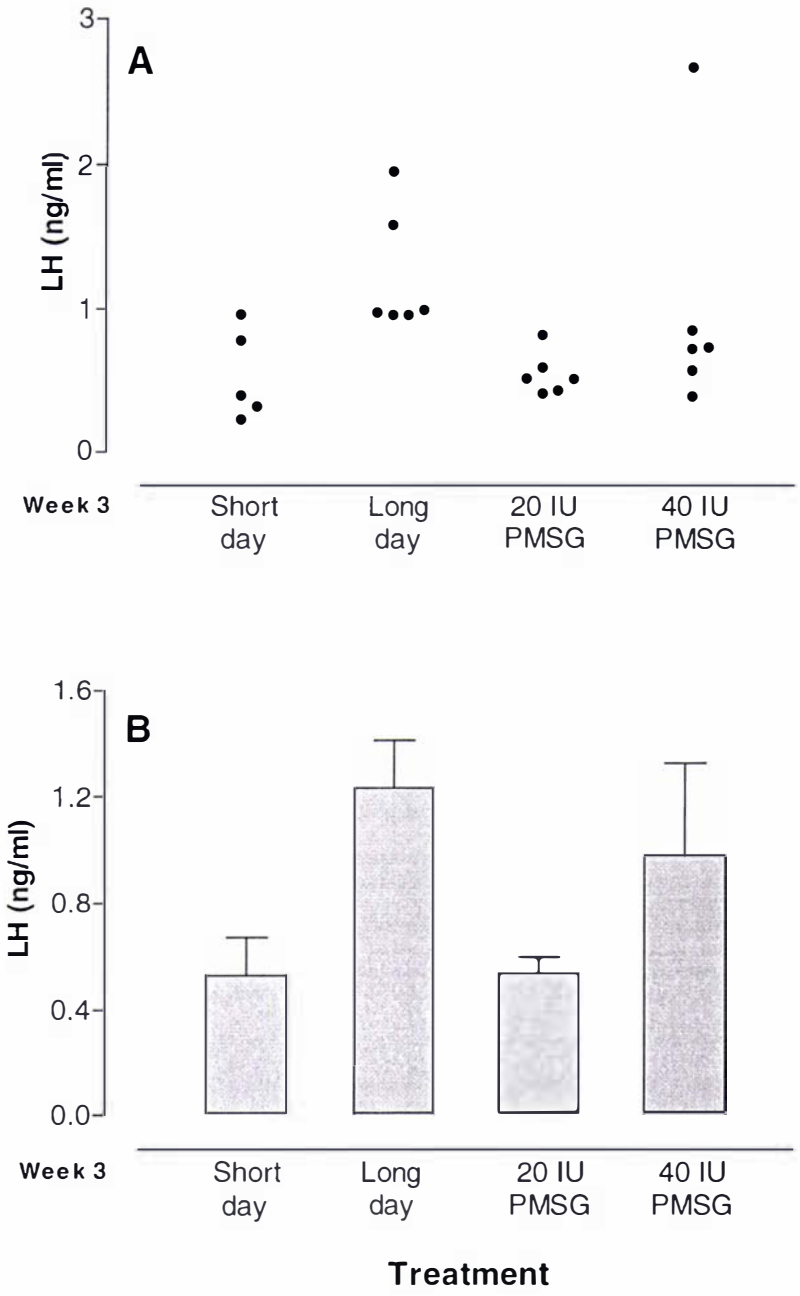

Figure 3.23. Raw data (A) and mean (B) plasma LH concentration of female Japanese quail after three weeks on short days, long days, or short days plus treatment with daily injections of 10 IU PMSG for one week, 20 IU for the second week, and 20 or 40 IU PMSG for the third week.

There were significant positive correlations between plasma estradiol concentrations and both ovary weight and oviduct weight for treated groups and untreated controls (Figure 3.22, see Table 3.19 for statistics).

Table 3.19. Summary of linear regression relationship between ovary weight and plasma estradiol concentration, and between oviduct weight and plasma estradiol concentration for all control and treated groups in experiment 3.

Group	Ovary weight			Oviduct weight		
	r^2	Slope	*p*	r^2	Slope	*p*
Untreated controls	0.2153	0.006 ± 0.002	0.0129	0.2865	0.008 ± 0.002	0.0033
PMSG treated	0.3238	0.004 ± 0.001	<0.0001	0.2371	0.003 ± 0.001	0.0011

3.3.3.5 Plasma luteinising hormone

Plasma luteinising hormone concentrations were measured in some groups to ensure that endogenous LH levels had not increased in response to PMSG. There was no significant difference in plasma LH concentration between groups (Figure 3.23, see Table 3.20 for statistics). However, linear contrasts showed that there was a significant difference in mean plasma LH concentration between short day and long day controls, and also between long day controls and birds receiving daily injections of 20 IU PMSG during the final week of treatment.

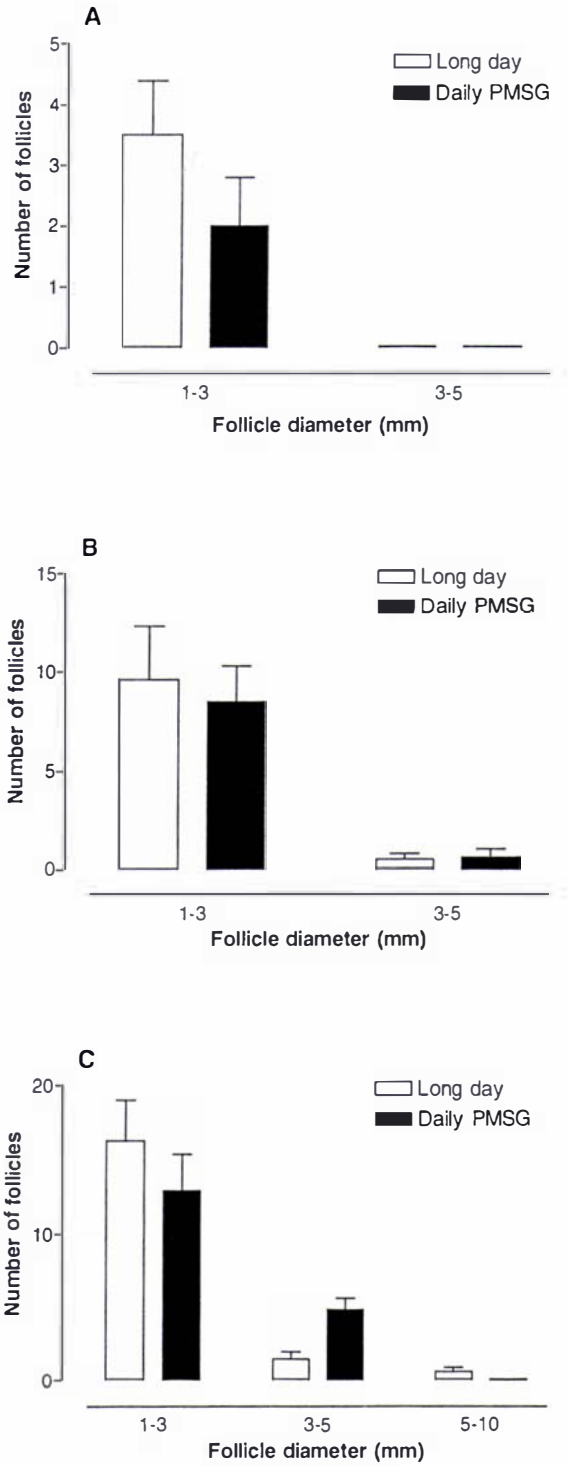

Figure 3.24. Distribution of follicle sizes in 0.00 – 0.15 g (A), 0.16 – 0.50 g (B) and 0.51 – 1.00 g (C) ovaries of female Japanese quail held on long days for one to three weeks, or receiving daily PMSG injections for one to three weeks.

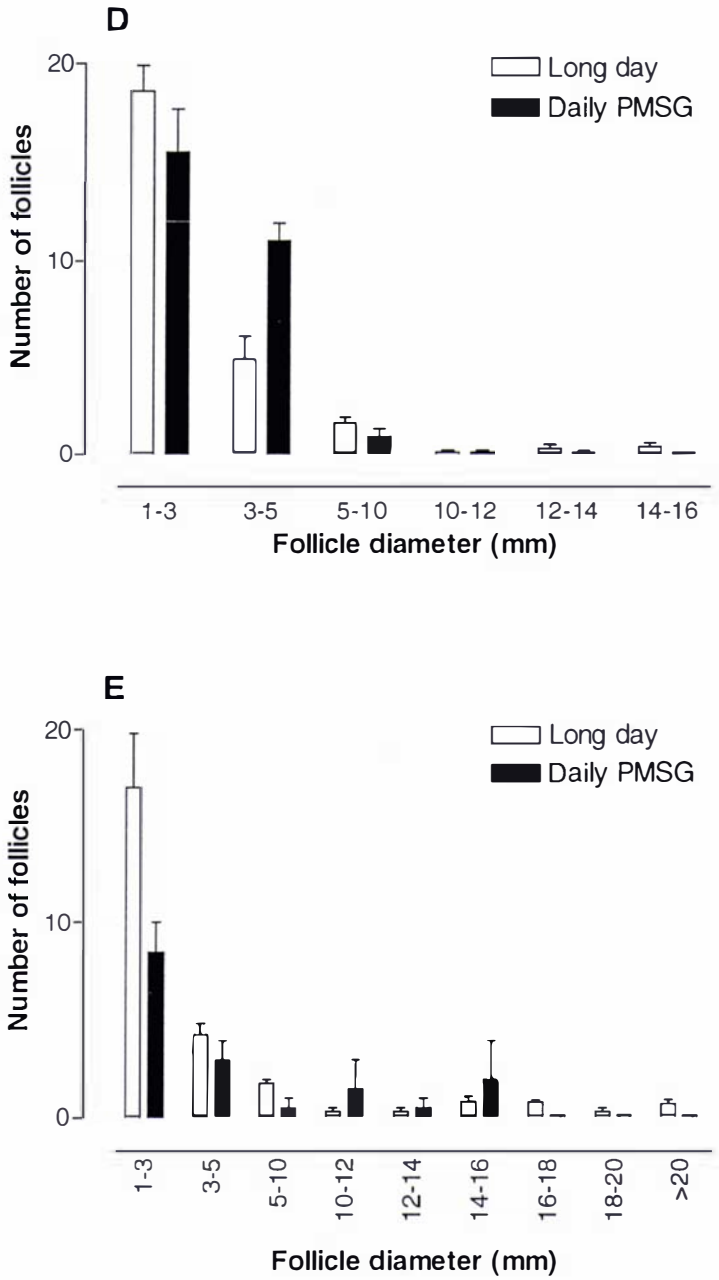

Figure 3.25. Distribution of follicle sizes in 1.01 – 5.00 g (D) and 5.01 – 9.00 g (E) ovaries of female Japanese quail held on long days for one to three weeks, or receiving daily PMSG injections for one to three weeks.

Table 3.20. Summary of one-way ANOVA and linear contrasts comparing plasma luteinising hormone concentrations between groups at the end of experiment 3.

ANOVA	F	df	*p*
	2.632	3, 19	0.080
Linear contrasts	**F**	**df**	***p***
Long day vs short day	5.109	1, 19	0.036
Short days vs 10 IU/20 IU/20 IU PMSG	2.687	1, 19	0.118
Long days vs 10 IU/20 IU/20 IU PMSG	5.540	1, 19	0.030
Short day vs 10 IU/20 IU/40 IU PMSG	2.101	1, 19	0.163
Long days vs 10 IU/20 IU/40 IU PMSG	0.723	1, 19	0.406

3.3.4 Distribution of follicle sizes

The number of follicles in each size class for ovaries weighing less than 0.50 g were similar in long day control birds and birds that received daily PMSG treatment (Figure 3.24, see Table 3.21 for statistics). In ovaries weighing 0.51 - 1.00 g and 1.01 – 5.00 g there were similar numbers of follicles in long day control birds and PMSG treated birds for each follicle size class except for follicles 3-5 mm diameter (Figures 3.24 and 3.25). There were some follicles measuring 5-10 mm in ovaries of long day birds weighing 0.51 – 1.00 g, but there were no follicles in this size class in PMSG treated birds. Similarly, the ovaries of long day birds weighing 1.01 – 5.00 g contained follicles that were 14 – 16 mm in diameter, but these were not present in the same sized ovaries of PMSG treated birds. There were similar numbers of follicles in long day birds and PMSG treated birds in each size class for ovaries that weighed 5.01 - 9.00 g, except for follicles 5 – 10 mm in diameter (Figure 3.25). There were numerous follicles greater than 16 mm in the ovaries of long day

control birds in this ovarian weight range, but no follicles greater than 16 mm in PMSG treated birds.

Table 3.21. Summary of two sample Student's t-tests comparing the number of follicles in each ovarian size class between birds held on long days and birds treated daily with PMSG. Tests could not be performed when one or both groups had no follicles in a particular size class (indicated by "No test").

Ovary size (g)	Follicle diameter (mm)	t	df	*p*
0.00 – 0.15	1 – 3	1.296	18	0.211
0.00 – 0.15	3 – 5		No test	
0.16 – 0.50	1 – 3	0.356	19	0.726
0.16 – 0.50	3 – 5	0.160	19	0.875
0.51 – 1.00	1 – 3	0.922	16	0.370
0.51 – 1.00	3 – 5	-3.505	16	0.003
0.51 – 1.00	5 – 10		No test	
1.01 – 5.00	1 – 3	1.146	16	0.268
1.01 – 5.00	3 – 5	-4.233	16	0.001
1.01 – 5.00	5 – 10	1.341	16	0.199
1.01 – 5.00	10 – 12	0.158	16	0.876
1.01 – 5.00	12 – 14	0.816	16	0.426
1.01 – 5.00	14 – 16		No test	
5.01 – 9.00	1 – 3	1.322	10	0.215
5.01 – 9.00	3 – 5	0.909	10	0.385
5.01 – 9.00	5 – 10	2.621	10	0.026
5.01 – 9.00	10 – 12	-1.907	10	0.086
5.01 – 9.00	12 – 14	-0.506	10	0.624
5.01 – 9.00	14 – 16	-1.240	10	0.243
5.01 – 9.00	16 – 18		No test	
5.01 – 9.00	18 – 20		No test	
5.01 – 9.00	> 20		No test	

3.4 Discussion

This is the first experiment in which ovarian development has been stimulated in Japanese quail using daily injections of PMSG. The effectiveness of daily injections of PMSG was similar to continuous PMSG treatment with osmotic pumps. The best combination of injection doses for stimulating ovarian development was 10 IU daily for one week, followed by 20 IU daily for one week, and then either 20 or 40 IU for a third week. Although ovarian growth occurred in some PMSG treated birds, ovulation and egg laying were not induced in any of these birds. In the birds that did development large yellow follicles in the ovary, these were not arranged in a hierarchy. Several birds did not respond to PMSG treatment and showed little or no ovarian growth, indicating that the current doses may not be high enough to stimulate follicular development in some birds, or that the treatment period may need to be extended.

The effectiveness of daily injections and osmotic pumps for delivering PMSG to stimulate ovarian development has not previous been compared in an avian species. Wakabayashi *et al.* (1996) stimulated ovarian development in Japanese quail using osmotic pumps delivering PMSG, but did not compare osmotic pumps with daily injections. Daily injections and implantation of osmotic pumps were equally effective for administering PMSG to Japanese quail. There were no significant differences in development of the ovary or oviduct between the two delivery methods. Therefore, when deciding which delivery method to use in future experiments, factors such as cost, ease of use, and other practicalities of the project must be considered. Although minor surgery is involved, osmotic pumps are advantageous as once they are implanted, the bird does not need to be captured and handled again until the pump is removed. This would be useful in situations where it is difficult or highly stressful for the bird to be captured on a regular basis. Injections require regular capture and handling of birds, but do allow for more control over dose, frequency and duration of treatment. The Japanese quail used in the current experiment and future experiments are housed in small, individual cages, and are therefore easily captured. Experiments in our laboratory have shown that Japanese quail do not

initiate a corticosterone response when handled for less than two minutes, which is sufficient time to administer a single injection. For our studies we considered injections to be the most suitable means of administering PMSG to Japanese quail, and daily injections were used in experiments 2 and 3.

This series of experiments was designed to find a combination of PMSG doses that would stimulate ovarian growth and development of a follicular hierarchy. Treatment with high PMSG doses (40 or 80 IU) during the first week of treatment caused the ovary to grow to a weight significantly larger than in birds held on long days for the same period. This suggests that daily doses of 40 IU PMSG or higher are not suitable for initiating normal ovarian development in Japanese quail. The reproductive organs of females treated with the lowest dose of PMSG (5 IU) were similar in size and weight to untreated or saline-treated control birds, indicating that this dose is too low to stimulate normal ovarian and oviductal growth. Doses of PMSG lower than 20 IU per day have not previously been used to stimulate development of the reproductive tract in Japanese quail. Wakabayashi *et al.* (1996) implanted female Japanese quail with osmotic pumps delivering 20 IU PMSG per day for seven days. However, they implanted a second pump containing a higher dose following expiry of the first pump, and did not collect information on the effects of daily treatment with 20 IU PMSG after seven days. Domestic chickens have been treated with relatively low doses of PMSG. Hosoda *et al.* (1955) showed that daily treatment with 10 or 20 IU PMSG could prevent a drop in serum vitelline levels in fasting birds and induce follicular growth in some hens. A dose of 5 IU PMSG was insufficient to prevent the drop in serum vitelline caused by fasting, and did not promote growth of new follicles (Hosoda *et al.*, 1955). Results from the current experiments indicate that doses of PMSG lower than 10 IU are unlikely to stimulate significant ovarian development in Japanese quail. Daily treatment with 10 IU PMSG daily does initiate ovarian growth, but is insufficient to stimulate follicular development. However, after one week of daily treatment with 10 IU PMSG, mean ovary and oviduct weights were similar to those of birds held on long days for one week. This dose was therefore chosen as the most suitable to stimulate a normal rate of ovarian development for the first week of treatment.

To stimulate a rate of ovarian growth similar to that which occurs in birds held on long days, the best results were achieved by increasing the daily dose of PMSG at weekly intervals. Treatment with 10 IU PMSG for one week, followed by 20 IU PMSG daily for the second week and 20 or 40 IU PMSG for the third week stimulated development of the ovary and oviduct to the same extent as in long day controls. Low doses of PMSG may be required to initiate ovarian growth and steroidogenesis, with higher doses necessary to facilitate entry of follicles into a hierarchy.

Although considerable follicular development occurred in some of the PMSG treated birds, many birds did not develop any yellow follicles, even after three weeks of treatment. This may simply be due to an insufficient period of treatment. In previous experiments (see Chapter 2) all birds that were transferred to long days started laying eggs within three weeks of transfer. Interestingly, in this series of experiments, relatively few of the birds transferred to a long day photoperiod ovulated or laid any eggs. Previous experiments with the New Zealand strain of Japanese quail have shown that three weeks of long days is sufficient to stimulate egg laying (Chua and Hessell, unpublished results). However, in previous experiments reproductively active male Japanese quail were held in rooms nearby, and their calls could be heard in the room that housed the females. Guyomarc'h *et al.* (1980) showed that exposure to male vocalizations caused a significant increase in ovarian weight in quail held under a long day photoperiod. This was particularly marked when calls were heard during the dark period. No male birds were held in the animal rooms during any of the experiments discussed in this chapter, which may explain why females held under a long day photoperiod for three weeks usually did not reach full sexual maturity. A less likely cause of this difference between experiments is the effect of other extraneous noise. Millam *et al.* (1985) and Li *et al.* (1987) have shown that radio music played for 3–12 hours per day can lead to an increase in ovarian and testicular weight in birds held on short days. Extraneous noises could be heard from within the quail rooms during the previous experiments, but it is unlikely that this would have affected gonadal growth under long day conditions.

Experiments that were carried out concurrently with the experiments discussed here investigated the metabolic clearance rate of PMSG in Japanese quail. Results from these experiments showed that PMSG persists in the plasma of Japanese quail up to 96 hours after a single 500 IU injection (Girling *et al.*, in press). Numerous studies using bioassays have shown that PMSG persists in the blood of rabbits (Cole *et al.*, 1967), rats (Parlow and Ward, 1961) and mice (Sasamoto *et al.*, 1972) for at least 20 hours after intravenous injection. McIntosh *et al.* (1975) measured the clearance rate of PMSG in sheep, and found that a single dose of 38 000 IU PMSG took up to 120 hours to be completely removed from the circulatory system and had a half-life of 20 hours. In contrast, LH is removed quickly from the circulatory system of both birds and mammals with a half-life of 17-30 minutes in rats (Parlow, 1968, cited in Scanes and Follett, 1973), 20-30 minutes in chickens, and 20 minutes in Japanese quail (Scanes and Follett, 1973). In previous experiments in which quail, chickens or other avian species have been treated with PMSG to stimulate ovarian development, PMSG has been delivered either by daily injection or continuously by osmotic pump. Only one published paper reports treating birds with PMSG less often than daily (Zadworny and Etches, 1988). The frequency of PMSG treatment may need to be adjusted based on the metabolic clearance rate of PMSG. Daily injections of higher doses of PMSG might lead to a gradual increase of PMSG concentration in the blood.

When Japanese quail were transferred to a long day photoperiod for one week, and then back to a short day photoperiod for the second week, the ovary continued to develop, and was similar in size to that of birds held on long days for two weeks. This indicates that one week of a long day photoperiod is sufficient to "switch on" the hypothalamo-pituitary-gonadal axis, which remains switched on during the second week in the absence of continued photostimulation. In contrast, in birds that were given 10 IU PMSG for one week, and then given lower doses of 0 or 5 IU for the second week, the ovary regressed, and returned to a size similar to that of short day controls. This suggests that the ovarian response to PMSG is physiologically different to the response to a stimulatory photoperiod. This is not surprising, as PMSG acts directly on the ovary, whereas the response to increasing daylength is mediated in the hypothalamus.

Although the distributions of follicle sizes were similar between long day control groups and PMSG treated groups for each ovarian weight class, there were often significantly more 3 – 5 mm follicles in PMSG treated birds than in long day controls. The Japanese quail used in these experiments are bred primarily for meat. Selection for growth rate may have favoured genes for growth factors or their receptors, which are active or transcribed in the ovary, and these may interact to modify the effect of pituitary hormones, which could result in increased recruitment of follicles into the hierarchy (Onagbesan *et al.*, 1994; Peddie *et al.*, 1994; Roberts *et al.*, 1994; Hocking and McCormack, 1995). PMSG stimulated ornithine decarboxylase activity (ODA) to 9.5 times that of basal levels in large white follicles (5-6 mm) of broiler hens, compared to layer hens in which ODA was only increased three-fold (Hocking and McCormack, 1995). The effect of PMSG on small white (2-3 mm) and small yellow (8-10 mm) follicles was similar for both strains, with an increase in ODA only two-three times that of basal levels. It may be that follicles that have just been incorporated into the hierarchy in broiler hens are more sensitive to exogenous and endogenous pituitary hormones than the follicles of layer hens.

Measurement of the cloacal opening diameter proved to be a useful external indicator during the early stages of ovarian and oviductal growth. Cloacal diameter correlated positively with both oviduct and ovary weight for both control and PMSG treated groups. Cloacal diameter may be a useful tool for adjusting dosage and timing of treatments for individual birds in order to overcome some of the variation that occurs between birds receiving the same treatment.

In conclusion, PMSG treatment with doses ranging from 5-80 IU can stimulate ovarian and oviductal development after seven, 14 or 21 days of treatment. Ovarian and oviductal weights did not differ significantly between birds implanted with osmotic pumps, and those receiving daily injections. Dosage, frequency and duration can be easily varied with daily injections, and therefore PMSG will be administered to Japanese quail using injections in future experiments. Low doses of PMSG (10 IU) are best for initiating ovarian development, but higher doses (20 – 40 IU PMSG) are required to stimulate ovarian follicular development. There was variation between birds in ovarian response to PMSG,

with some birds showing excessive follicular development and others showing little or no ovarian growth. The length of the treatment period may not have been sufficient to stimulate ovarian growth in all of the birds. Daily injections of PMSG may have caused a gradual increase in PMSG concentrations in the blood leading to overstimulation of follicular development in those birds that did respond to treatment. The results from this and previous experiments have been used to plan the next experiment which will investigate the effect of PMSG injections given every two, three or four days over a period of one, two or three weeks.

4 Effect of PMSG treatment at intervals of one, two, three or four days on ovarian development

4.1 Introduction

Previous experiments have shown that continuous or daily treatment with PMSG can stimulate ovarian development and ovulation in Japanese quail. However, there is considerable variation in response between birds, with some birds showing overstimulation of follicular growth and breakdown of the hierarchy, and some showing little or no ovarian response to treatment. PMSG has a longer half-life than LH and FSH, and the ovarian overstimulation seen in some birds might be related to this long half-life.

The clearance rate of LH and FSH from the circulatory system is relatively rapid. LH has a half-life of 17-30 minutes in mice and rats (Parlow, 1968, cited in Scanes and Follett, 1973) and 20-30 minutes in chickens (Scanes and Follett, 1973) and Japanese quail (Davies *et al.*, 1976). The clearance rate of FSH is more variable. In mice, various preparations of FSH were compared, and they were shown to have half-lives of 11-30 minutes (Phillips *et al.*, 1993). The metabolic clearance rate of FSH is slower in sheep and humans, with FSH persisting in ovine plasma for approximately two hours (Fry *et al.*, 1987), and in human plasma for up to five hours (Urban *et al.*, 1991). In contrast, PMSG persists in the blood of rats (Parlow and Ward, 1961), mice (Sasamoto *et al.*, 1972) and rabbits (Cole *et al.*, 1967) for at least 20 hours after a single intravenous injection. McIntosh *et al.* (1975) measured the clearance rate of PMSG in sheep, and found that a single dose of PMSG took up to 120 hours to be completely removed from the circulatory system. Experiments carried out in our laboratory have shown that PMSG persists in the circulatory system of Japanese quail for up to 96 hours after treatment with a single injection (Girling *et al.*, in press). Daily treatment of quail with PMSG may therefore be too frequent and could lead to persistently elevated plasma concentrations of this gonadotropic hormone. The frequency of PMSG

treatment may need to be adjusted to allow for the relatively slow metabolic clearance rate of PMSG. Unfortunately, there are few examples of previous experiments in which ovarian development has been stimulated by periodic treatment with PMSG or other gonadotropic hormones. Zadworny and Etches (1988) are the only researchers to report treating an avian species with PMSG less often than daily. They treated incubating and out-of-lay turkeys with PMSG every two days and induced ovarian growth and ovulation in several birds.

Mammalian studies in which reproductive activity has been stimulated using exogenous gonadotropins have used single injections, intermittent injections or continuous infusion of GnRH, LH, FSH, hCG or PMSG (Yavas and Walton, 2000). Often a single injection with exogenous gonadotropin is sufficient to induce follicular growth or ovulation in mammalian species. A single injection of GnRH stimulated ovulation in cattle (Taponen *et al.*, 1999) and similarly a single treatment with PMSG has been used to stimulate follicular development in pigs (Duanyai and Srikandakumar, 1998), and induce ovulation in deer (Waldhalm *et al.*, 1989) and possums (Glazier, 1998). When injections of gonadotropin are given periodically to mammals, this is usually designed to mimic natural GnRH or LH pulses. This technique has been used predominantly to induce ovulation in cattle (McNatty *et al.*, 1993; Hamilton *et al.*, 1999; Vizcarra *et al.*, 1999) and requires treatment with GnRH, LH or FSH every one to four hours for several days. Martinez *et al.* (1999) treated cattle with GnRH or porcine LH once every three days for ten days to induce ovulation. This treatment schedule was designed to correspond with specific stages of the first follicular wave (growing, early-static and late-static phases). There are no published studies in which PMSG alone has been administered periodically to a mammalian species to stimulate follicular growth and induce ovulation.

The current experiment was designed to investigate the effect on ovarian development and ovulation of PMSG treatment at intervals of one or more days. Treatment with PMSG every two, three or four days may increase the number of birds which respond positively to treatment, and decrease the incidence of overstimulation of follicular development.

4.2 Materials and Methods

4.2.1 Animals

Female Japanese quail (n= were purchased at three weeks of age from our normal commercial supplier (Rangitikei Game Farms). Before the start of the experiment, birds were maintained under short day conditions (8L:16D, 10°C) as described in Chapter 2.

4.2.2 Hormone preparation and administration

Pregnant mare serum gonadotropin (PMSG; Intervet) was kindly donated by Pharmaco New Zealand.

PMSG was dissolved in saline and administered using injections. Injections were administered subcutaneously in the abdomen using a 0.5 ml insulin syringe and a 27 gauge, ½ inch needle. Each bird received a volume of 200 µl of solution per injection, containing saline or 20 or 40 IU PMSG.

4.2.3 Experimental design

At six weeks of age, birds were divided into 26 treatment groups (n=6 per group, see Table 4.1).

Group one was euthanased and dissected at the start of the treatment period to determine the size of the ovary and oviduct prior to treatment. Groups two to four were transferred to long day conditions (16L:8D, 20°C) and dissected on days 7, 14 or 21. The amount of oviductal and ovarian development stimulated by a long day photoperiod provided a target for the PMSG treatment to achieve. Groups five and six remained under short day conditions until dissection on day 21. Group five received no injections while group six received daily injections of 0.9% saline. Groups seven to 16 received

injections of 20 IU PMSG daily or every two, three or four days, and groups 17 to 26 received injections of 40 IU PMSG daily or every two, three or four days. Groups were dissected at day 7, 14 or 21 as shown in Table 4.1.

Table 4.1. Experimental groups.

Group	Photoperiod	PMSG dosage (IU)	Interval between injections	Day of dissection
1	-	-	-	0
2	Long day	-	-	7
3	Long day	-	-	14
4	Long day	-	-	21
5	Short day	-	-	21
6	Short day	Saline	1	21
7	Short day	20	1	7
8	Short day	20	2	7
9	Short day	20	3	7
10	Short day	20	4	7
11	Short day	20	2	14
12	Short day	20	3	14
13	Short day	20	4	14
14	Short day	20	2	21
15	Short day	20	3	21
16	Short day	20	4	21
17	Short day	40	1	7
18	Short day	40	2	7
19	Short day	40	3	7
20	Short day	40	4	7
21	Short day	40	2	14
22	Short day	40	3	14
23	Short day	40	4	14
24	Short day	40	2	21
25	Short day	40	3	21
26	Short day	40	4	21

Regular measurements (every two days) of cloacal opening diameter were made during the experiment as an indicator of development of the oviduct. Body weight was also measured regularly to ensure birds maintained a healthy weight.

Birds were euthanased on the appropriate days and the ovary and oviduct excised and weighed, and body weight and cloacal opening diameter recorded. Ovaries were divided into six size classes (0.00-0.15 g, 0.16-0.50 g, 0.51–1.00 g, 1.01–5.00 g, 5.01–9.00 g and 9.01 – 13.00 g) and the numbers and sizes of yellow follicles present in each ovary were recorded. The follicular distribution within each ovarian weight class was compared between control and treated groups. Blood samples were collected and stored at -20°C for radioimmunoassay of plasma estradiol.

All experimental procedures were approved by the Massey University Animal Ethics Committee.

4.2.4 Radioimmunoassay of plasma estradiol

Plasma samples were assayed for estradiol using the method described in Chapter 2.

4.2.5 Statistics

All variables were tested for homogeneity of variance using Levene's test. When variances were homogeneous across groups for a single variable, ANOVA followed by linear contrasts were used to analyse data. Linear contrasts were used rather than Bonferroni's posthoc tests due to the large number of groups that were compared. When variances were not homogeneous across groups, Kruskal-Wallis non-parametric ANOVA followed by Mann-Whitney U tests were performed to determine differences between groups. One-way ANOVA and Student's t-tests were used to analyse follicular size frequency distribution data. Repeated measures ANOVA was used to analyse changes in cloacal diameter over time. Relationships between variables were investigated using linear regression to calculate r^2 values.

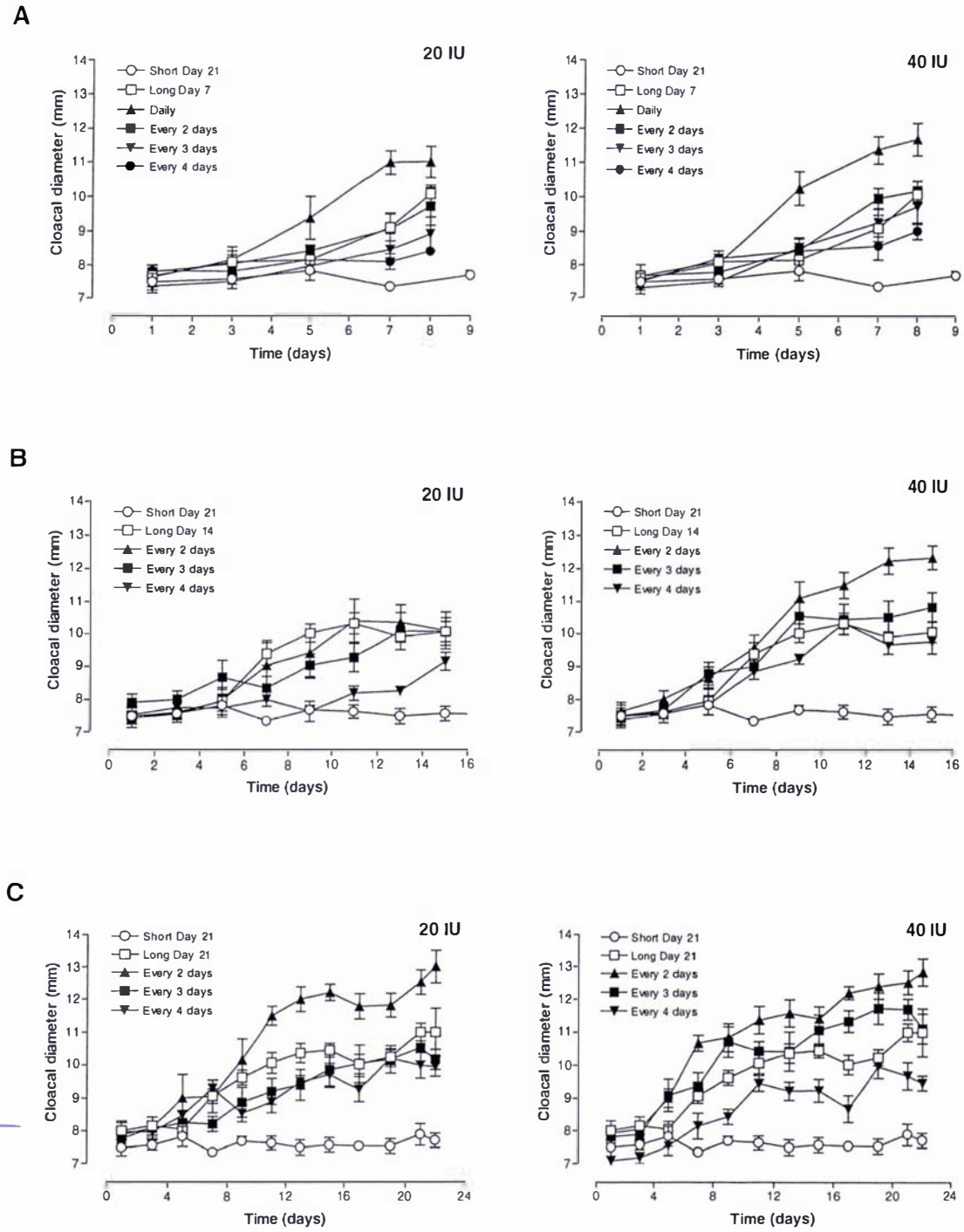

Figure 4.1. Changes in cloacal diameter in birds held under a short day photoperiod and treated with 20 or 40 IU PMSG daily or every two, three or four days for one (A), two (B) or three (C) weeks, or held on short days for three weeks or long days for one, two or three weeks receiving no treatment.

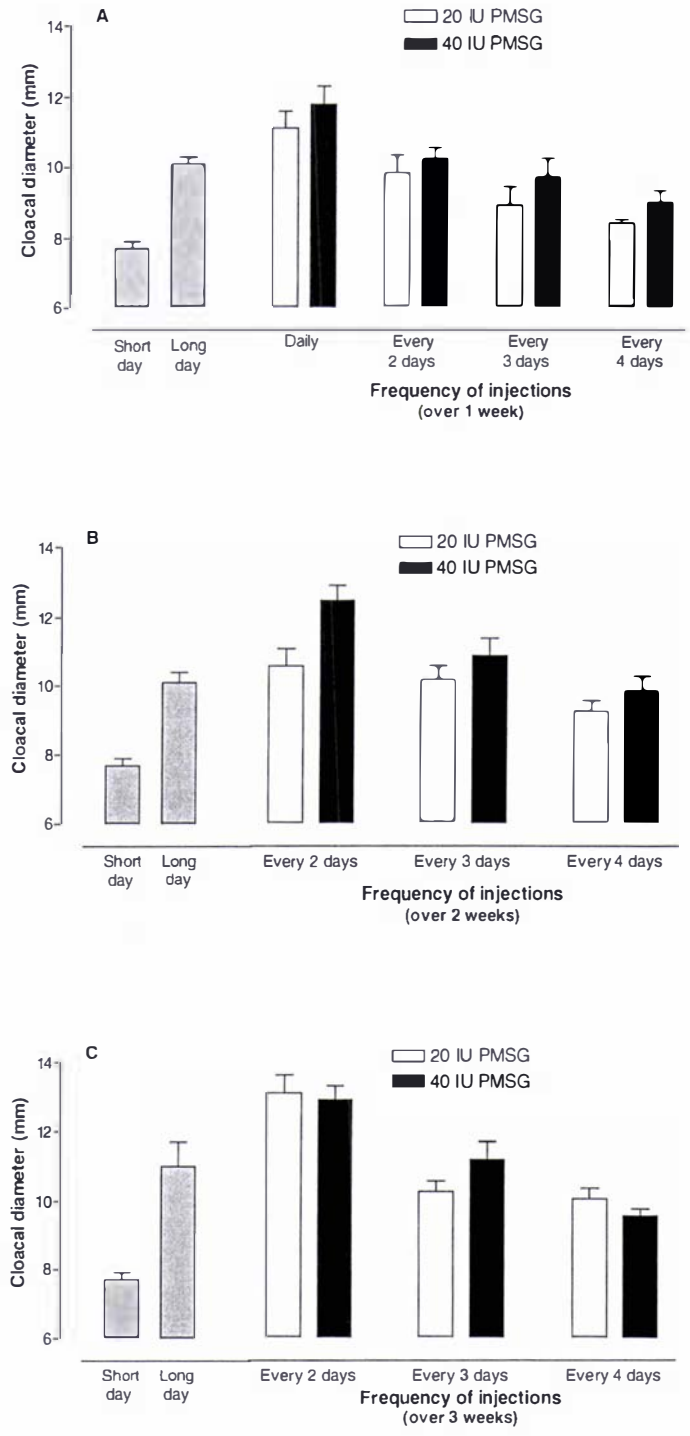

Figure 4.2. Mean cloacal diameter for birds on short days, long days, and short days plus treatment with 20 or 40 IU PMSG daily or every two, three or four days after one (A), two (B) or three (C) weeks.

ANOVAs and non-parametric equivalents were carried out using Systat Version 8.0 (SPSS Inc., 1988), and relationships between variables (linear regression) were analysed using GraphPad Prism Version 3.0 (GraphPad Software Inc., 1999). Data were transformed to logarithms where necessary. Data are presented as individual points or as means ± standard error.

4.3 Results

4.3.1 Cloacal opening

Cloacal diameter changed significantly in all PMSG treated groups during one, two or three weeks of treatment, except for the group treated with 20IU PMSG every 4 days for one week (Figure 4.1, see Table 4.2 for statistics). There were also significant increases in cloacal diameter in birds held on long days for one, two or three weeks. There were no significant changes in the cloacal diameter of birds held on short days for three weeks receiving no treatment, or in saline treated birds.

After one, two or three weeks of treatment, the mean cloacal diameters of long day controls were significantly larger than short day controls. After one week of treatment, the mean cloacal diameters of all PMSG treated groups were significantly larger than short day controls, except those treated with 20 IU PMSG every four days (Figure 4.2, see Table 4.3 for statistics). The mean cloacal diameters of PMSG treated birds were not significantly different to long day controls after one week, except for birds treated daily with 40 IU PMSG which had significantly larger cloacal openings, and birds treated every three or four days with 20 IU PMSG which had smaller cloacal diameters. All PMSG treated groups had mean cloacal diameters significantly larger than short day controls but similar to that of long day controls after two weeks, except for birds treated with 40 IU PMSG every two days which had a mean cloacal diameter significantly larger than both short day and long day controls. The mean cloacal diameters of birds treated with 20 or 40 IU PMSG every two days for three weeks were significantly larger than long day controls. All other PMSG treated groups had mean cloacal diameters that were not significantly different from long day controls after three weeks, except for

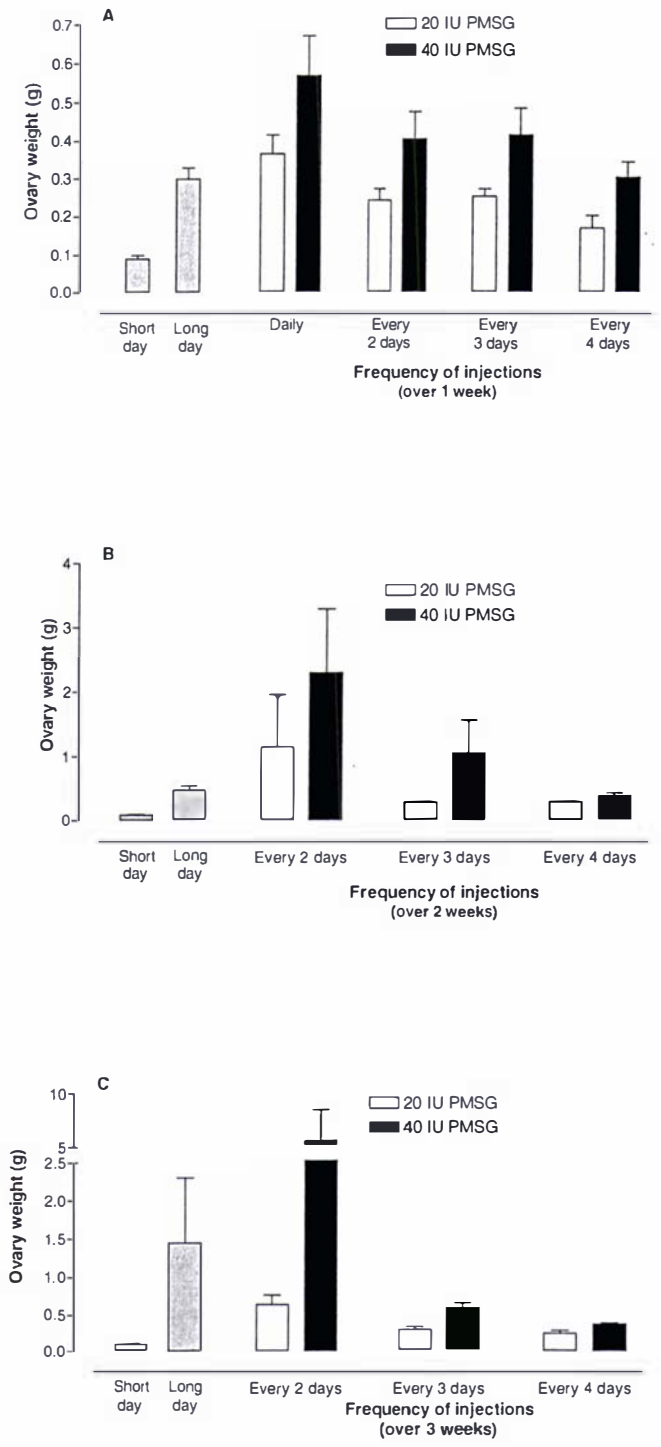

Figure 4.3. Mean ovary weight for birds on short days, long days, and short days plus treatment with 20 or 40 IU PMSG daily or every two, three of four days after one (A), two (B) or three (C) weeks.

birds treated with 40 IU PMSG every four days which had a smaller mean cloacal diameter than long day controls.

Table 4.2. Summary of one-way repeated measures ANOVA comparing changes in cloacal opening diameter in each group during one, two or three weeks of PMSG treatment given daily, every two, three or four days, or one, two or three weeks under a long day photoperiod receiving no treatment.

	One week			Two weeks			Three weeks		
Group	**F**	**df**	***p***	**F**	**df**	***p***	**F**	**df**	***p***
Short day	-	-	-	-	-	-	0.782	11,44	0.656
Long day	11.23	3, 12	0.001	27.95	7, 28	0.000	13.22	11,44	0.000
Saline	-	-	-	-	-	-	1.01	11,44	0.450
20IU PMSG daily	41.33	3, 15	0.000	-	-	-	-	-	-
40IU PMSG daily	53.51	3, 15	0.000	-	-	-	-	-	-
20IU every 2 days	5.99	3, 15	0.007	11.63	7, 35	0.000	35.75	11,55	0.000
40IU every 2 days	23.62	3, 15	0.000	35.90	7, 35	0.000	35.76	11,55	0.000
20IU every 3 days	8.68	3, 15	0.001	14.49	7, 35	0.000	14.79	11,55	0.000
40IU every 3 days	34.80	3, 15	0.000	27.74	7, 35	0.000	22.25	11,55	0.000
20IU every 4 days	2.06	3, 15	0.148	7.31	7, 35	0.000	14.56	11,55	0.000
40IU every 4 days	4.20	3, 15	0.024	32.98	7, 35	0.000	17.31	11,55	0.000

4.3.2 Ovary weight

Mean ovary weights were significantly larger in long day control groups and all PMSG treated groups compared to short day controls after one, two and three weeks of treatment (Figure 4.3, see Table 4.4 for statistics). After one week of treatment, birds given 20 IU PMSG daily, 20 or 40 IU every two or three days, or 40 IU every four days had similar mean ovary weights to long day controls. Birds treated with 40 IU PMSG daily had a mean ovary weight significantly larger than long day controls. After two weeks of treatment, birds receiving 20 IU PMSG every three or four days had mean ovary weights that were significantly smaller than long day controls, whilst birds treated with 20 IU PMSG every 2 days or 40 IU every three or four days had ovary weights

similar to long day controls, and birds given 40 IU PMSG every two days had a mean ovary weight larger than birds held on long days. Treatment with 20 IU PMSG every three or four days, or 40 IU every four days for three weeks was insufficient to stimulate ovarian growth to the same extent as found in long day controls. Mean ovary weights of birds treated with 20 IU PMSG every two days or 40 IU every two or three days were not significantly different from the mean ovary weight of long day controls.

Table 4.3. Summary of one-way ANOVA and linear contrasts comparing cloacal opening diameter between groups after one, two or three weeks of PMSG treatment given daily, every two, three or four days, or one, two or three weeks under a long day photoperiod receiving no treatment.

	One week			Two weeks			Three weeks		
	F	**df**	***p***	**F**	**df**	***p***	**F**	**df**	***p***
ANOVA	9.86	10, 51	0.000	14.17	8, 41	0.000	30.17	9, 45	0.000
Linear contrasts	**F**	**df**	***p***	**F**	**df**	***p***	**F**	**df**	***p***
SD vs LD	16.01	1, 51	0.000	17.34	1, 41	0.000	42.18	1, 45	0.000
SD vs Sham	-	-	-	-	-	-	0.59	1, 45	0.446
SD vs 20 IU daily	33.58	1, 51	0.000	-	-	-	-	-	-
LD vs 20 IU daily	2.612	1, 51	0.112	-	-	-	-	-	-
SD vs 40 IU daily	48.93	1, 51	0.000	-	-	-	-	-	-
LD vs 40 IU daily	7.928	1, 51	0.007	-	-	-	-	-	-
SD vs 20 IU, 2 days	13.81	1, 51	0.001	22.27	1, 41	0.000	101.9	1, 45	0.000
LD vs 20 IU, 2 days	0.21	1, 51	0.646	0.76	1, 41	0.388	10.99	1, 45	0.002
SD vs 40 IU, 2 days	19.02	1, 51	0.000	73.46	1, 41	0.000	97.52	1, 45	0.000
LD vs 40 IU, 2 days	0.03	1, 51	0.857	17.82	1, 41	0.000	9.56	1, 45	0.003
SD vs 20 IU, 3 days	4.56	1, 51	0.037	19.03	1, 41	0.000	28.80	1, 45	0.000
LD vs 20 IU, 3 days	4.17	1, 51	0.046	0.00	1, 41	0.990	2.01	1, 45	0.163
SD vs 40 IU, 3 days	12.75	1, 51	0.001	33.35	1, 41	0.000	48.84	1, 45	0.000
LD vs 40 IU, 3 days	0.37	1, 51	0.545	2.03	1, 41	0.162	0.08	1, 45	0.784
SD vs 20 IU, 4 days	1.51	1, 51	0.225	7.14	1, 41	0.011	24.50	1, 45	0.000
LD vs 20 IU, 4 days	8.70	1, 51	0.005	2.81	1, 41	0.101	3.36	1, 45	0.073
SD vs 40 IU, 4 days	5.35	1, 51	0.025	14.51	1, 41	0.000	16.05	1, 45	0.000
LD vs 40 IU, 4 days	3.49	1, 51	0.068	0.29	1, 41	0.592	7.71	1, 45	0.008

Table 4.4. Summary of one-way ANOVA or Kruskal-Wallis nonparametric ANOVA and linear contrasts or Mann-Whitney U tests comparing ovary weight between groups after one, two or three weeks of PMSG treatment given daily, every two, three or four days, or one, two or three weeks under a long day photoperiod receiving no treatment.

	One week			Two weeks			Three weeks		
	F	df	*p*	K-W	df	*p*	K-W	df	*p*
ANOVA	15.20	10, 51	0.000	41.56	8	0.000	48.27	9	0.000
Linear contrasts	**F**	**df**	***p***	**M-W**	**df**	***p***	**M-W**	**df**	***p***
SD vs LD	31.47	1, 51	0.000	0.0	1	0.009	0.0	1	0.009
SD vs Saline	-	-	-	-	-	-	8.0	1	0.347
SD vs 20 IU daily	41.80	1, 51	0.000	-	-	-	-	-	-
LD vs 20 IU daily	0.37	1, 51	0.547	-	-	-	-	-	-
SD vs 40 IU daily	71.92	1, 51	0.000	-	-	-	-	-	-
LD vs 40 IU daily	6.88	1, 51	0.011	-	-	-	-	-	-
SD vs 20 IU, 2 days	22.78	1, 51	0.000	0.0	1	0.006	0.0	1	0.006
LD vs 20 IU, 2 days	1.18	1, 51	0.283	22.0	1	0.199	16.0	1	0.855
SD vs 40 IU, 2 days	46.81	1, 51	0.000	0.0	1	0.006	0.0	1	0.006
LD vs 40 IU, 2 days	0.97	1, 51	0.330	2.0	1	0.017	7.0	1	0.144
SD vs 20 IU, 3 days	24.71	1, 51	0.000	0.0	1	0.006	0.0	1	0.006
LD vs 20 IU, 3 days	0.79	1, 51	0.379	30.0	1	0.006	26.0	1	0.045
SD vs 40 IU, 3 days	49.39	1, 51	0.000	0.0	1	0.006	0.0	1	0.006
LD vs 40 IU, 3 days	1.37	1, 51	0.248	7.0	1	0.141	12.0	1	0.584
SD vs 20 IU, 4 days	9.26	1, 51	0.004	0.0	1	0.006	1.0	1	0.011
LD vs 20 IU, 4 days	7.93	1, 51	0.007	28.0	1	0.017	30.0	1	0.006
SD vs 40 IU, 4 days	31.06	1, 51	0.000	0.0	1	0.006	0.0	1	0.006
LD vs 40 IU, 4 days	0.08	1, 51	0.776	22.0	1	0.199	26.0	1	0.045

Large yellow follicles were present on the ovary of only one long day control bird (held on long days for three weeks) and four treated birds given 40 IU PMSG every two days for two or three weeks. One of the PMSG treated birds laid an egg, and this was relatively small (10.3g compared to normal egg weight: 12.5 ± 0.3 g) and incompletely pigmented. None of the long day control birds laid eggs in this experiment.

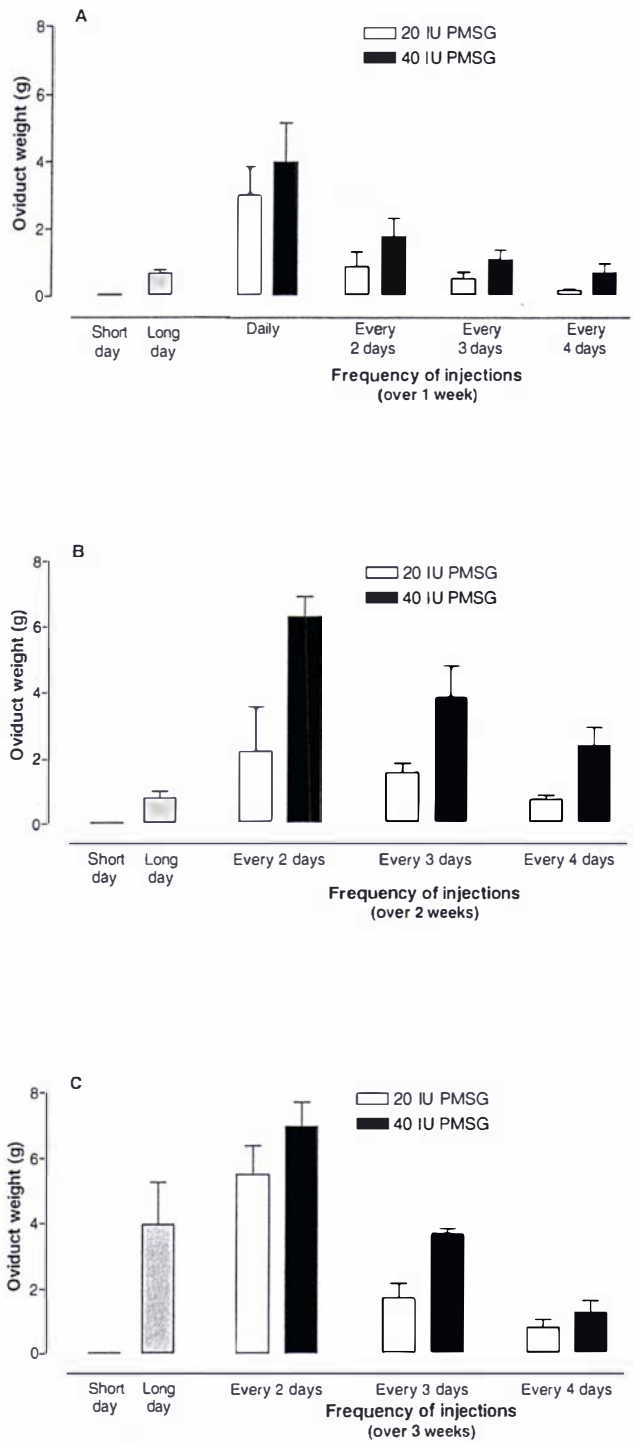

Figure 4.4. Mean oviduct weight for birds on short days, long days, and short days plus treatment with 20 or 40 IU PMSG daily or every two, three of four days after one (A), two (B) or three (C) weeks.

4.3.3 Oviduct weight

Table 4.5. Summary of Kruskal-Wallis nonparametric ANOVA and Mann-Whitney U tests comparing oviduct weight between groups after one, two or three weeks of PMSG treatment given daily, every two, three or four days, or one, two or three weeks under a long day photoperiod receiving no treatment.

	One week			Two weeks			Three weeks		
	K-W	**df**	***p***	**K-W**	**df**	***p***	**K-W**	**df**	***p***
Kruskal-Wallis	41.70	10	0.000	36.65	8	0.000	47.33	9	0.000
Mann-Whitney	**M-W**	**df**	***p***	**M-W**	**df**	***p***	**M-W**	**df**	***p***
SD vs LD	0.0	1	0.009	0.0	1	0.009	0.0	1	0.009
SD vs Saline	-	-	-	-	-	-	10.0	1	0.602
SD vs 20 IU daily	0.0	1	0.006	-	-	-	-	-	-
LD vs 20 IU daily	6.0	1	0.100	-	-	-	-	-	-
SD vs 40 IU daily	0.0	1	0.006	-	-	-	-	-	-
LD vs 40 IU daily	3.0	1	0.028	-	-	-	-	-	-
SD vs 20 IU, 2 days	0.0	1	0.006	0.0	1	0.006	0.0	1	0.006
LD vs 20 IU, 2 days	19.0	1	0.465	9.0	1	0.273	10.5	1	0.410
SD vs 40 IU, 2 days	0.0	1	0.006	0.0	1	0.006	0.0	1	0.006
LD vs 40 IU, 2 days	8.0	1	0.201	0.0	1	0.006	4.0	1	0.045
SD vs 20 IU, 3 days	0.0	1	0.006	0.0	1	0.006	0.0	1	0.006
LD vs 20 IU, 3 days	18.0	1	0.584	3.0	1	0.028	21.0	1	0.273
SD vs 40 IU, 3 days	0.0	1	0.006	0.0	1	0.006	0.0	1	0.006
LD vs 40 IU, 3 days	9.0	1	0.272	4.0	1	0.045	18.0	1	0.583
SD vs 20 IU, 4 days	4.0	1	0.045	0.0	1	0.006	0.0	1	0.006
LD vs 20 IU, 4 days	29.0	1	0.011	12.0	1	0.584	24.0	1	0.100
SD vs 40 IU, 4 days	0.0	1	0.006	0.0	1	0.006	0.0	1	0.006
LD vs 40 IU, 4 days	17.0	1	0.715	3.0	1	0.028	22.0	1	0.201

Mean oviduct weights were significantly larger in long day control groups and all PMSG treated groups compared to short day controls after one, two and three weeks of treatment (Figure 4.4, see Table 4.5 for statistics). Mean oviduct weights of birds given

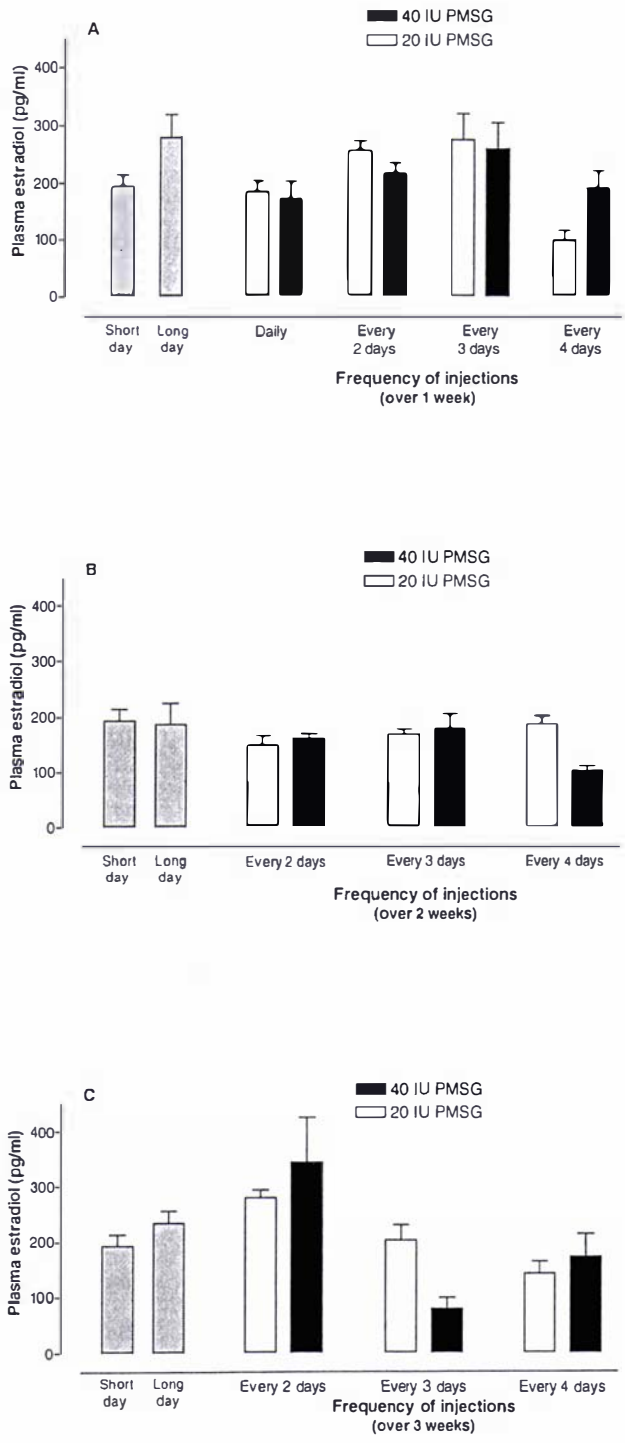

Figure 4.5. Mean plasma estradiol concentration for birds on short days, long days, and short days plus treatment with 20 or 40 IU PMSH daily or every two, three of four days after one (A), two (B) or three (C) weeks.

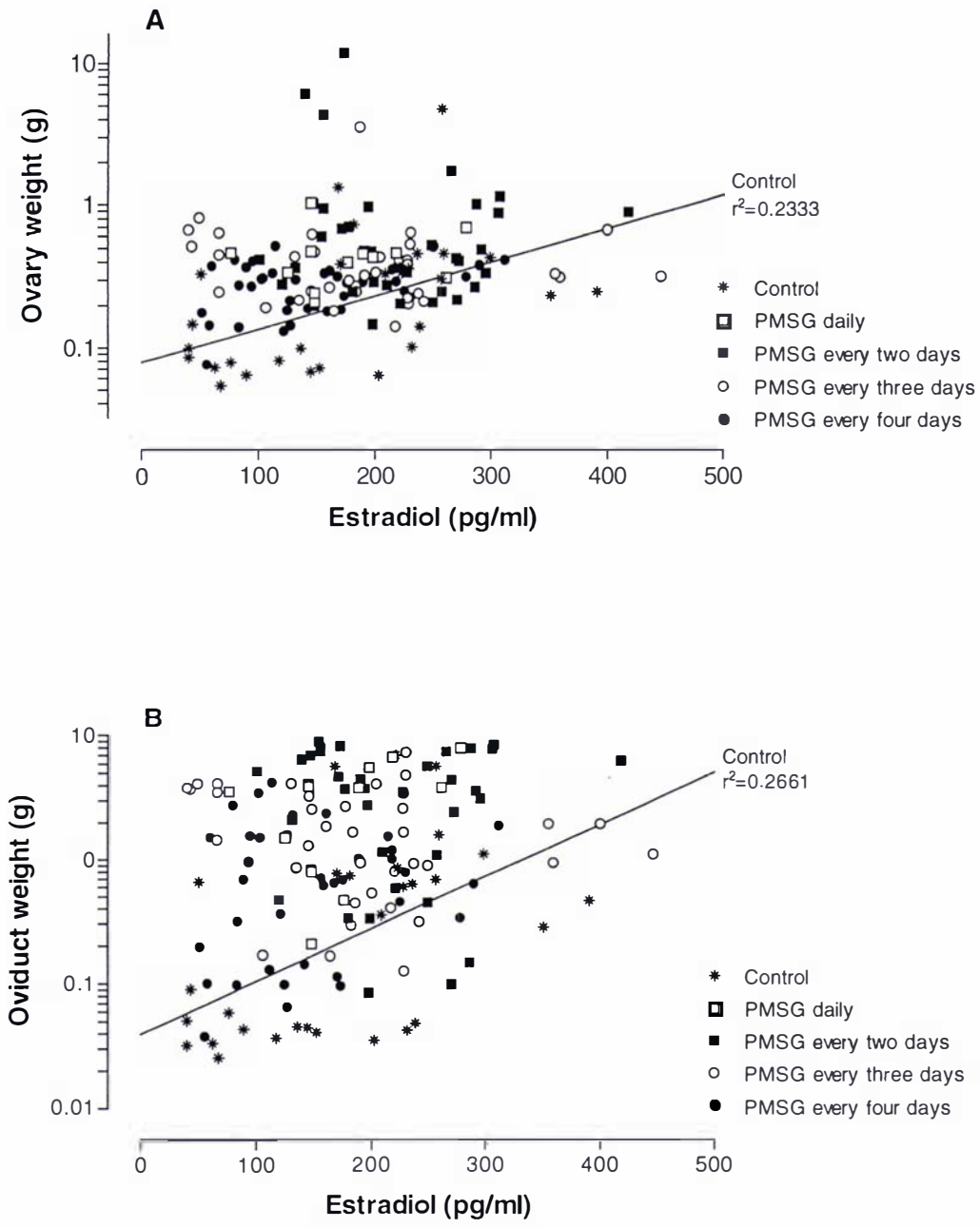

Figure 4.6. Relationships between plasma estradiol concentration and ovary and oviduct weight in control birds (untreated birds held on short days or long days) and birds held on short days while receiving treatment with 20 or 40 IU PMSG daily or every two, three or four days for one, two or three weeks.

20 IU PMSG daily or every two or three days, or 40 IU PMSG every two, three or four days for one week were similar to long day controls after one week. Birds treated daily with 40 IU PMSG had a significantly larger mean oviduct weight after one week than long day controls. After two weeks of treatment, birds receiving 20 IU PMSG every two or four days had mean oviduct weights that were not significantly different to long day controls, and birds given 20 IU PMSG every three days, or 40 IU every two, three or four days had mean oviduct weights larger than birds held on long days. All groups treated with PMSG had mean oviduct weights that were not significantly different to long day controls after three weeks, except for birds treated with 40 IU PMSG every two days which had a significantly larger mean oviduct weight.

4.3.4 Plasma estradiol

The mean plasma estradiol concentration of short day controls was not significantly different to that of long day controls after one, two or three weeks (Figure 4.5, see Table 4.6 for statistics). After one week of treatment, the mean plasma estradiol concentration of birds treated with 20 IU PMSG every four days was significantly lower than both long day and short day controls. All other PMSG treated groups had similar mean plasma estradiol concentrations to long day and short day control groups. A similar pattern was evident after two weeks of treatment, with the group treated with 40 IU PMSG every four days having a significantly lower mean plasma estradiol concentration than long day and short day controls. After three weeks of PMSG treatment, plasma estradiol concentrations were highest in birds treated with PMSG every two days, but mean values were not significantly different from long day or short day controls. Birds treated with 20 IU PMSG every four days for three weeks had a significantly lower mean plasma estradiol concentration than long day and short day controls.

There was no correlation between plasma estradiol concentration and ovary or oviduct weight for any of the PMSG treated groups (Figure 4.6). There was a relatively weak correlation between plasma estradiol concentration and ovary or oviduct weight in long day and short day controls (p=0.008 for ovary weight and p=0.004 for oviduct weight).

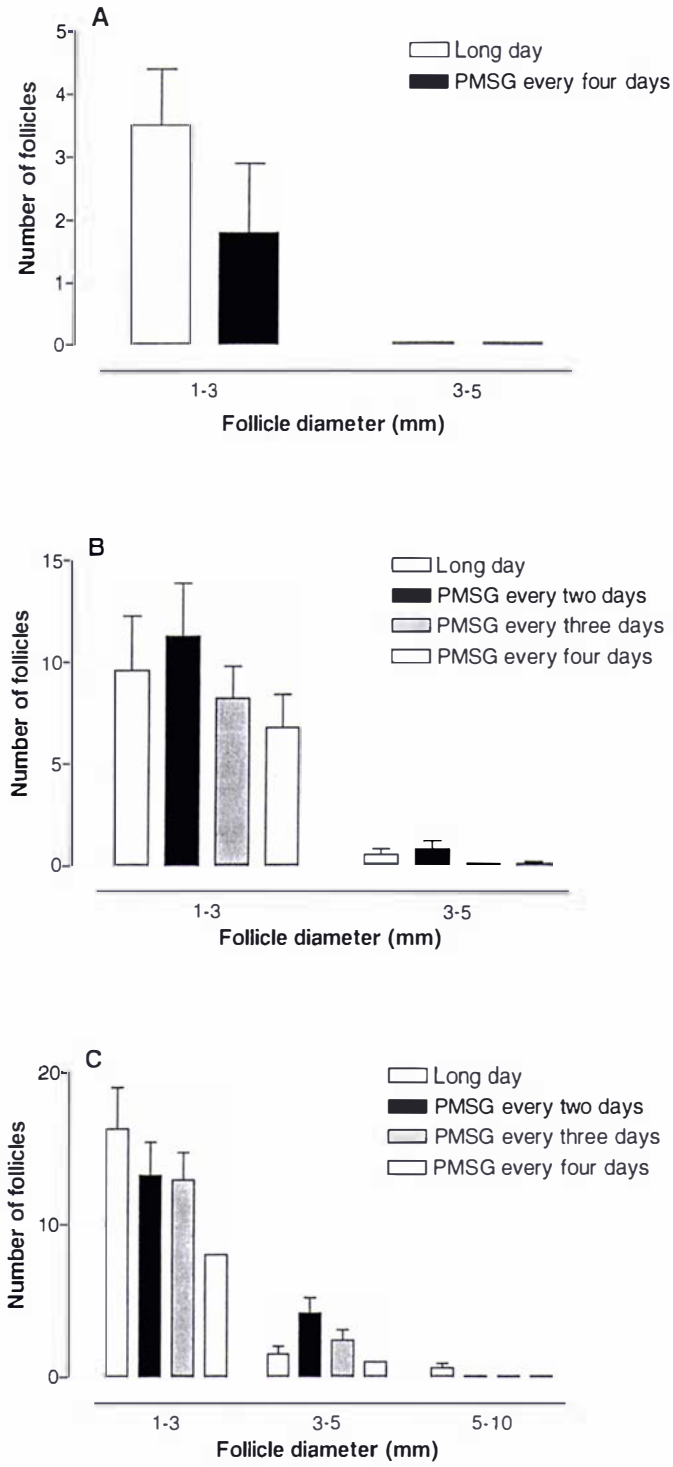

Figure 4.7. Distribution of follicle sizes in 0.00 – 0.15 g (A), 0.16 – 0.50 g (B) and 0.51 – 1.00 g (C) ovaries of Japanese quail held on long days for one, two or three weeks, or receiving 20 or 40 IU PMSG every two, three or four days for one, two or three weeks.

Table 4.6. Summary of one-way ANOVA and linear contrasts comparing plasma estradiol concentration between groups after one, two or three weeks of PMSG treatment given daily, every two, three or four days, or one, two or three weeks under a long day photoperiod receiving no treatment.

	One week			Two weeks			Three weeks		
	F	**df**	***p***	**F**	**df**	***p***	**F**	**df**	***p***
ANOVA	5.98	10, 51	0.000	3.30	8, 41	0.005	10.16	9, 45	0.000
Linear contrasts	**F**	**df**	***p***	**F**	**df**	***p***	**F**	**df**	***p***
SD vs LD	2.30	1, 51	0.135	0.05	1, 41	0.818	0.51	1, 45	0.477
SD vs Saline	-	-	-	-	-	-	26.64	1, 45	0.000
SD vs 20 IU daily	0.10	1, 51	0.749	-	-	-	-	-	-
LD vs 20 IU daily	3.64	1, 51	0.062	-	-	-	-	-	-
SD vs 40 IU daily	0.81	1, 51	0.373	-	-	-	-	-	-
LD vs 40 IU daily	6.17	1, 51	0.016	-	-	-	-	-	-
SD vs 20 IU, 2 days	1.61	1, 51	0.210	3.36	1, 41	0.074	1.97	1, 45	0.167
LD vs 20 IU, 2 days	0.10	1, 51	0.754	2.53	1, 41	0.120	0.43	1, 45	0.516
SD vs 40 IU, 2 days	0.241	1, 51	0.626	1.70	1, 41	0.200	3.15	1, 45	0.083
LD vs 40 IU, 2 days	1.20	1, 51	0.279	1.12	1, 41	0.295	1.05	1, 45	0.310
SD vs 20 IU, 3 days	1.86	1, 51	0.179	1.17	1, 41	0.286	0.01	1, 45	0.928
LD vs 20 IU, 3 days	0.05	1, 51	0.825	0.70	1, 41	0.407	0.71	1, 45	0.406
SD vs 40 IU, 3 days	0.99	1, 51	0.325	0.38	1, 41	0.541	15.18	1, 45	0.000
LD vs 40 IU, 3 days	0.35	1, 51	0.557	0.14	1, 41	0.711	21.57	1, 45	0.000
SD vs 20 IU, 4 days	13.50	1, 51	0.001	0.11	1, 41	0.747	2.02	1, 45	0.162
LD vs 20 IU, 4 days	27.66	1, 51	0.000	0.01	1, 41	0.934	4.71	1, 45	0.035
SD vs 40 IU, 4 days	0.12	1, 51	0.727	12.17	1, 41	0.001	1.01	1, 45	0.321
LD vs 40 IU, 4 days	3.745	1, 51	0.059	10.54	1, 41	0.002	3.07	1, 45	0.087

4.3.5 Distribution of follicle sizes

The numbers of follicles in each size class for ovaries weighing less than 1.00 g were similar in long day control birds and birds that received PMSG treatment (Figure 4.7, see Tables 4.7 and 4.8 for statistics). In ovaries weighing 0.51 – 1.00 g there were some

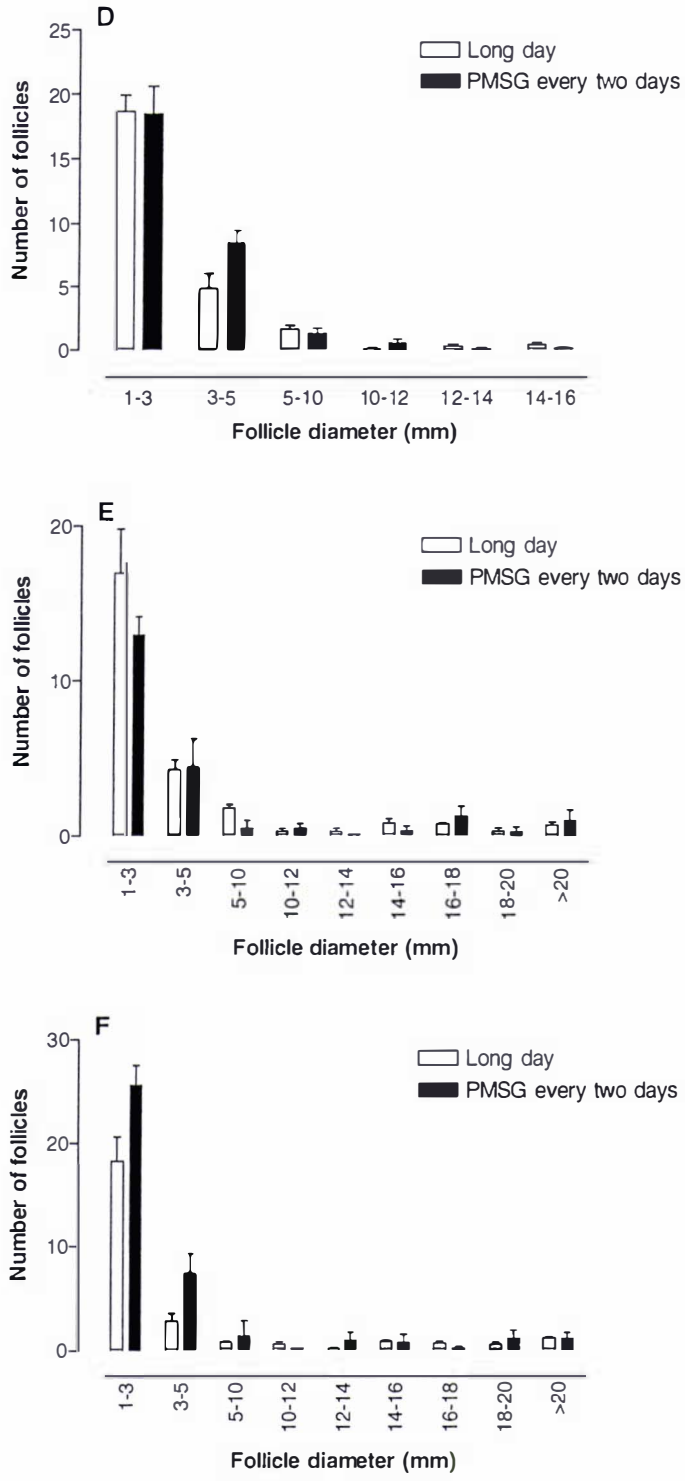

Figure 4.8. Distribution of follicle sizes in 1.01 – 5.00 g (D), 5.01 – 9.00 g (E) and 9.01 – 13.00 g (F) ovaries of Japanese quail held on long days for one, two or three weeks, or receiving 20 or 40 IU PMSG every two, three or four days for one, two or three weeks.

follicles measuring 5 – 10 mm in birds held on long days, but these were not present in the ovaries of any PMSG treated birds. In ovaries weighing 1.01 - 5.00 g and 9.01 – 13.00 g there were similar numbers of follicles in long day control birds and PMSG treated birds for each follicle size class except for follicles 3-5 mm in diameter (Figure 4.8, see Table 4.9 for statistics) for which there were significantly more follicles in PMSG treated birds. There were similar numbers of follicles in long day birds and PMSG treated birds in each size class for ovaries that weighed 5.01 - 9.00 g, except for follicles 5 – 10 mm in diameter (Figure 4.8, see Table 4.9 for statistics).

Table 4.7. Summary of two sample Student's t-tests comparing the number of follicles in ovarian size class 0.00-0.15 g between birds held on long days and birds treated with PMSG every four days. Tests could not be performed when one or both groups had no follicles in a particular size class (indicated by "No test").

Ovary size (g)	Follicle diameter (mm)	t	df	*p*
0.00 – 0.15	1 – 3	1.171	13	0.263
0.00 – 0.15	3 – 5		No test	

Table 4.8. Summary of one-way ANOVA comparing the number of follicles in ovarian size classes 0.16-0.50 g and 0.51–1.00 g between birds held on long days and birds treated with PMSG every two, three or four days. Tests could not be performed when one or both groups had no follicles in a particular size class (indicated by "No test").

Ovary size (g)	Follicle diameter (mm)	F	df	*p*
0.16 – 0.50	1 – 3	0.693	3, 36	0.562
0.16 – 0.50	3 – 5	2.007	3, 35	0.131
0.51 – 1.00	1 – 3	0.699	3, 24	0.562
0.51 – 1.00	3 – 5	2.237	3, 24	0.110
0.51 – 1.00	5 – 10		No test	

Table 4.9. Summary of two sample Student's t-tests comparing the number of follicles in ovarian size classes 1.01–5.00 g, 5.01–9.00 g and 9.01 – 13.00 g between birds held on long days and birds treated with PMSG every two days. Tests could not be performed when one or both groups had no follicles in a particular size class (indicated by "No test").

Ovary size (g)	Follicle diameter (mm)	t	df	*p*
1.01 – 5.00	1 – 3	0.082	16	0.935
1.01 – 5.00	3 – 5	2.295	16	0.036
1.01 – 5.00	5 – 10	0.585	16	0.567
1.01 – 5.00	10 – 12	0.193	16	0.250
1.01 – 5.00	12 – 14	0.816	16	0.426
1.01 – 5.00	14 – 16	1.392	16	0.183
5.01 – 9.00	1 – 3	0.884	12	0.394
5.01 – 9.00	3 – 5	-0.137	12	0.893
5.01 – 9.00	5 – 10	2.963	12	0.012
5.01 – 9.00	10 – 12	-0.665	12	0.519
5.01 – 9.00	12 – 14		No test	
5.01 – 9.00	14 – 16	1.114	12	0.287
5.01 – 9.00	16 – 18	-1.230	12	0.242
5.01 – 9.00	18 – 20	0.173	12	0.865
5.01 – 9.00	> 20	-0.553	12	0.591
9.01 – 13.00	1 – 3	-2.054	13	0.061
9.01 – 13.00	3 – 5	-2.877	13	0.013
9.01 – 13.00	5 – 10	-0.618	13	0.547
9.01 – 13.00	10 – 12		No test	
9.01 – 13.00	12 – 14	-1.428	13	0.177
9.01 – 13.00	14 – 16	0.178	13	0.862
9.01 – 13.00	16 – 18	1.933	13	0.075
9.01 – 13.00	18 – 20	-1.013	13	0.330
9.01 – 13.00	> 20	0.000	13	1.000

4.4 Discussion

PMSG administered to Japanese quail once every two, three, or four days for three weeks stimulated ovarian growth in several birds. However, yellow follicles were present in less than 6% of the PMSG treated birds, and only one of these birds ovulated and produced an egg. In previous experiments in which birds were treated with PMSG by daily injections or continuously by osmotic pump for three weeks, over 25% of treated birds had large yellow follicles in the ovary, and small yellow follicles had developed in over 50% of PMSG treated birds. It is noteworthy that in the previous experiments quail held under long days for three weeks showed much greater ovarian development than the long day controls in the current experiment (no long day females laid eggs, whereas in previous experiments 50-100% of birds had started laying after three weeks of long days). The current results therefore do not necessarily indicate that PMSG treatment at intervals of greater than one day is insufficient to stimulate follicular growth. If this is the case, the fact that few of the PMSG treated birds developed large yellow follicles indicates that treatment every two, three or four days more successfully mimics long day conditions than daily treatment with PMSG. Unfortunately, there have been few experiments in which the frequency of treatment with PMSG or other gonadotropins has been varied. Zadworny and Etches (1988) treated turkeys with PMSG every two days, but did not treat any birds with daily injections for comparison. Treatment every two days did stimulate ovarian and oviductal development in turkeys, but large yellow follicles were not arranged in a hierarchy.

Daily treatment with PMSG was used in the first week of this experiment to show that this treatment is not suitable for stimulating a normal rate of ovarian development. Although birds treated daily with 20 IU PMSG for one week had ovary and oviduct weights that were not significantly different from long day controls, the mean ovary and oviduct weights tended to be larger in these birds than those held on long days. Treatment with PMSG once every four days did initiate ovarian and oviductal development in the first two weeks of treatment, but failed to stimulate further growth during the third week of treatment. The ovary and oviduct weights of birds treated

every two or three days with PMSG were generally similar to those of long day controls, suggesting that these treatments are the most suitable for mimicking the ovarian response to long day conditions.

Previous experiments in which Japanese quail have been treated with PMSG to stimulate reproduction have shown that more satisfactory results are achieved when the dose of PMSG given is changed at weekly intervals. The results of this experiment suggest that changing the time between injections at different stages of treatment may also lead to better results. After one week of PMSG treatment every three or four days, ovary weights were generally similar to long day controls, but after three weeks of treatment every three or four days, ovary weights were significantly smaller than long day controls in all but the group treated with 40 IU PMSG every three days. Similarly, birds treated every two days with 40 IU PMSG for one week had similar ovary weights to birds held on long days. However, after two weeks of treatment, the mean ovary weight of birds treated every two days with 40 IU PMSG was significantly larger than long day controls. It may be that less frequent treatment is required to initiate the early stages of ovarian development, but more regular injections of PMSG are required to stimulate follicular growth. Wells *et al.* (1985) found that PMSG has 60% FSH activity and only 40% LH activity in birds. Onagbesan and Peddie (1988b) showed that FSH acts predominantly on the small follicles. This finding is supported by the fact that the number of FSH receptors in the granulosa cells decreases as follicles move through the hierarchy (Etches and Cheng, 1981; Ritzhaupt and Bahr, 1987), there is a progressive loss of FSH-stimulated progesterone production by granulosa cells of chickens as the follicles mature (Hammond *et al.*, 1981), and the highest amount of rat FSH binding per granulosa cell in hierarchical follicles occurs in F5 follicles (Masuda *et al.*, 1984). The high FSH activity of PMSG may explain why less frequent injections are able to initiate ovarian growth, but not stimulate development of large follicles.

The similarity in follicular size distribution between PMSG treated birds and long day controls was slightly better in this experiment than in previous experiments. However, as with previous experiments, there were significantly more 3–5 mm follicles in the ovaries of PMSG treated birds than in long day controls, and some potential reasons for this have been discussed in the previous chapter. Hammond *et al.* (1981) showed that avian LH caused a more potent steroidogenic response from incubated granulosa cells

from chickens than mammalian LH. Conversely, mammalian FSH was more effective in promoting progesterone production from granulosa cells than avian FSH. As discussed above, PMSG has a higher FSH activity than LH activity, and if the mammalian gonadotropin elicits a more potent response than avian FSH, this may lead to excessive follicular development in the small size classes.

In the four PMSG treated birds that did develop large yellow follicles in the ovary, these were not arranged in a hierarchy. As discussed in previous chapters, normal steroid feedback mechanisms may not be operating in PMSG treated birds, and as a result, changes in receptor numbers and affinity that occur as a follicle moves through the hierarchy may be disrupted. In normal laying birds, there is a change in number and affinity of LH receptors during the last three days of follicular maturation (F3 to F1). In the theca, although affinity of LH receptors increases three fold from F2 to F1, the number of receptors and receptor binding decreases abruptly (Kikuchi and Ishii, 1992). In Japanese quail treated with a chicken pituitary extract there was a slight change in LH receptor binding from F2 to F1, but this was not as marked as that which occurs in normal laying birds (Wakabayashi *et al.*, 1996). LH binding to granulosa cells increases gradually from F3 to F1 in normal laying hens, but in birds treated with chicken pituitary extract, there was little or no change in LH binding to the granulosa (Wakabayashi *et al.*, 1996). The disruption to changes in LH receptor numbers and LH binding may be even more pronounced in PMSG treated birds.

Variation in ovarian response between birds may be due potential variation in the amount of PMSG that enters the circulatory system following subcutaneous injection. Most experiments in which birds have been treated with injections of exogenous gonadotropins have used intramuscular sites of administration (Opel and Nalbandov, 1961a; Mitchell, 1966; 1967a; 1967b; Imai, 1972; Imai *et al.*, 1972; Reeves *et al.*, 1973) rather than intravenous or subcutaneous sites. Some studies have used intramuscular injections to stimulate ovarian growth, followed by intravenous treatments to induce ovulation (Opel and Nalbandov, 1961a; Mitchell, 1966; 1967b). Preliminary testing carried out by Phillips (1943) compared the effectiveness of subcutaneous and intramuscular injections of gonadotropin preparations. Phillips (1943) found that subcutaneous injections of gonadotropin produced a less pronounced response than intramuscular injections, and there was more variation in ovarian response between

birds when treated with intramuscular injections. Van Tienhoven and Schally (1972) injected laying hens with porcine LHRH to induce premature ovulation. They compared the effectiveness of intravenous injections with intracarotid injections and stated that there was no evidence that intracarotid injections were more effective than intravenous injections. However, the data presented suggested that intravenous injections were more likely to stimulate premature ovulation than intracarotid injections delivering the same dose of LHRH.

Plasma estradiol concentrations are difficult to interpret as there were no clear patterns and no significant differences between short day and long day controls. This may be due to the fact that very few of the treated or long day control birds had large yellow follicles present in the ovary. However, the birds that did have yellow follicles in the ovary did not have higher estradiol concentrations than birds with no yellow follicles. There was a weak relationship between plasma estradiol concentrations and ovary weight in control birds, but no relationship in PMSG treated birds. In contrast, Zadworny and Etches (1988) found a positive linear relationship between plasma estradiol concentration and ovary and oviduct weight in PMSG treated turkeys. They also showed that plasma estradiol concentrations were higher in birds treated with higher doses of PMSG. A similar relationship was not evident in this experiment.

PMSG alone may not be sufficient to stimulate development of a normal ovarian hierarchy in Japanese quail. Other hormones may need to be given in conjunction with PMSG to stimulate normal follicular development. There are no published examples in which an avian species has been treated with PMSG plus another hormone, but this technique has been used in several mammalian species. Naqvi and Gulyani (1998) found that treatment with PMSG in conjunction with four micrograms of Buserelin (GnRH) increased the ovulation rate in adult sheep above that observed when PMSG was used alone. However, PMSG given in conjunction with FSH (0.5 U ovagen) had no effect on ovulation. PMSG plus progesterone treatment in medroxyprogesterone acetate-primed sheep resulted in normal luteal function, whereas PMSG treatment alone caused premature luteal regression in 80% of treated females (Leyva *et al.*, 1998). PMSG antibodies have been used in conjunction with PMSG to induce superovulation in goats (Pintado *et al.*, 1998) and cattle (Dieleman *et al.*, 1993; González *et al.*, 1994a, 1994b), and resulted in a significantly larger number of viable embryos than PMSG

treatment alone. Similar treatments have been used in non-domesticated species. Sawyer Steffan *et al.* (1983) used a combination of high doses of PMSG and hCG to induce ovulation in bottlenose dolphins (*Tursiops truncatus*). These results show clearly that PMSG treatment in conjunction with another hormone is more successful than PMSG alone for initiating reproductive activity in mammalian species.

Excessive follicular development has occurred in some birds in all experiments described so far, and is one of the predominant anomalies that occur in response to PMSG treatment in Japanese quail. There are many potential ways of altering the current methodology to overcome the breakdown of the follicular hierarchy, and one of these is to restrict the amount of food made available to the birds during the treatment period. Feed restriction is commonly used by the commercial poultry industry to restrict follicular development and increase the rate of lay in broiler breeders. When fed *ad libitum*, broiler breeders show excessive follicular growth and develop multiple hierarchies. This often leads to multiple ovulations, reduced egg production and poor egg quality (Costa, 1981). Restricting feed intake in broilers until the onset of lay increases egg production and improves egg quality. Potentially, feed restriction could reduce the number of ovarian follicles that enter the rapid growth phase in PMSG treated birds, leading to development of a follicular hierarchy. A decrease in food intake may exert an influence on follicular development in a number of different ways. Reduced food intake provides fewer precursors that can be synthesised into yolk by the liver, leading to a decrease in the amount of yolk that can be transported and incorporated into developing follicles (Hosoda *et al.*, 1955). A decrease in feed intake may lead to a decline in GnRH production from the hypothalamus, which may result in a decrease in LH and FSH secretion from the pituitary gland. Feed restriction is known to increase plasma corticosterone concentrations in quail (Scott *et al.*, 1983). Corticosterone may act on the pituitary gland to inhibit LH and FSH production, both of which are involved in the initiation and maintenance of follicular growth. Corticosterone may also alter the way yolk precursors are synthesised by the liver. One or all of these factors could contribute to a decrease in the number of developing follicles in hormone treated female quail on a restricted diet.

In summary, treatment with PMSG every two, three or four days stimulate a more natural rate of ovarian development than daily treatment. However, further work is

required to determine appropriate changes in PMSG dose and treatment frequency to increase the number of birds that produce large yellow follicles. Further research is also required to determine the effects of other hormones or a restricted feeding regimen on ovarian development in Japanese quail treated with PMSG, and to establish a way of predicting an individual bird's response to PMSG prior to treatment.

5 Effect of restricted feed and PMSG treatment on ovarian development

5.1 Introduction

Treatment with PMSG is able to stimulate ovarian development in Japanese quail, as described in previous experiments. However, when yellow follicles develop in response to PMSG treatment, they are not arranged in a hierarchy. Several experiments have been carried out exploring the effect of changing the doses of PMSG that are given, and changing the frequency of treatment, but breakdown of the follicular hierarchy still occurs in almost all birds that develop large ovarian follicles. It may be that PMSG treatment alone will not stimulate development of a normal follicular hierarchy in Japanese quail.

Restricting the feed intake of PMSG treated Japanese quail is one potential way of improving the development of a follicular hierarchy. This method is used routinely by the commercial poultry industry to increase egg production in broiler chickens (Costa, 1981). Modern broiler breeders have been selected for faster growth and rapid weight gain, which has resulted in a sharp increase in food consumption. The rapid increase in body weight coincides with development of multiple hierarchies in the ovary, consisting of large follicles that are similar in size. There are usually 10-14 large yellow follicles, with 2-3 follicles at each stage in the hierarchy (Jaap and Muir, 1968; Hocking *et al.*, 1987, 1989). This results in excessive or multiple ovulations, increased incidence of erratic ovipositions, and production of defective eggs (Jaap and Muir, 1968). Multiple ovulations can be internal or lead to multiple-yolk eggs (Conrad and Warren, 1940), or result in soft, thin or poorly shelled eggs (Hocking, 1993). These are consequences of excessive follicular recruitment from the white follicles into the hierarchy of yellow follicles (Hocking *et al.*, 1987; 1989).

Broiler breeders are reared on a restricted feeding regimen from three weeks of age until the onset of lay, which decreases body weight in commercial flocks by up to 40% compared with *ad lib* fed birds (Costa, 1981; Yu *et al.*, 1992b; Hocking, 1996). The reduction in body weight is concurrent with a decrease in ovary weight and the development of fewer large yellow follicles that are arranged in a single hierarchy (Hocking *et al.*, 1989; Hocking and Robertson, 2000). This leads to regular, single ovulations, improved egg quality, and an overall increase in egg production (Hocking *et al.*, 1989).

It is not clear why *ad lib* fed broilers produce too many follicles, or how feed restriction controls this process. It may simply be that reduced feed intake provides fewer precursors for yolk synthesis, and consequently, excessive follicular development as seen in *ad lib* feed broiler breeders is not possible. Hosoda *et al.* (1955) showed that serum vitellogenin concentrations drop significantly in laying chickens after only two days of fasting, and after seven days of fasting there were no yolk filled follicles present in the ovary.

It is likely that feed restriction affects the functioning of the hypothalamic-pituitary axis in birds. In mammals, it is well known that under-nutrition leads to delayed puberty in young animals, mainly due to suppressed GnRH levels, which decrease gonadotropin concentrations in the pituitary and plasma (Bronson, 1986; Landefeld *et al.*, 1989; Wade *et al.*, 1996). Dunn *et al.* (1990) found baseline concentrations of LH to be higher in feed restricted broiler breeders than in *ad lib* fed ones. In contrast, Bruggeman *et al.* (1998a) showed that feed restriction reduces GnRH synthesis in the median eminence and causes a decrease in plasma LH concentrations in broiler breeder hens. GnRH injections resulted in similar increase in plasma LH, regardless of feeding regimen, suggesting that food restriction exerts its affect at the hypothalamic level or increases the clearance rate of LH (Buonomo *et al.*, 1982). However, Bruggeman *et al.* (1998b) showed that there is a greater LH production in response to GnRH in sexually mature restricted fed birds, but a reduced LH response in birds receiving restricted feed during sexual maturation. The precise

mechanisms by which restricted feeding modifies ovarian development thus remains unclear.

The Japanese quail used in the experiments described here have been selected for increased body weight. As mentioned in Chapter 3, selection for body weight and rapid growth rate may have favoured genes for growth hormones and other associated hormones and metabolites, which may interact to modify production of, or response to, gonadotropins (Onagbesan *et al.*, 1994; Peddie *et al.*, 1994; Roberts *et al.*, 1994, Hocking and McCormack, 1995). Hocking and McCormack (1995) have shown that ornithine decarboxylase activity (indicator of cell proliferation and differentiation, and hence, rate of tissue growth) in response to PMSG treatment is significantly higher in broiler chickens than in layer chickens. Although Japanese quail selected for body weight do not show excessive follicular development as found in broiler chickens, the changes in endocrine function that occur as a result of selection for body weight may be similar in chickens and quail. Therefore, restricting the feed intake of PMSG treated Japanese quail may lead to development of an ovarian follicular hierarchy as it does in broiler chickens.

A pilot experiment was carried out to determine an appropriate level of feed restriction for female Japanese quail that would allow the birds to maintain a healthy body weight. A second experiment then examined the effect of a restricted feeding regime on ovarian follicular development in Japanese quail treated with PMSG.

5.2 Materials and Methods

5.2.1 Animals

Female Japanese quail (n=142) were purchased at three weeks of age from our normal commercial supplier (Rangitikei Game Farms). Before the start of the experiment, birds were maintained under short day conditions (8L:16D, 10°C) as described in Chapter 2.

5.2.2 Hormone preparation and administration

Pregnant mare serum gonadotropin (PMSG; Intervet) was kindly donated by Pharmaco New Zealand.

PMSG was dissolved in saline and administered using injections. Injections were administered subcutaneously in the abdomen using a 0.5 ml insulin syringe and a 27 gauge, ½ inch needle. Each bird received a volume of 200 µl of solution per injection, containing saline or 40 or 80 IU PMSG.

5.2.3 Experimental design

A small pilot experiment was carried out to determine by how much the daily feed intake of female Japanese quail could be restricted. Thirty-two, six week old female Japanese quail were held with *ad libitum* access to feed and water under a short day photoperiod (LD 8:16) at 10°C for one week. The food intake of each individual bird was then measured for one week. A premeasured quantity (150 g) of quail pellets was placed in each feed tray (one feed tray/bird) at 9 am each day. At 9 am on the following day, the amount of feed remaining in each tray was weighed and recorded, and the tray filled again with 150 g of pellets. The body weight and cloacal opening diameter of each bird was measured daily. This process was continued daily for one week, after which the average daily feed intake for each individual bird was calculated.

At eight weeks of age, birds were divided into groups as shown in Table 5.1. Group 1 continued to receive an *ad libitum* feed regime, and daily food intake was recorded. Individuals in groups 2-4 received 80%, 60% or 40% of their average daily feed intake. The designated quantity of food for each bird was placed in the feed tray at 9 am each day. At 9 am the following day, any remaining food was weighed and recorded, and the tray refilled with the amount of food allocated to that bird. Body weight, cloacal opening

diameter and egg production were recorded daily for all birds. Each group received the allocated level of feed restriction for 16 days. Birds were then euthanased using the standard method of stunning and decapitation. Peritoneal fat, pectoral muscle, ovaries and oviducts were excised from all birds and weighed. Blood samples for radioimmunoassay of plasma estradiol were collected immediately following decapitation.

Table 5.1. Feeding schedule for pilot experiment.

Age	Group 1	Group 2	Group 3	Group 4
7 weeks	*Ad lib* feed	*Ad lib* feed	*Ad lib* feed	*Ad lib* feed
8 weeks	*Ad lib* feed	80% feed	60% feed	40% feed
9 weeks	*Ad lib* feed	80% feed	60% feed	40% feed
10 weeks	*Ad lib* feed	80% feed	60% feed	40% feed

Using the results from the pilot experiment, an experiment incorporating restricted feed as well as PMSG treatment to stimulate ovarian development was designed. One hundred and ten, four week old female Japanese quail were held with *ad libitum* access to feed and water under a short day photoperiod (LD 8:16) at 10°C for one week. The farm was unable to supply 110 four week old female Japanese quail in one batch, so 46 birds were collected one week, and 64 birds the following week. The day after they were brought from the farm, a blood sample was taken from the wing vein of each of the birds collected on the second week. At eight weeks of age, the food intake of each individual bird was measured as described for the pilot experiment. At nine weeks of age, birds were divided into groups as shown in Table 5.2.

Table 5.2. Experimental groups.

Group number	Photoperiod	% of daily feed intake given	Dose of PMSG/injection
1	Short day	Ad lib	-
2	Short day	Ad lib	None
3	Long day	Ad lib	None
4	Long day	80	None
5	Long day	60	None
6	Short day	Ad lib	40 IU
7	Short day	80	40 IU
8	Short day	60	40 IU
9	Short day	Ad lib	80 IU
10	Short day	80	80 IU
11	Short day	60	80 IU

All birds remained under a short day photoperiod at 10°C from 9-11 weeks of age and received one of three feeding regimes; 60% normal daily intake, 80% normal daily intake or *ad lib* feed. The amount of food provided to the 60% and 80% groups was calculated in relation to the mean daily feed intake of each bird. The designated quantity of food was placed in feed trays at 9 am each day. At 9 am the following day, any remaining food was weighed and recorded, and the tray refilled with the amount of food allocated to that bird. All birds were weighed every second day. If an individual lost condition to the point where their health was seriously impaired, then they were removed from the experiment. Birds were maintained on this feeding regime for two weeks before being administered with PMSG or being transferred to long days. At 11 weeks of age, six groups started receiving PMSG treatment, either one injection of 40 IU PMSG every two days, or 80 IU PMSG every two days, as well as the allocated feed regimen. Daily food intake, body weight and

cloacal opening diameter were recorded. At the same time, three groups were transferred to long days at 20°C while still receiving the allocated amount of feed. One group of birds (Group 2) remained on short days with *ad lib* feed as a control. A second group (Group 1) was euthanased at the start of the PMSG treatment period.

After three weeks of PMSG treatment and/or restricted feed, all birds were euthanased. The following measurements were taken: ovary weight, oviduct weight, pectoral muscle weight, peritoneal fat weight and metatarsus length. A condition index was calculated for each bird using the formula:

$$\text{Condition index} = \text{body weight (g)} / [\text{tarsometatarsus length (cm)}]^3$$

Ovaries were divided into five size classes (0.00-0.15 g, 0.16-0.50 g, 0.51–1.00 g, 1.01–5.00 g, and 5.01–9.00 g) and the numbers and sizes of yellow follicles present in each ovary were recorded. The follicular distribution within each ovarian weight class was compared between control and treated groups receiving restricted feed. Terminal blood samples were taken for radioimmunoassay of plasma estradiol, progesterone and corticosterone.

All experimental procedures were approved by the Massey University Animal Ethics Committee.

5.2.4 Radioimmunoassay for plasma estradiol

Plasma samples were assayed for estradiol using the method described in Chapter 2.

5.2.5 Radioimmunoassay for plasma progesterone

5.2.5.1 Extraction of progesterone from plasma

Progesterone was extracted from plasma as described for estradiol in Chapter 2. However, n-hexane was used instead of dichloromethane during the extraction procedure.

Due to large variations between samples in extraction efficiency, the recovery of progesterone during the extraction process was measured for all samples by adding 20 µl of tritiated progesterone solution (5 000 cpm) to each sample, with individual efficiencies used in calculations of plasma progesterone concentrations. The mean percentage recovery for progesterone in quail plasma was 54.0% ± 0.7% (n=384) with a range from 21.9% to 90.2%.

5.2.5.2 Radioimmunoassay of progesterone

Progesterone concentrations were measured in extracted quail plasma by radioimmunoassay using the same procedure as described for estradiol (Chapter 2) but with progesterone antibody (Dr. R. J. Etches, University of Guelph, Ontario, Canada; 1:4 000 final dilution) and progesterone label (tritiated progesterone, approximately 5 000 cpm, Amersham, UK).

The sensitivity of the radioimmunoassay for progesterone was determined as the hormone concentration at the mean minus two standard deviations from the percentage bound of the zero hormone tubes. The sensitivity was 62.8 pg/ml on the standard curve, which was equivalent to a progesterone concentration in quail plasma of 0.622 ng/ml (n=7 assays). Serial dilutions of extracted quail plasma in assay buffer (PBSG) were parallel to the progesterone standard curve (n=3). Recovery of progesterone added to quail plasma was 90.9 ± 3.9%, 93.3 ± 3.6% and 90.6 ± 3.1% for three different samples (W.H. Chua, pers. comm.).

Solutions of progesterone in PBSG at concentrations that gave approximately 20, 50 and 80% binding on the standard curve were used as high, medium and low quality controls in every assay. The mean concentrations of these solutions were 2384.2 ± 197.8, 682.7 ± 56.7

and 270.9 ± 46.5 pg/ml respectively. Intra-assay coefficients of variation for progesterone were determined by conducting an assay with ten duplicates of each quality control. The intra-assay coefficients of variation for estradiol were 6.7%, 7.7% and 13.9% for high, medium and low quality controls respectively. Inter-assay coefficients of variation were calculated from duplicates of the quality controls included at the beginning and end of each assay. The inter-assay coefficients of variation for seven assays were 8.3%, 8.3% and 17.2% for high, medium and low quality controls respectively.

The cross-reactivity of the progesterone antibody was reported by Etches and Croze (1983) as follows: 5α-pregnane-3,20-dione (15.6%), 5β-pregnane-3,20-dione (6.7%), 11β-hydroxyprogesterone (5.4%), and cholesterol, testosterone, 17β-estradiol, cortisol, corticosterone, deoxycorticosterone, 17α-hydroxyprogesterone, pregnenolone, 5α-pregnan-3α-ol-20-one, 4-pregnene-20β-ol-3-one, 4-pregnen-20α-ol-3-one, 5β-pregnan-3α-ol-20-one, 5β-pregnan-3β-ol-20-one, 5α-pregnan-3β-ol-20-one (all <1.0%).

5.2.6 Radioimmunoassay for plasma corticosterone

5.2.6.1 Extraction of corticosterone from plasma

Corticosterone was extracted from plasma as described for estradiol in Chapter 2. The recovery of corticosterone during the extraction process was measured by adding 20 µl of tritiated corticosterone solution (5 000 cpm) to 48 samples in a single extraction. The mean percentage recovery for corticosterone in quail plasma was 101.0% ± 1.7%.

5.2.6.2 Radioimmunoassay of corticosterone

Plasma corticosterone radioimmunoassays were carried out by Mrs. Janis Bridges. Corticosterone concentrations were measured in extracted quail plasma by radioimmunoassay using the same procedure as described for estradiol (Chapter 2) but with

corticosterone antibody (Dr. R. J. Etches, University of Guelph, Ontario, Canada; 1:18 000 final dilution) and corticosterone label (tritiated corticosterone, approximately 5 000 cpm, Amersham, UK).

The sensitivity of the radioimmunoassay for corticosterone was determined as the hormone concentration at the mean minus two standard deviations from the percentage bound of the zero hormone tubes. The sensitivity was 30.4 pg/ml on the standard curve, which was equivalent to a corticosterone concentration in quail plasma of 0.73 ng/ml (n=12 assays). Serial dilutions of extracted quail plasma in assay buffer (PBSG) were parallel to the corticosterone standard curve (n=3). Recovery of corticosterone added to quail plasma was 93.9 ± 4.9%, 98.6 ± 7.3% and 88.9 ± 5.5% for three different samples (D. C. Adams, pers. comm.).

Solutions of corticosterone in PBSG at concentrations that gave approximately 20, 50 and 80% binding on the standard curve were used as high, medium and low quality controls in every assay. The mean concentrations of these solutions were 1414.6 ± 54.3, 309.6 ± 13.2 and 60.5 ± 10.0 pg/ml respectively. Intra-assay coefficients of variation for corticosterone were determined by conducting an assay with ten duplicates of each quality control. The intra-assay coefficients of variation for corticosterone were 13.2%, 14.8% and 13.4% for high, medium and low quality controls respectively. Inter-assay coefficients of variation were calculated from duplicates of the quality controls included at the beginning and end of each assay. The inter-assay coefficients of variation for five assays were 11.9%, 16.2% and 19.5% for high, medium and low quality controls respectively.

The cross-reactivity of the corticosterone antibody with other steroids was reported by Etches (1976) as follows: deoxycorticosterone (27.9%), cortisol (6.9%), progesterone (37.6%), 11β-hydroxyprogesterone (21.3%) and estradiol, testosterone, 17α-hydroxyprogesterone, 1-deoxycortisol, aldosterone and pregnenolone (<5%). Although this antibody was found to cross-react with progesterone and 11β-hydroxyprogesterone, these hormones are poorly extracted in dichloromethane, so corticosterone is the predominant hormone measured by this antibody.

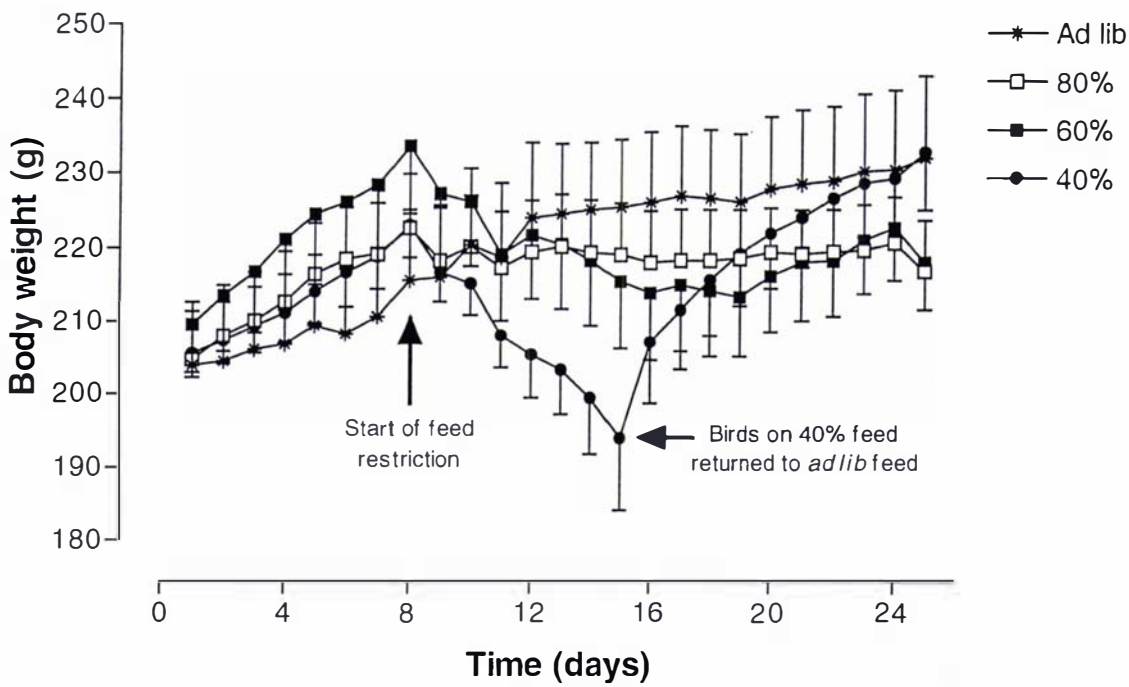

Figure 5.1. Changes in body weight in female Japanese quail held on short days for while receiving *ad libitum* feed or 80%, 60% or 40% of their normal daily feed intake.

5.2.7 Statistics

All variables were tested for homogeneity of variance using Levene's test. When variances were homogeneous across groups for a single variable, ANOVA followed by linear contrasts were used to analyse data. Linear contrasts were used rather than Bonferroni's posthoc tests due to the large number of comparisons. When variances were not homogeneous across groups, Kruskal-Wallis non-parametric ANOVA followed by Mann-Whitney U tests were performed to determine differences between groups. One-way ANOVA or Student's t-tests were used to analyse follicular size frequency distribution data. Repeated measures ANOVA was used to analyse changes in cloacal diameter over time. Relationships between variables were investigated using linear regression to calculate r^2 values.

ANOVAs and non-parametric equivalents were carried out using Systat Version 8.0 (SPSS Inc., 1988), and relationships between variables (linear regression) were analysed using GraphPad Prism Version 3.0 (GraphPad Software Inc., 1999). Data were transformed to logarithms where necessary. Data are shown as mean ± standard error.

5.3 Results

5.3.1 Pilot experiment

An individual female Japanese quail eats approximately 35 grams/day of food when under a short day photoperiod. Each bird eats a similar amount each day, and the average daily intake for birds on short days ranges from 20 g to 65 g. The pilot experiment showed that 40% of a bird's normal daily feed intake is not sufficient to maintain a healthy weight and body condition (Figure 5.1). The body weight of birds receiving *ad lib* feed continued to increase during the experiment. Birds receiving 60% or 80% of their normal daily feed

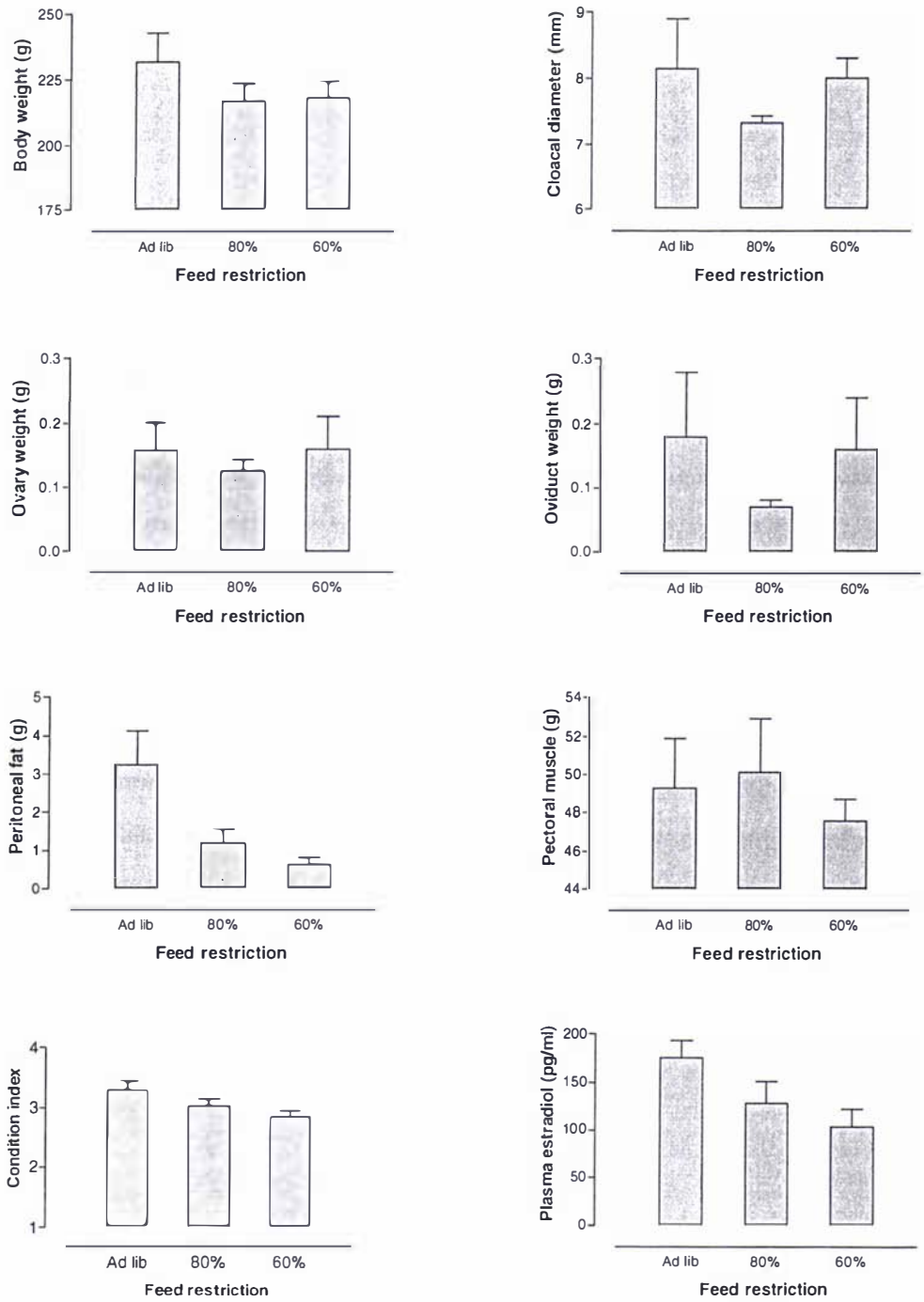

Figure 5.2. Body weight, cloacal diameter, ovary weight, oviduct weight, pectoral muscle weight, peritoneal fat weight, condition index and plasma estradiol concentration in female Japanese quail after 16 days receiving ad lib, 80% or 60 % of normal daily feed intake during the pilot experiment.

intake lost weight initially, but then stabilised after approximately eight days on restricted feed. Birds that were receiving 40% of their daily feed intake lost weight rapidly, and were returned to *ad lib* feeding after only six days on restricted feed.

Table 5.3. Summary of one-way ANOVA comparing body weight, cloacal diameter, ovary weight, oviduct weight, peritoneal fat weight, pectoral muscle weight, condition index and plasma estradiol concentration between groups receiving *ad lib* feed, 80% or 60 of normal daily feed intake for 16 days.

Variables	F	df	*p*
Body weight	0.588	2, 19	0.565
Cloacal diameter	0.876	2, 19	0.433
Ovary weight	0.220	2, 19	0.804
Oviduct weight	0.655	2, 19	0.531
Peritoneal fat weight	5.280	2, 19	0.015
Pectoral muscle weight	0.236	2, 19	0.729
Condition index	1.269	2, 19	0.304
Plasma estradiol	3.249	2, 19	0.061

A feeding regime of 80% or 60% of normal daily feed intake did not have any significant effect on body weight, cloacal diameter, ovary weight, oviduct weight, pectoral muscle weight, condition index or plasma estradiol concentration (Figure 5.2; Table 5.3). There was a significant difference in the peritoneal fat weight between birds receiving *ad lib* feed and those receiving 60% of normal daily intake ($p = 0.023$).

Results from the pilot experiment showed that restricted feeding regimes of 60 or 80% of normal daily feed intake do not adversely affect the health of female Japanese quail, and therefore could be used in the next experiment.

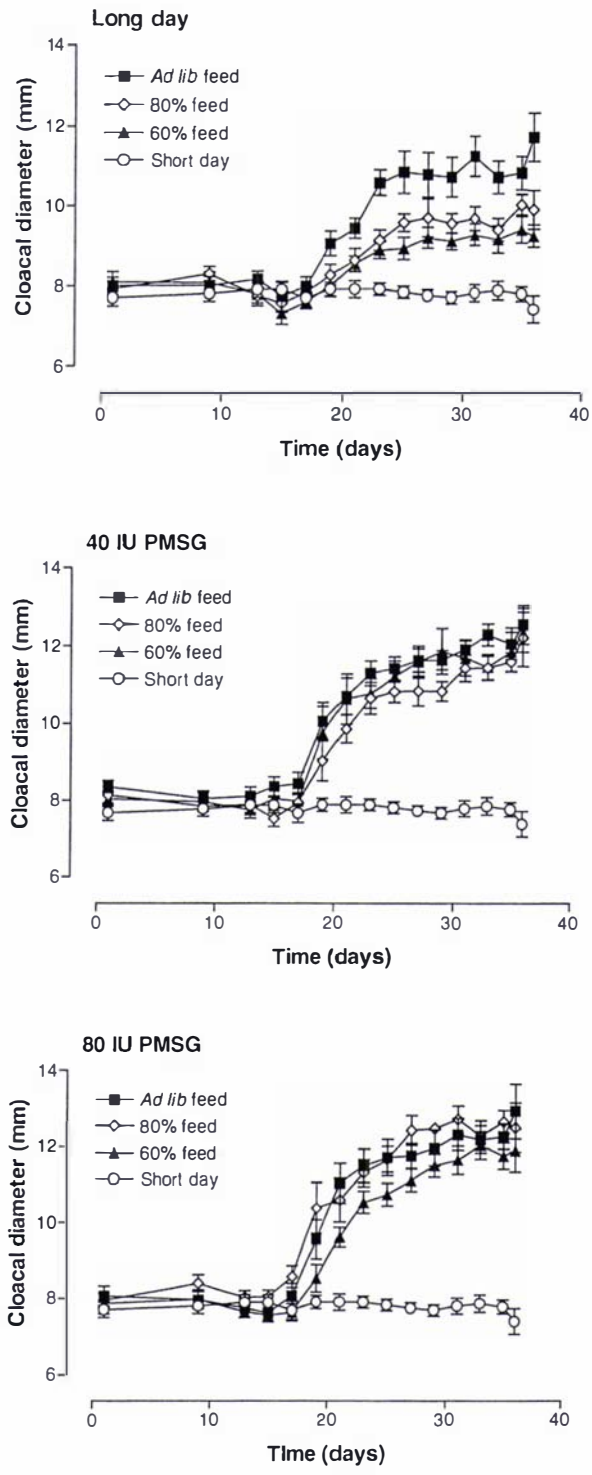

Figure 5.3. Changes in cloacal diameter in birds held under a short day photoperiod receiving *ad lib*, 80% or 60% of normal daily feed intake and treated with 40 or 80 IU PMSG every two days for three weeks, or held on short days or long days for three weeks receiving no treatment. Day 15 was the first day of PMSG treatment or long days.

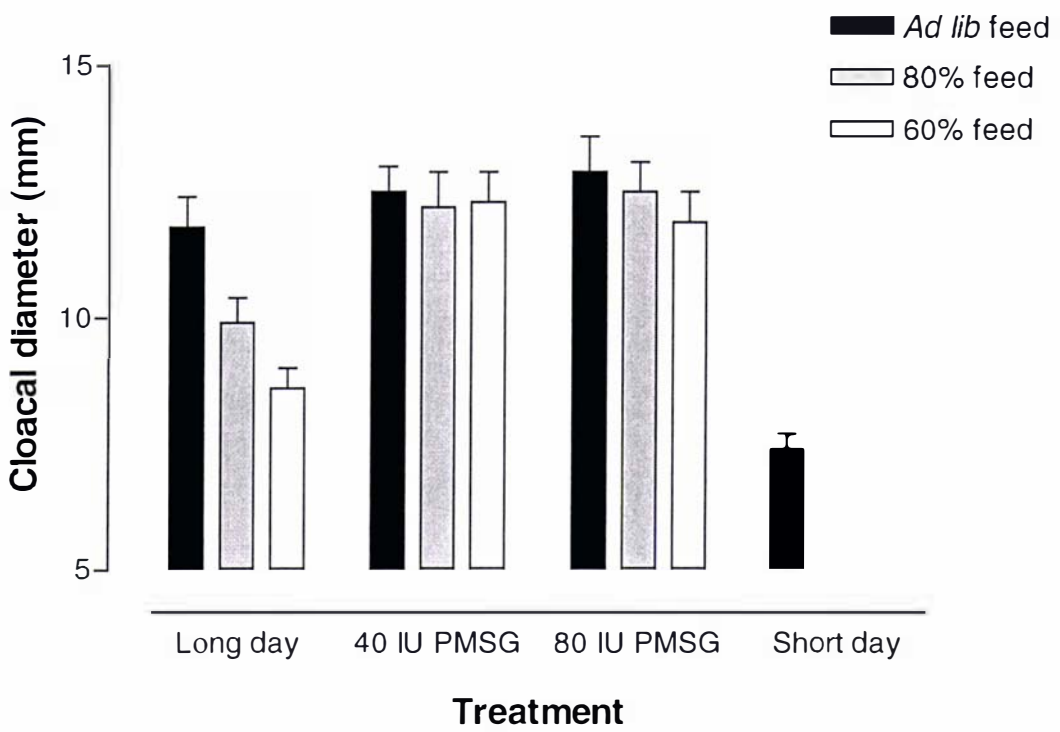

Figure 5.4. Mean cloacal diameter of female Japanese quail after three weeks on short days, long days receiving *ad lib*, 80% or 60% normal daily feed intake, or short days receiving *ad lib*, 80% or 60% normal daily feed intake plus treatment with 40 or 80 IU PMSG every two days.

5.3.2 Cloacal opening diameter

There was little or no change in cloacal opening diameter during the two weeks in which birds received restricted feeding regimes but no PMSG treatment (Figure 5.3, see Table 5.4 for statistics). Cloacal diameter increased in PMSG treated groups and groups transferred to long days during the treatment period. There was no increase in cloacal diameter in birds held on short days receiving no treatment.

Table 5.4. Summary of one-way repeated measures ANOVA comparing changes in cloacal opening diameter in each group during two weeks of ad lib, 80% or 60% normal daily feed intake alone, and three weeks receiving an allocated feeding regime and treatment every two days with 40 or 80 IU PMSG, or transfer to a long day photoperiod.

Group for three week PMSG treatment period	Two weeks before PMSG treatment			Three week PMSG treatment period		
	F	df	*p*	F	df	*p*
Short day	0.431	3, 24	0.733	1.100	11, 88	0.371
Long day ad lib feed	0.448	3, 27	0.720	22.535	11, 99	0.000
Long day 80% feed	2.614	3, 27	0.072	13.976	11, 99	0.000
Long day 60% feed	3.266	3, 27	0.037	13.795	11, 99	0.000
40 IU PMSG ad lib feed	1.279	3, 27	0.302	25.312	11, 99	0.000
40 IU PMSG 80% feed	2.984	3, 27	0.049	21.254	11, 99	0.000
40 IU PMSG 60% feed	1.178	3, 27	0.337	26.015	11, 99	0.000
80 IU PMSG ad lib feed	2.658	3, 27	0.068	35.525	11, 99	0.000
80 IU PMSG 80% feed	1.285	3, 27	0.300	22.991	11, 99	0.000
80 IU PMSG 60% feed	1.887	3, 27	0.156	34.920	11, 88	0.000

At the end of the experiment there were significant differences between groups in the mean cloacal opening diameters (Figure 5.4, see Table 5.5 for statistics). The mean cloacal

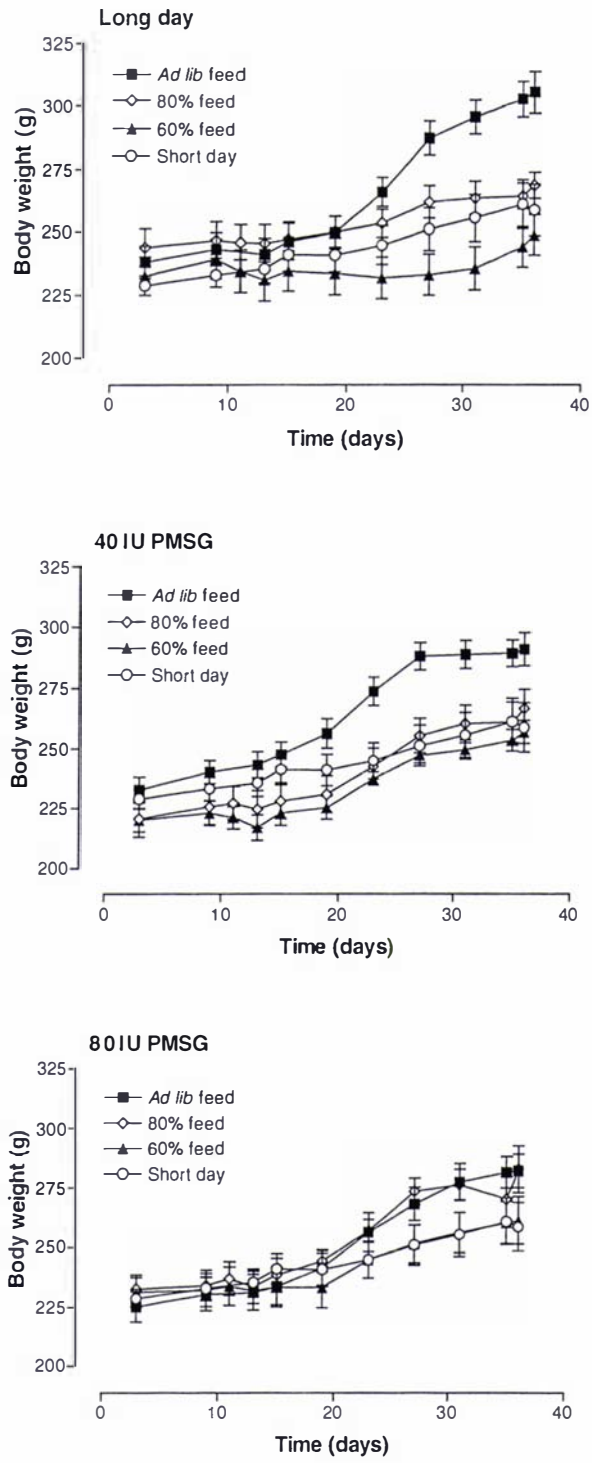

Figure 5.5. Changes in body weight in birds held under a short day photoperiod receiving *ad lib*, 80% or 60% of normal daily feed intake for and treated with 40 or 80 IU PMSG every two days for three weeks, or held on short days or long days for three weeks receiving no treatment. Day 15 was the first day of PMSG treatment or long days.

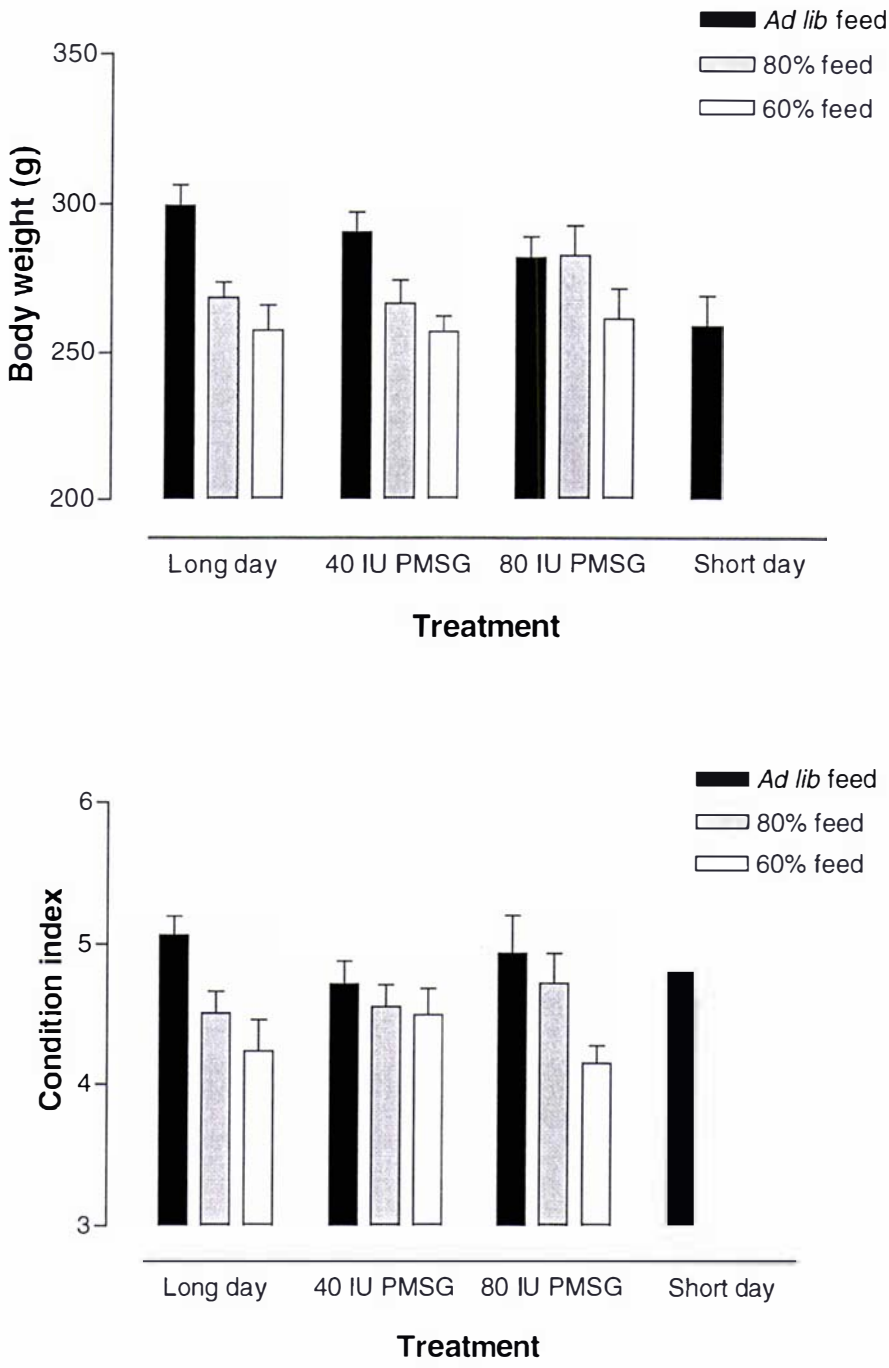

Figure 5.6. Mean body weight and condition index of female Japanese quail after three weeks on short days, long days receiving *ad lib*, 80% or 60% normal daily feed intake, or short days receiving *ad lib*, 80% or 60% normal daily feed intake plus treatment with 40 or 80 IU PMSG every two days.

diameter of birds receiving 60% feed was significantly smaller than birds receiving *ad lib* feed. The mean cloacal diameters of birds treated with 40 or 80 IU PMSG were significantly larger than those of birds held on long days, regardless of feeding regime. All PMSG treated groups had cloacal diameters similar to birds held on long days receiving *ad lib* feed. The mean cloacal diameters of birds on long days receiving 80% or 60% feed were significantly smaller than birds receiving *ad lib* feed on long days. However, there was no difference between the 60% and 80% groups on long days. There was no effect of feeding regime on cloacal opening diameter in any of the PMSG treated groups.

5.3.3 Body weight and condition index

The mean body weights of all groups receiving *ad lib* or 80% feed significantly increased during the first two weeks (Figure 5.5, see Table 5.6 for statistics). During this period, the three groups receiving 60% of normal daily feed intake showed no significant increase in body weight. The mean body weights of all groups increased significantly during the last three weeks on all photoperiods, levels of feeding and PMSG treatments.

A condition index was calculated to allow for differences in body size between birds. Body weight data are also shown to indicate how body weight results alone may be misinterpreted. There were significant differences in mean body weights and condition indices between all groups at the end of the experiment (Figure 5.6; see Table 5.7 for statistics). *Ad lib* fed birds had significantly larger mean condition indices than birds given 80% or 60% feed. There were no differences in mean body conditions between birds held on long days and birds treated with 40 or 80 IU PMSG regardless of feeding regime. The mean condition index of birds held on long days receiving *ad lib* feed was significantly larger than any of the groups receiving 60% feed. *Ad lib* fed long day birds also had a significantly greater body condition than birds given 80% feed on long days. There was no effect of feeding regime on body condition within the groups treated with 40 IU PMSG. Birds treated with 80 IU PMSG receiving *ad lib* or 80% feed had significantly greater body conditions than birds given 60% feed and 80 IU PMSG.

Table 5.5. Summary of one-way ANOVA and linear contrasts comparing cloacal opening diameter between groups at the end of the experiment.

ANOVA	**F**	**df**	***p***
All groups	12.886	10, 95	0.000
Linear contrasts	**F**	**df**	***p***
All *Ad lib* feed vs All 80% feed	3.891	1, 95	0.051
All *Ad lib* feed vs All 60% feed	10.089	1, 95	0.002
All 80% feed vs All 60% feed	1.579	1, 95	0.212
All Long day vs All 40 IU PMSG	24.999	1, 95	0.000
All Long day vs All 80 IU PMSG	27.087	1, 95	0.000
All 40 IU PMSG vs All 80 IU PMSG	0.042	1, 95	0.838
Long day *ad lib* feed vs Short day	30.235	1, 95	0.000
Long day *ad lib* feed vs 40 IU PMSG *ad lib* feed	1.027	1, 95	0.314
Long day *ad lib* feed vs 40 IU PMSG 80% feed	0.342	1, 95	0.560
Long day *ad lib* feed vs 40 IU PMSG 60% feed	0.526	1, 95	0.470
Long day *ad lib* feed vs 80 IU PMSG *ad lib*	2.350	1, 95	0.129
Long day *ad lib* feed vs 80 IU PMSG 80% feed	0.949	1, 95	0.332
Long day *ad lib* feed vs 80 IU PMSG 60% feed	0.037	1, 95	0.849
Long day *ad lib* feed vs Long day 80% feed	5.901	1, 95	0.017
Long day *ad lib* feed vs Long day 60% feed	15.518	1, 95	0.000
Long day 80% feed vs Long day 60% feed	2.480	1, 95	0.119
40 IU PMSG *ad lib* feed vs 40 IU PMSG 80% feed	0.184	1, 95	0.669
40 IU PMSG *ad lib* feed vs 40 IU PMSG 60% feed	0.068	1, 95	0.794
40 IU PMSG 80% feed vs 40 IU PMSG 60% feed	0.024	1, 95	0.876
80 IU PMSG *ad lib* feed vs 80 IU PMSG 80% feed	0.588	1, 95	0.445
80 IU PMSG *ad lib* feed vs 80 IU PMSG 60% feed	1.692	1, 95	0.196
80 IU PMSG 80% feed vs 80 IU PMSG 60% feed	0.573	1, 95	0.451

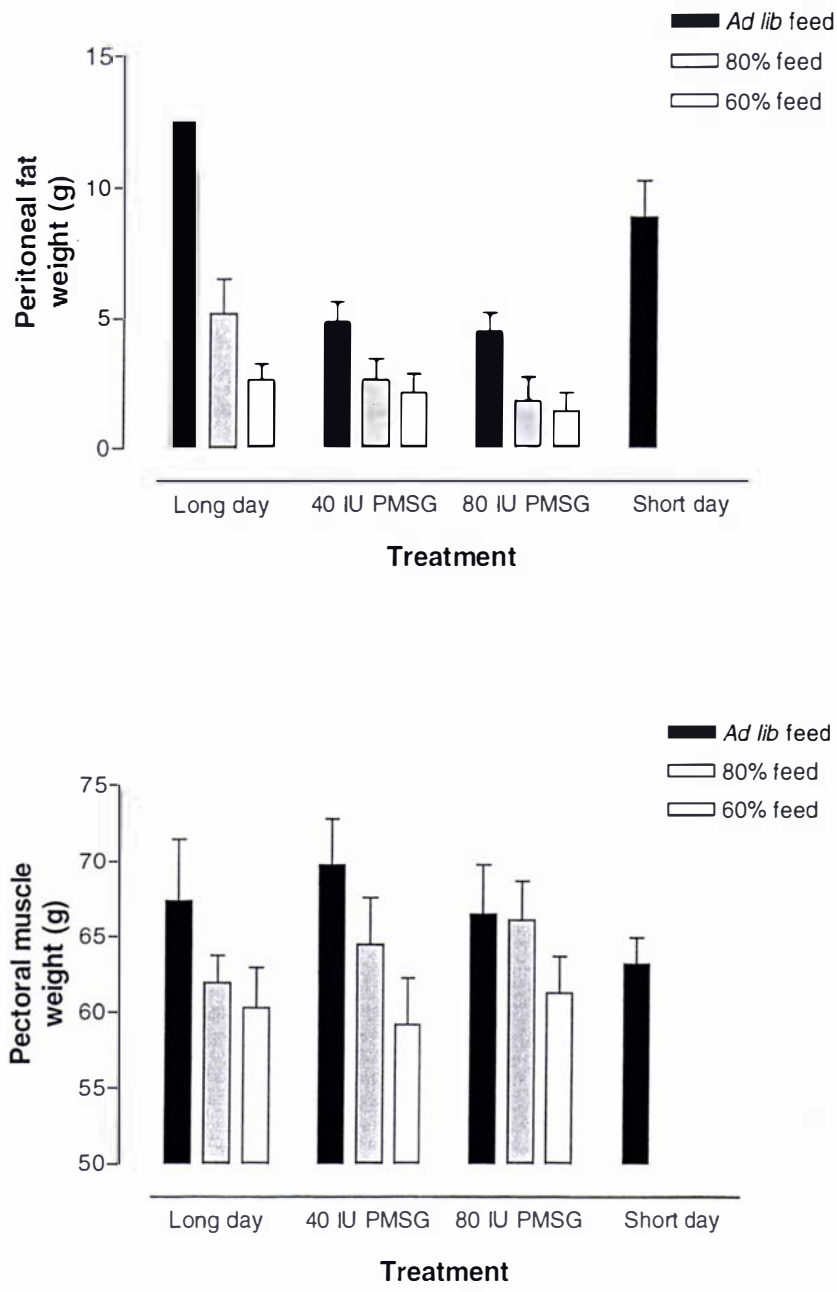

Figure 5.7. Mean peritoneal fat weight and pectoral muscle weight of female Japanese quail after three weeks on short days, long days receiving *ad lib*, 80% or 60% normal daily feed intake, or short days receiving *ad lib*, 80% or 60% normal daily feed intake plus treatment with 40 or 80 IU PMSG every two days.

Table 5.6. Summary of one-way ANOVA comparing changes in body weight in each group during two weeks of ad lib, 80% or 60% normal daily feed intake alone, and three weeks receiving an allocated feeding regime and treatment every two days with 40 or 80 IU PMSG, or transfer to a long day photoperiod.

Group for three week PMSG treatment period	Two weeks before PMSG treatment			Three week PMSG treatment period		
	F	df	*p*	F	df	*p*
Short day	9.308	4, 32	0.000	29.791	5, 40	0.000
Long day ad lib feed	9.541	4, 36	0.000	36.973	5, 45	0.000
Long day 80% feed	2.809	4, 36	0.040	8.570	5, 45	0.000
Long day 60% feed	2.479	4, 36	0.061	17.936	5, 45	0.000
40 IU PMSG ad lib feed	39.916	4, 36	0.000	24.404	5, 45	0.000
40 IU PMSG 80% feed	9.958	4, 36	0.000	21.440	5, 45	0.000
40 IU PMSG 60% feed	2.250	4, 36	0.083	16.682	5, 45	0.000
80 IU PMSG ad lib feed	17.020	4, 36	0.000	24.731	5, 45	0.000
80 IU PMSG 80% feed	8.928	4, 36	0.000	14.336	5, 45	0.000
80 IU PMSG 60% feed	0.193	4, 36	0.941	14.239	5, 40	0.000

5.3.4 Peritoneal fat weight

There were significant differences in mean peritoneal fat weights between all groups at the end of the experiment (Figure 5.7, see Table 5.8 for statistics). In long day groups and groups treated with PMSG, a*d lib* fed birds had significantly more peritoneal fat than birds given 80% or 60%. The mean fat weights of birds held on long days were significantly greater than those of birds held on short days treated with PMSG. Birds held on long days and given *ad lib* feed had significantly more peritoneal fat that any of the PMSG treated groups, except for birds treated with 40 IU PMSG receiving *ad lib* feed. There was no difference in mean peritoneal fat weight between long day and short day controls.

Table 5.7. Summary of one-way ANOVA and linear contrasts comparing body weight and condition index between groups at the end of the experiment.

	Body weight			Condition index		
ANOVA	**F**	**df**	***p***	**F**	**df**	***p***
All groups	5.411	9, 86	0.000	2.216	9, 86	0.028
Linear contrasts	**F**	**df**	***p***	**F**	**df**	***p***
All *Ad lib* feed vs All 80% feed	8.989	1, 86	0.003	4.201	1, 86	0.043
All *Ad lib* feed vs All 60% feed	25.920	1, 86	0.000	15.633	1, 86	0.000
All 80% feed vs All 60% feed	4.722	1, 86	0.032	3.837	1, 86	0.053
All Long day vs All 40 IU PMSG	0.478	1, 86	0.491	0.007	1, 86	0.934
All Long day vs All 80 IU PMSG	0.004	1, 86	0.949	0.000	1, 86	0.999
All 40 IU PMSG vs All 80 IU PMSG	0.394	1, 86	0.532	0.007	1, 86	0.935
Long day *ad lib* feed vs Short day	14.737	1, 86	0.000	3.007	1, 86	0.086
Long day *ad lib* feed vs 40 IU PMSG *ad lib* feed	0.984	1, 86	0.324	1.742	1, 86	0.190
Long day *ad lib* feed vs 40 IU PMSG 80% feed	10.354	1, 86	0.002	3.772	1, 86	0.055
Long day *ad lib* feed vs 40 IU PMSG 60% feed	15.903	1, 86	0.000	4.459	1, 86	0.038
Long day *ad lib* feed vs 80 IU PMSG *ad lib*	3.199	1, 86	0.077	0.236	1, 86	0.628
Long day *ad lib* feed vs 80 IU PMSG 80% feed	2.959	1, 86	0.089	1.669	1, 86	0.200
Long day *ad lib* feed vs 80 IU PMSG 60% feed	12.911	1, 86	0.001	11.669	1, 86	0.001
Long day *ad lib* feed vs Long day 80% feed	9.216	1, 86	0.003	4.503	1, 86	0.037
Long day *ad lib* feed vs Long day 60% feed	15.554	1, 86	0.000	9.475	1, 86	0.003
Long day 80% feed vs Long day 60% feed	0.978	1, 86	0.325	1.026	1, 86	0.314
40 IU PMSG *ad lib* feed vs 40 IU PMSG 80% feed	4.953	1, 86	0.028	0.387	1, 86	0.535
40 IU PMSG *ad lib* feed vs 40 IU PMSG 60% feed	9.133	1, 86	0.003	0.684	1, 86	0.410
40 IU PMSG 80% feed vs 40 IU PMSG 60% feed	0.732	1, 86	0.394	0.049	1, 86	0.825
80 IU PMSG *ad lib* feed vs 80 IU PMSG 80% feed	0.005	1, 86	0.946	0.649	1, 86	0.423
80 IU PMSG *ad lib* feed vs 80 IU PMSG 60% feed	3.431	1, 86	0.067	8.660	1, 86	0.004
80 IU PMSG 80% feed vs 80 IU PMSG 60% feed	3.682	1, 86	0.058	4.660	1, 86	0.034

Table 5.8. Summary of one-way ANOVA and linear contrasts comparing peritoneal fat weight between groups at the end of the experiment.

ANOVA	F	df	*p*
All groups	6.559	9, 86	0.000
Linear contrasts	**F**	**df**	***p***
All *Ad lib* feed vs All 80% feed	20.121	1, 86	0.000
All *Ad lib* feed vs All 60% feed	29.598	1, 86	0.000
All 80% feed vs All 60% feed	1.154	1, 86	0.286
All Long day vs All 40 IU PMSG	4.220	1, 86	0.043
All Long day vs All 80 IU PMSG	8.167	1, 86	0.005
All 40 IU PMSG vs All 80 IU PMSG	0.645	1, 86	0.424
Long day *ad lib* feed vs Short day	0.033	1, 86	0.856
Long day *ad lib* feed vs 40 IU PMSG *ad lib* feed	3.614	1, 86	0.061
Long day *ad lib* feed vs 40 IU PMSG 80% feed	17.650	1, 86	0.000
Long day *ad lib* feed vs 40 IU PMSG 60% feed	17.849	1, 86	0.000
Long day *ad lib* feed vs 80 IU PMSG *ad lib*	3.958	1, 86	0.050
Long day *ad lib* feed vs 80 IU PMSG 80% feed	22.091	1, 86	0.000
Long day *ad lib* feed vs 80 IU PMSG 60% feed	25.400	1, 86	0.000
Long day *ad lib* feed vs Long day 80% feed	7.651	1, 86	0.007
Long day *ad lib* feed vs Long day 60% feed	15.564	1, 86	0.000
Long day 80% feed vs Long day 60% feed	1.569	1, 86	0.214
40 IU PMSG *ad lib* feed vs 40 IU PMSG 80% feed	5.291	1, 86	0.024
40 IU PMSG *ad lib* feed vs 40 IU PMSG 60% feed	5.638	1, 86	0.020
40 IU PMSG 80% feed vs 40 IU PMSG 60% feed	0.018	1, 86	0.892
80 IU PMSG *ad lib* feed vs 80 IU PMSG 80% feed	7.306	1, 86	0.008
80 IU PMSG *ad lib* feed vs 80 IU PMSG 60% feed	9.631	1, 86	0.003
80 IU PMSG 80% feed vs 80 IU PMSG 60% feed	0.223	1, 86	0.638

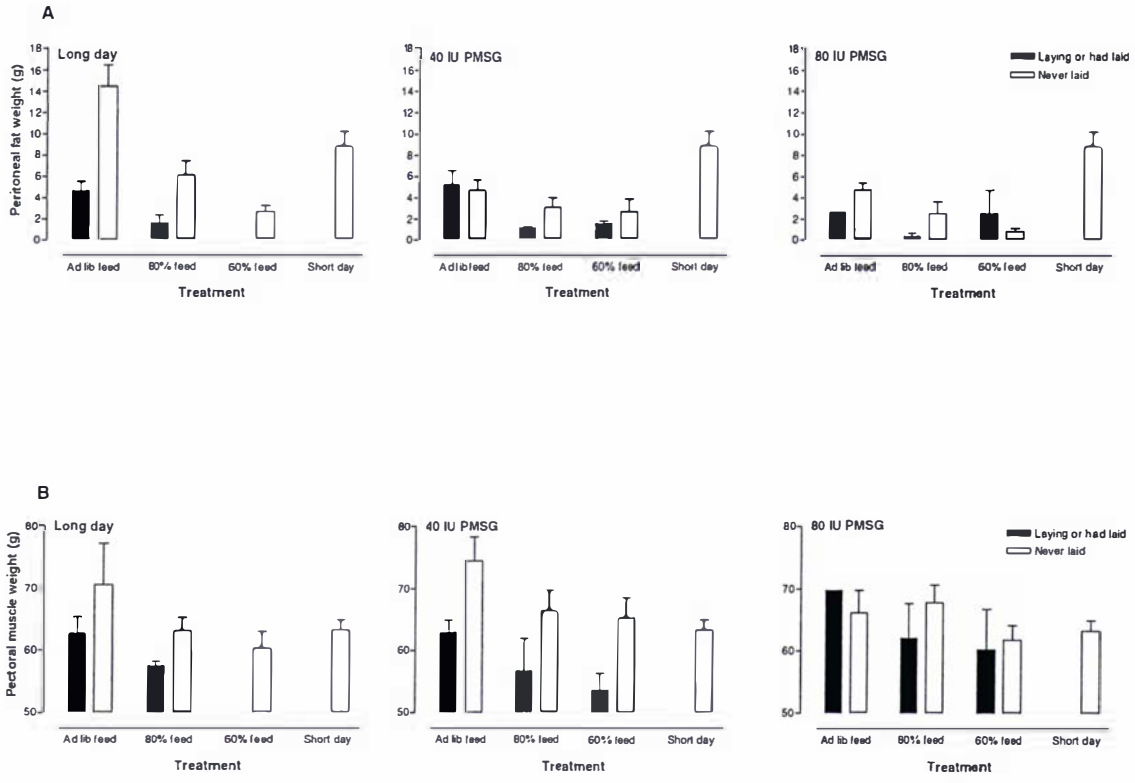

Figure 5.8. Mean peritoneal fat weight (A) and pectoral muscle weight (B) of female Japanese quail, divided into those that had laid and those that had not laid after three weeks on short days, long days receiving *ad lib*, 80% or 60% normal daily feed intake, or short days receiving *ad lib*, 80% or 60% normal daily feed intake plus treatment with 40 or 80 IU PMSG every two days.

There were no significant differences in mean peritoneal fat weights between birds that had laid and those that had not laid in the long day groups or either of the PMSG treated groups. (Figure 5.8, see Table 5.9 for statistics).

Table 5.9. Summary of t-tests comparing peritoneal fat weight and pectoral muscle weight in birds that laid with those that did not lay.

	Peritoneal fat weight			Pectoral muscle weight		
Group	**t**	**df**	***p***	**t**	**df**	***p***
All long day	1.320	27	0.198	0.665	27	0.512
All 40 IU PMSG	0.548	27	0.588	3.387	27	0.002
All 80 IU PMSG	0.985	27	0.334	0.499	27	0.622

5.3.5 Pectoral muscle weight

The mean pectoral muscle weights did not differ significantly between groups at the end of the experiment ($F_{9, 86}$ =1.406, p=0.198; Figure 5.7), although linear contrasts showed that *ad lib* fed groups had significantly larger mean pectoral muscle weights than groups given 60% feed ($F_{1, 86}$ =10.499, p=0.002).

There were no significant differences in mean pectoral muscle weights between birds that laid and those that had not laid in groups held on long days or treated with 80 IU PMSG (Figure 5.8, see Table 5.9 for statistics). In groups treated with 40 IU PMSG, there was a significant difference in pectoral muscle weight between birds that had laid and those that had not laid.

5.3.6 Ovulation and oviposition

A total of 86 shelled eggs were either laid during the experiments or removed from the oviduct at the time of dissection (Tables 5.10 and 5.11). Three oocytes were also found in the peritoneal cavity of birds at the time of dissection. PMSG treated birds produced 33 shelled eggs, with almost five times more eggs from birds treated with 40 IU than with 80 IU. There was a marked effect of feed intake on egg production in birds held on long days. Birds receiving 80% feed produced half as many eggs as *ad lib* fed birds, with birds receiving 60% feed not laying at all on long days. Long day birds laid twice as many eggs as birds treated with 40 IU PMSG.

Eggs laid by birds held on long days were within the normal size and weight range for the strain of Japanese quail. All eggs were fully shelled and pigmented. Eggs laid by PMSG treated birds varied considerably in quality. Several of the eggs were undersized and incompletely shelled or pigmented. The birds that started laying in response to a long day photoperiod laid an egg every day in sequences of about six to ten eggs. This was not the case for PMSG treated birds in which eggs were laid sporadically, with no identifiable pattern in oviposition.

Table 5.10. Egg laying record for individual birds on long days (LD) or receiving 40 IU or 80 IU PMSG every two days.

		Day of treatment														
Treat-ment	**Feed**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**Ovid***	**Total**
LD	*ad lib*				1	1	1	1	1	1	1	1	1	1	1	11
LD	*ad lib*						1	1	1	1	1	1		1	1	8
LD	*ad lib*							1	1	1	1	1	1	1	1	8
LD	*ad lib*								2	1	1	1	1	1	1	8
LD	80%					1	1	1		1	1	1	1	1	1	9
LD	80%						1	1	1	1	1	1	1	1	1	9
40 IU	*ad lib*			1		2		1								4
40 IU	*ad lib*							2		1						3
40 IU	*ad lib*									1		1		1	2	5
40 IU	*ad lib*									1	2					3
40 IU	80%														1	1
40 IU	80%														1	1
40 IU	60%	1			1	1				1						4
40 IU	60%							1		1						2
40 IU	60%													1	1	2
40 IU	60%														1	1
40 IU	60%														1	1
80 IU	*ad lib*											1				1
80 IU	80%			1												1
80 IU	80%									1		1			1	3
80 IU	80%														1	1
80 IU	60%						1		1							2
80 IU	60%								1					1		2
80 IU	60%													1		1

*Egg in oviduct on dissection.

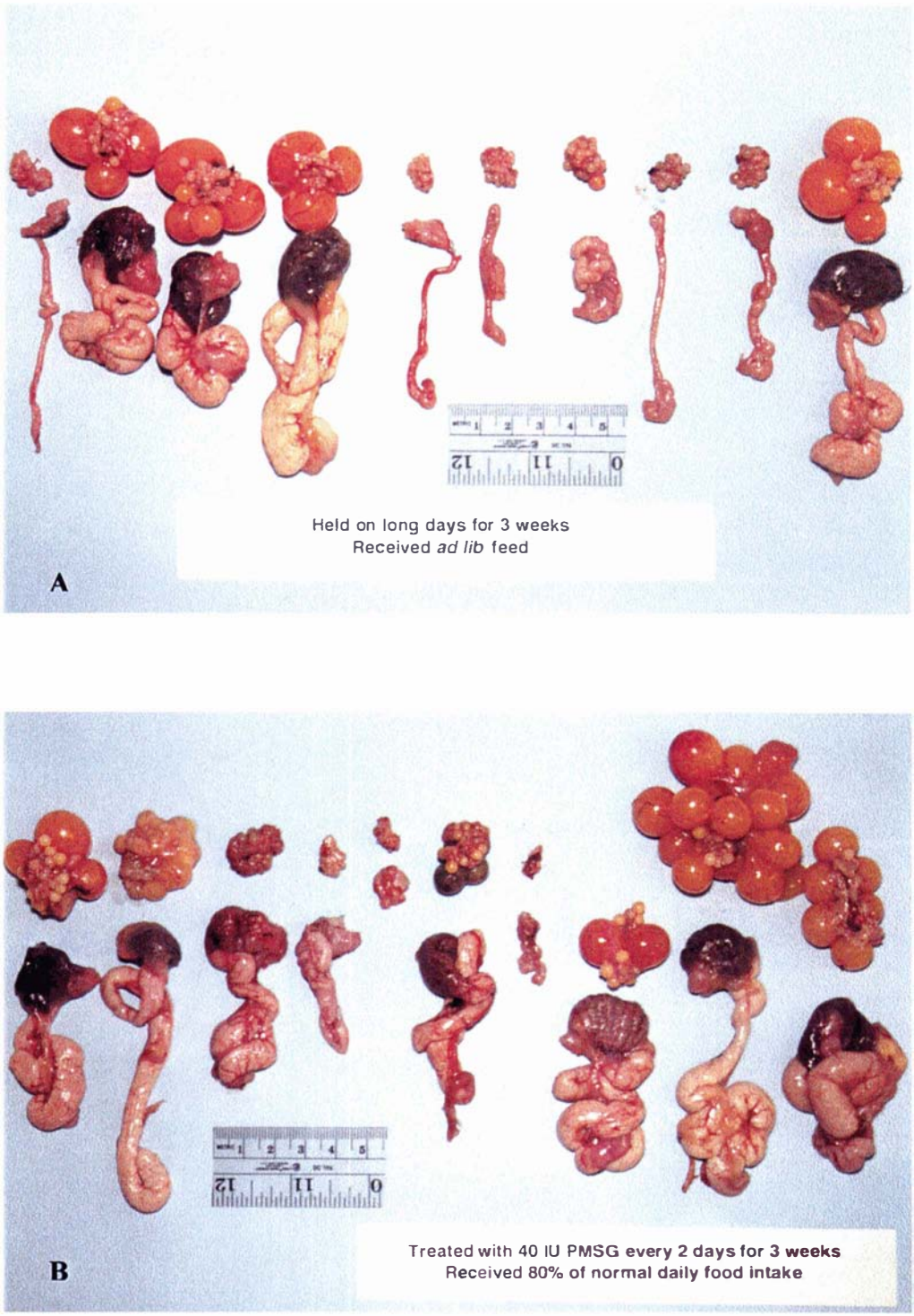

Figure 5.9. Variation in ovarian and oviductal development in Japanese quail held on a long day photoperiod receiving *ad lib* feed (A) and treated with 40 IU PMSG every two days for three weeks while receiving 80% of normal daily feed intake (B).

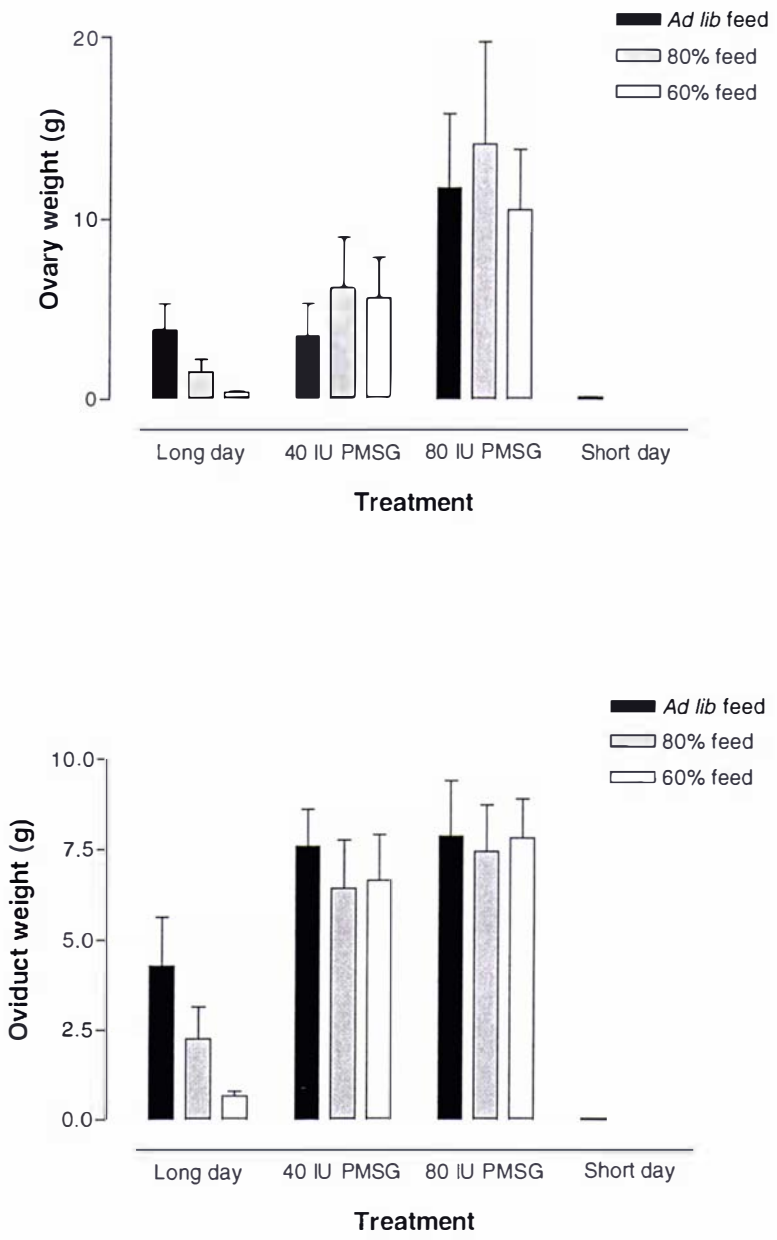

Figure 5.10. Mean ovary weight and oviduct weight of Japanese quail after three weeks on short days, long days receiving *ad lib*, 80% or 60% normal daily feed intake, or short days receiving *ad lib*, 80% or 60% normal daily feed intake plus treatment with 40 or 80 IU PMSG every two days.

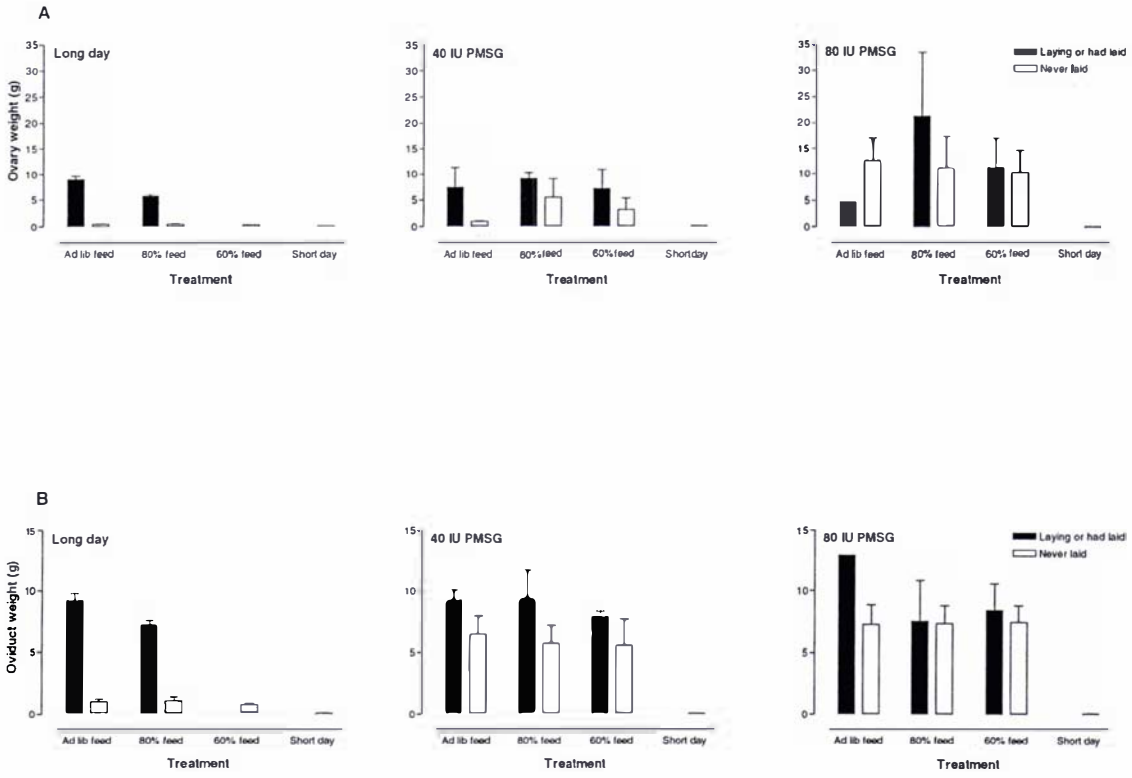

Figure 5.11. Mean ovary weight (A) and oviduct weight (B) of female Japanese quail, divided into those that had laid and those that had not laid after three weeks on short days, long days receiving *ad lib*, 80% or 60% normal daily feed intake, or short days receiving *ad lib*, 80% or 60% normal daily feed intake plus treatment with 40 or 80 IU PMSG every two days.

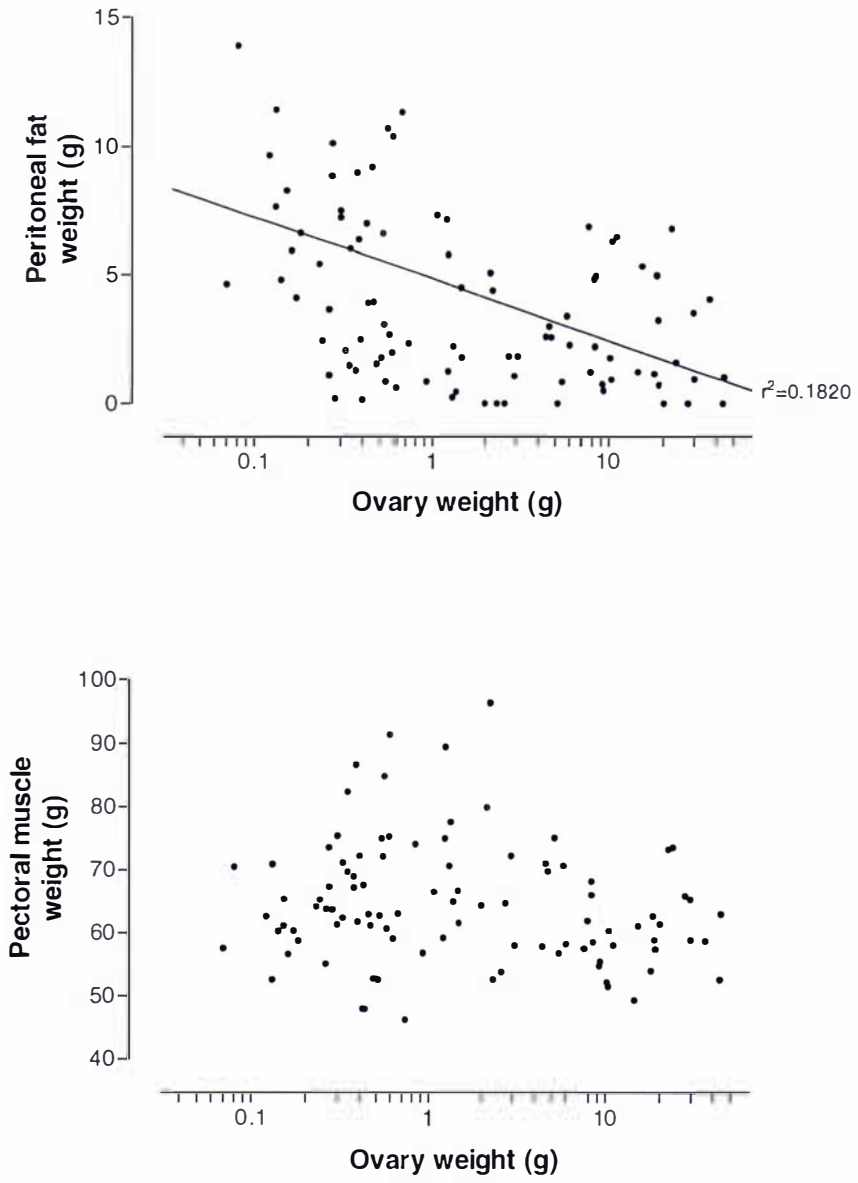

Figure 5.12. Relationships between ovary weight and peritoneal fat and pectoral muscle weight in Japanese quail after three weeks on short days, long days receiving *ad lib*, 80% or 60% normal daily feed intake, or short days receiving *ad lib*, 80% or 60% normal daily feed intake plus treatment with 40 or 80 IU PMSG every two days.

Table 5.11. Total number of eggs laid by birds on long days (LD) or receiving 40 IU or 80 IU PMSG every two days.

Treatment	Feed			Total
	Ad lib	80%	60%	
Long day	35	18	0	53
40 IU PMSG	15	2	10	27
80 IU PMSG	1	5	0	6

5.3.7 Ovarian development

Treatment with 40 or 80 IU PMSG stimulated ovarian development in Japanese quail but, as in previous experiments, there was considerable variation in ovarian growth between birds within the same group (Figure 5.9), and overstimulation of follicular development was evident in many birds. There was no effect of restricted feed on ovarian weight in PMSG treated birds (Figure 5.10, see Table 5.12 for statistics). Within the groups held on long days, birds receiving *ad lib* feed had significantly larger ovaries than those given 60% of normal daily feed intake. There was a significant difference in mean ovary weights between birds treated with 40 or 80 IU PMSG and those transferred to long days. The mean ovary weights of all PMSG treated groups were not significantly different to long day controls given *ad lib* feed. There were significant differences in mean ovary weight between birds that had laid and those that had not laid in long day groups (Figure 5.11, see Table 5.13 for statistics). This difference was not evident in either of the PMSG treated groups.

Peritoneal fat weight was inversely related to ovary weight (r^2=0.1820, p<0.0001; Figure 5.12) but there was no relationship between pectoral muscle weight and ovary weight (p=0.117).

Table 5.12. Summary of Kruskal-Wallis nonparametric ANOVA and Mann-Whitney U tests comparing ovary weight and oviduct weight between groups at the end of the experiment.

	Ovary weight			Oviduct weight		
Kruskal-Wallis	**K-W**	**df**	***p***	**K-W**	**df**	***p***
All groups	58.176	9	0.000	68.402	9	0.000
Mann-Whitney U tests	**F**	**df**	***p***	**F**	**df**	***p***
All *Ad lib* feed vs All 80% feed	416.5	1	0.620	355.5	1	0.162
All *Ad lib* feed vs All 60% feed	351.5	1	0.392	293.0	1	0.073
All 80% feed vs All 60% feed	378.5	1	0.672	385.5	1	0.731
All Long day vs All 40 IU PMSG	217.0	1	0.020	170.5	1	0.000
All Long day vs All 80 IU PMSG	193.0	1	0.000	103.5	1	0.000
All 40 IU PMSG vs All 80 IU PMSG	287.5	1	0.039	345.5	1	0.243
Long day *ad lib* feed vs Short day	0.0	1	0.000	0.0	1	0.000
Long day *ad lib* feed vs 40 IU PMSG *ad lib* feed	45.0	1	0.705	25.0	1	0.059
Long day *ad lib* feed vs 40 IU PMSG 80% feed	50.0	1	1.000	41.0	1	0.496
Long day *ad lib* feed vs 40 IU PMSG 60% feed	38.5	1	0.595	34.5	1	0.391
Long day *ad lib* feed vs 80 IU PMSG *ad lib*	37.0	1	0.326	22.0	1	0.034
Long day *ad lib* feed vs 80 IU PMSG 80% feed	34.5	1	0.241	25.0	1	0.059
Long day *ad lib* feed vs 80 IU PMSG 60% feed	23.0	1	0.072	20.0	1	0.041
Long day *ad lib* feed vs Long day 80% feed	68.5	1	0.162	71.0	1	0.112
Long day *ad lib* feed vs Long day 60% feed	76.0	1	0.011	73.0	1	0.022
Long day 80% feed vs Long day 60% feed	63.0	1	0.142	58.0	1	0.288
40 IU PMSG *ad lib* feed vs 40 IU PMSG 80% feed	42.0	1	0.545	62.0	1	0.364
40 IU PMSG *ad lib* feed vs 40 IU PMSG 60% feed	48.0	1	0.806	56.0	1	0.369
40 IU PMSG 80% feed vs 40 IU PMSG 60% feed	44.0	1	0.935	42.0	1	0.806
80 IU PMSG *ad lib* feed vs 80 IU PMSG 80% feed	45.0	1	0.705	58.0	1	0.545
80 IU PMSG *ad lib* feed vs 80 IU PMSG 60% feed	44.0	1	0.935	53.0	1	0.514
80 IU PMSG 80% feed vs 80 IU PMSG 60% feed	43.0	1	0.870	47.0	1	0.870

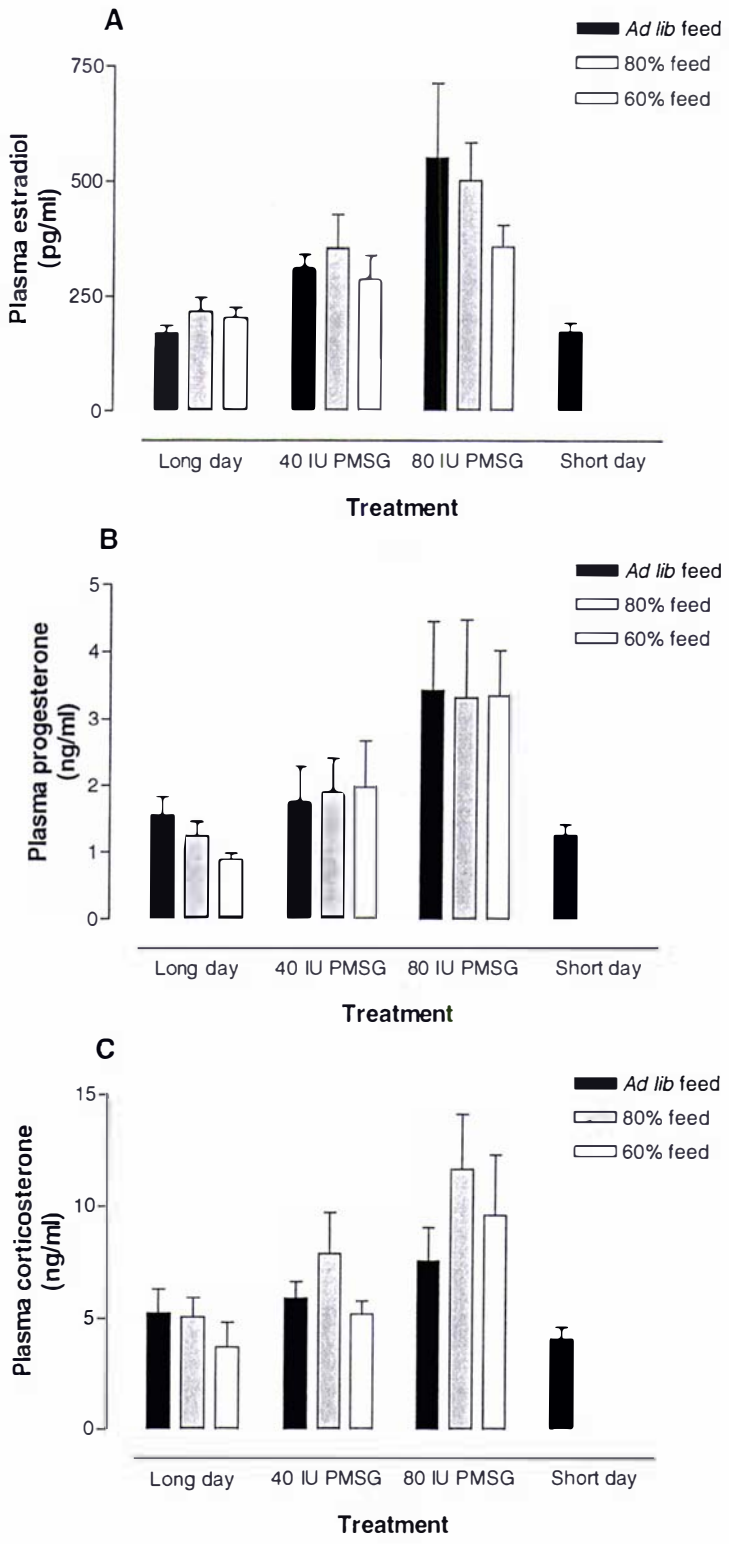

Figure 5.13. Mean plasma estradiol (A), plasma progesterone (B) and plasma corticosterone (C) concentrations in Japanese quail after three weeks on short days, long days receiving *ad lib*, 80% or 60% normal daily feed intake, or short days receiving *ad lib*, 80% or 60% normal daily feed intake plus treatment with 40 or 80 IU PMSG every two days.

Table 5.13. Summary of t-tests comparing ovary weight and oviduct weight in birds that laid with those that did not lay.

Group	Ovary weight			Oviduct weight		
	t	df	*p*	t	df	*p*
All long day	-18.419	27	0.000	-19.439	27	0.000
All 40 IU PMSG	-1.736	27	0.094	-2.156	27	0.040
All 80 IU PMSG	-0.313	27	0.757	-0.695	27	0.493

5.3.8 Oviduct weight

Oviductal development was stimulated in most birds treated with PMSG (Figure 5.10). As with ovarian development, there was no effect of restricted feed on oviduct weight in PMSG treated birds (see Table 5.12 for statistics). Within the groups held on long days, birds receiving *ad lib* feed had significantly larger oviducts than those given 60% of normal daily feed intake. There was a significant difference in mean oviduct weights between birds treated with PMSG and those transferred to long days, but no significant difference in mean oviduct weight between the two PMSG doses. The mean oviduct weights of PMSG treated groups were not significantly different to long day controls given *ad lib* feed, except for the groups treated with 80 IU PMSG receiving *ad lib* feed or 60% feed. There were significant differences in mean oviduct weight between birds that had laid and those that had not laid in long day groups and groups treated with 40 IU PMSG, but not within groups treated with 80 IU PMSG (Figure 5.11, see Table 5.13 for statistics).

5.3.9 Plasma estradiol, progesterone and corticosterone

Mean plasma estradiol concentrations varied significantly between groups (Figure 5.13 A, see Table 5.14 for statistics). There was no effect of restricted feeding on plasma estradiol

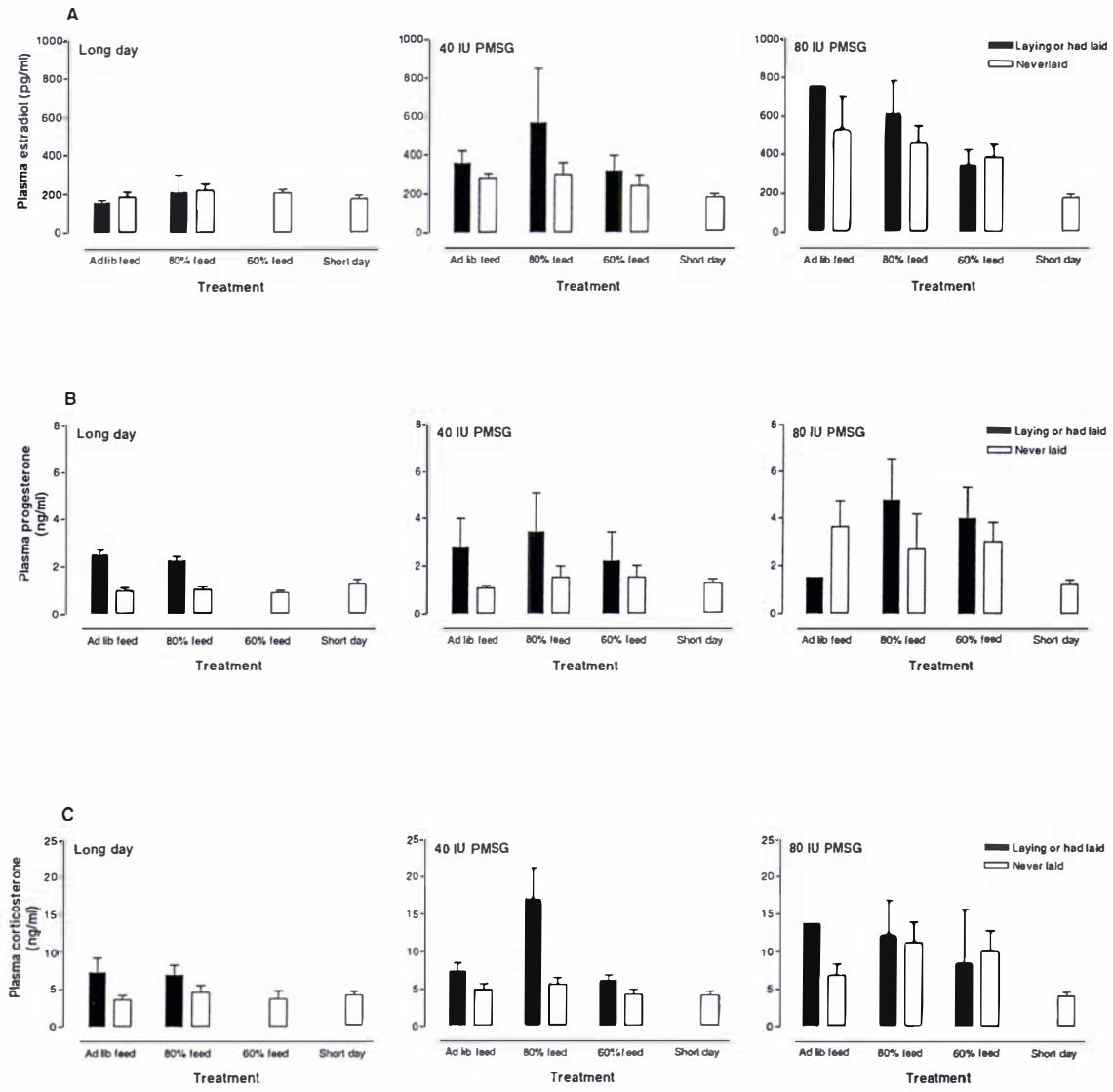

Figure 5.14. Mean plasma estradiol (A), plasma progesterone (B) and plasma corticosterone (C) concentrations in female Japanese quail, divided into those that had laid and those that had not laid after three weeks on short days, long days receiving *ad lib*, 80% or 60% normal daily feed intake, or short days receiving *ad lib*, 80% or 60% normal daily feed intake plus treatment with 40 or 80 IU PMSG every two days.

concentrations, although mean plasma estradiol concentrations of long day control birds were significantly different from those of each of the PMSG treatments. Mean plasma estradiol concentrations of each of the PMSG treated groups were significantly higher than long day birds receiving *ad lib* feed, except for the group receiving 40 IU PMSG and 60% normal daily feed intake. Mean plasma estradiol concentrations of long day and short day controls were not significantly different.

There were no significant differences in plasma progesterone concentrations between groups (Figure 5.13 B, Kruskal-Wallis test statistic = 18.237, p=0.051). However, Mann-Whitney U tests contrasts showed that mean plasma progesterone concentrations of birds treated with 80 IU PMSG differed significantly from long day birds (Mann-Whitney U test statistic = 232.0, p = 0.003) and birds treated with 40 IU PMSG (Mann-Whitney U test statistic = 293.0, p = 0.047).

Ad lib fed birds had similar mean plasma corticosterone levels to birds fed 80% or 60% feed (Figure 5.13 C, see Table 5.14 for statistics). There was a significant difference in plasma corticosterone concentration between each of the two feed restriction groups overall, although this difference was not evident within the long day, 40 IU or 80 IU PMSG groups when analysed separately. Groups given 80 IU PMSG every two days had significantly higher plasma corticosterone concentrations than birds held on long days. Only the group given 80 IU PMSG and 80% feed had significantly higher plasma corticosterone levels than long day birds given *ad lib* feed.

There were no significant differences in mean plasma estradiol concentrations between birds that had laid and those that had not laid in long day groups or PMSG treated groups (Figure 5.14 A, see Table 5.15 for statistics). There were significant differences in mean plasma progesterone and plasma corticosterone concentrations between birds that had laid and those that had not laid in long day groups and groups treated with 40 IU PMSG (Figure 5.14 B and C). This difference was not evident in groups treated with 80 IU PMSG.

Table 5.14. Summary of one-way ANOVA and linear contrasts comparing plasma estradiol and corticosterone concentrations between groups at the end of the experiment.

	Estradiol			Corticosterone		
Kruskal-Wallis	**F**	**df**	***p***	**F**	**df**	***p***
All groups	4.465	9, 86	0.000	2.229	9, 83	0.028
Mann-Whitney U tests	**F**	**df**	***p***	**F**	**df**	***p***
All ad lib feed vs All 80% feed	0.193	1, 86	0.661	1.643	1, 83	0.203
All ad lib feed vs All 60% feed	0.429	1, 86	0.514	0.627	1, 83	0.431
All 80% feed vs All 60% feed	1.173	1, 86	0.281	4.177	1, 83	0.044
All Long day vs All 40 IU PMSG	9.507	1, 86	0.003	3.558	1, 83	0.063
All Long day vs All 80 IU PMSG	31.786	1, 86	0.000	12.219	1, 83	0.001
All 40 IU PMSG vs All 80 IU PMSG	6.526	1, 86	0.012	2.754	1, 83	0.101
Long day *ad lib* feed vs Short day	0.006	1, 86	0.938	0.356	1, 83	0.553
Long day *ad lib* feed vs 40 IU PMSG *ad lib* feed	7.046	1, 86	0.009	0.353	1, 83	0.554
Long day *ad lib* feed vs 40 IU PMSG 80% feed	6.754	1, 86	0.011	1.183	1, 83	0.280
Long day *ad lib* feed vs 40 IU PMSG 60% feed	2.631	1, 86	0.108	0.042	1, 83	0.839
Long day *ad lib* feed vs 80 IU PMSG *ad lib*	16.724	1, 86	0.000	0.803	1, 83	0.373
Long day *ad lib* feed vs 80 IU PMSG 80% feed	17.206	1, 86	0.000	5.992	1, 83	0.016
Long day *ad lib* feed vs 80 IU PMSG 60% feed	9.630	1, 86	0.003	1.636	1, 83	0.204
Long day *ad lib* feed vs Long day 80% feed	0.575	1, 86	0.450	0.002	1, 83	0.969
Long day *ad lib* feed vs Long day 60% feed	0.495	1, 86	0.483	1.902	1, 83	0.172
Long day 80% feed vs Long day 60% feed	0.001	1, 86	0.973	2.114	1, 83	0.150
40 IU PMSG *ad lib* feed vs 40 IU PMSG 80% feed	0.003	1, 86	0.956	0.257	1, 83	0.613
40 IU PMSG *ad lib* feed vs 40 IU PMSG 60% feed	0.925	1, 86	0.339	0.148	1, 83	0.701
40 IU PMSG 80% feed vs 40 IU PMSG 60% feed	0.824	1, 86	0.366	0.771	1, 83	0.382
80 IU PMSG *ad lib* feed vs 80 IU PMSG 80% feed	0.003	1, 86	0.953	2.688	1, 83	0.105
80 IU PMSG *ad lib* feed vs 80 IU PMSG 60% feed	0.770	1, 86	0.383	0.173	1, 83	0.679
80 IU PMSG 80% feed vs 80 IU PMSG 60% feed	0.873	1, 86	0.353	1.457	1, 83	0.231

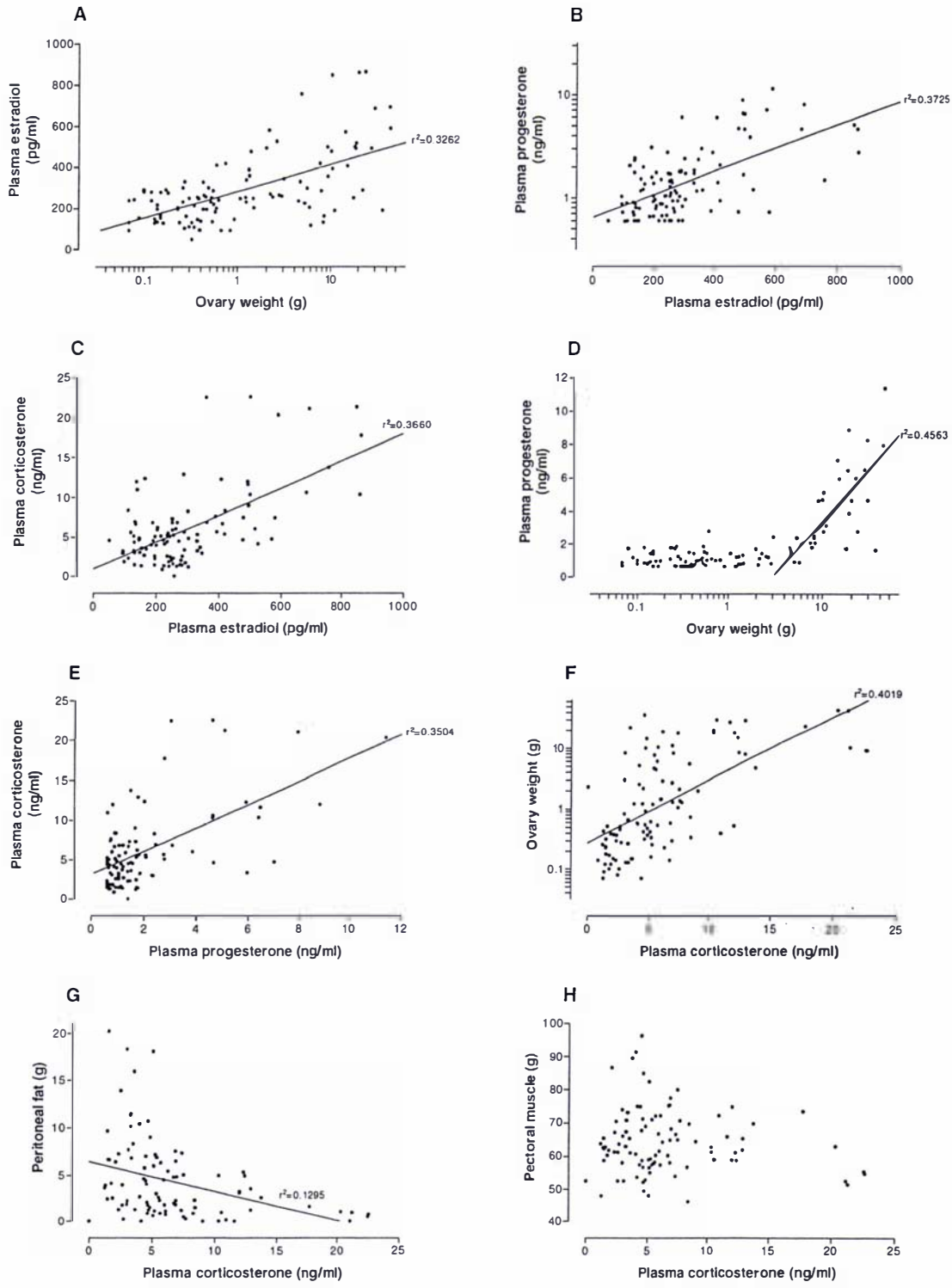

Figure 5.15. Relationships between plasma estradiol (A, B and C), plasma progesterone (D and E) or plasma corticosterone (F, G and H) concentrations and variables such as ovary weight, peritoneal fat and pectoral muscle weight in Japanese quail after three weeks on short days, long days receiving *ad lib*, 80% or 60% normal daily feed intake, or short days receiving *ad lib*, 80% or 60% normal daily feed intake plus treatment with 40 or 80 IU PMSG every two days.

Table 5.15. Summary of t-tests comparing plasma estradiol, plasma corticosterone and plasma progesterone concentrations in birds that laid with those that did not lay.

Group	Estradiol			Corticosterone			Progesterone		
	t	df	*p*	t	df	*p*	t	df	*p*
All long day	0.963	27	0.344	-2.448	26	0.021	-8.898	27	0.000
All 40 IU PMSG	-1.885	27	0.070	-2.905	27	0.007	-2.296	27	0.030
All 80 IU PMSG	-0.021	27	0.983	-0.685	25	0.499	-0.232	27	0.818

Plasma estradiol concentrations were correlated positively with ovary weight (log), plasma progesterone and plasma corticosterone (Figure 5.15 A, B and C, see Table 5.16 for statistics). There was also a linear relationship between plasma progesterone and ovary weight (ovary weight greater than 5 g; Figure 5.15 D) and a positive correlation between plasma progesterone and plasma corticosterone (Figure 5.15 E). There was a positive relationship between ovary weight and plasma corticosterone (Figure 5.15 F), a negative relationship between peritoneal fat and plasma corticosterone (Figure 5.15 G) and no relationship between plasma corticosterone and pectoral muscle weight (Figure 5.15 H).

Table 5.16. Summary of linear regressions comparing plasma estradiol, plasma progesterone and plasma corticosterone concentrations with other variables.

Correlation	r^2	Slope	*p*
A Plasma estradiol vs ovary weight (log)	0.3567	0.003 ± 0.001	<0.0001
B Plasma progesterone vs plasma estradiol	0.3725	0.001 ± 0.000	<0.0001
C Plasma corticosterone vs plasma estradiol	0.3660	0.017 ± 0.002	<0.0001
D Plasma progesterone vs ovary weight (log)	0.4563	0.298 ± 0.027	<0.0001
E Plasma corticosterone vs plasma progesterone	0.3181	8.548 ± 1.244	<0.0001
F Ovary weight vs plasma corticosterone	0.4019	3.866 ± 0.467	<0.0001
G Peritoneal fat weight vs plasma corticosterone	0.1295	-0.318 ± 0.085	0.0003
H Pectoral muscle weight vs plasma corticosterone	0.0153	-0.246 ± 0.204	0.2330

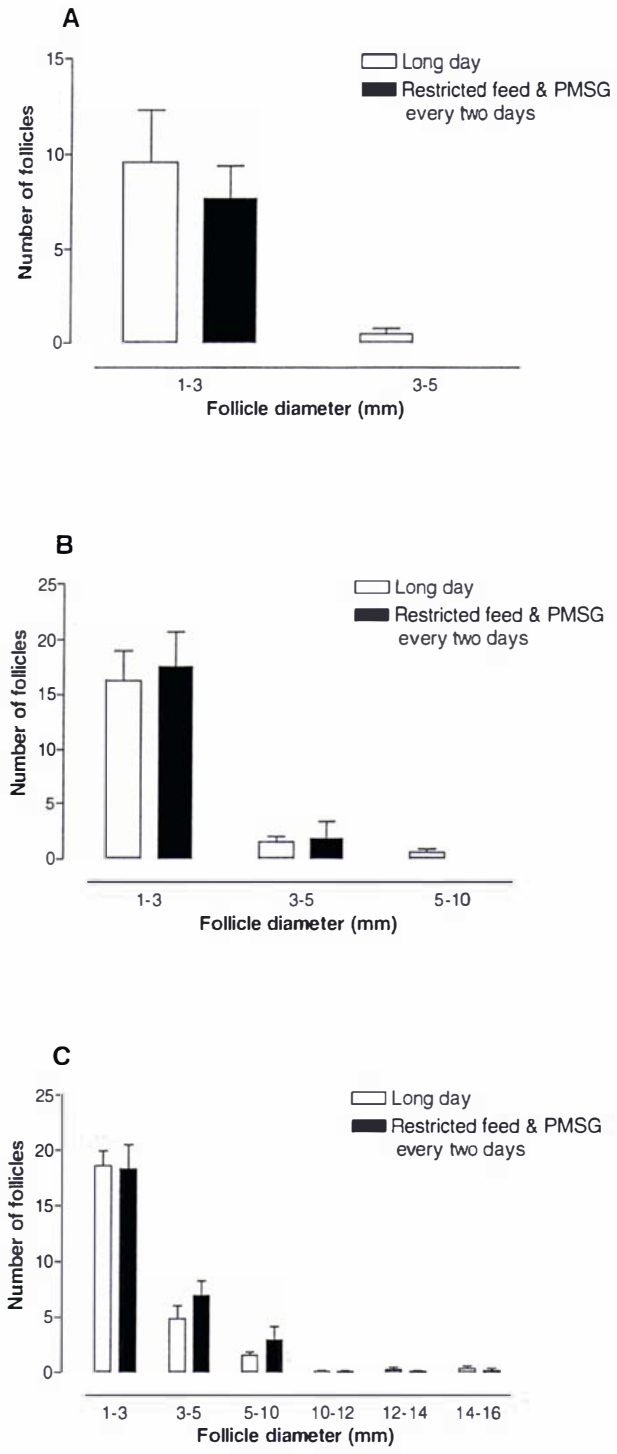

Figure 5.16. Distribution of follicle sizes in 0.16 – 0.50 g (A), 0.51 – 1.00 g (B) and 1.00 – 5.00 g (C) ovaries of female Japanese quail held on long days for three weeks, or given 20 IU or 40 IU PMSG injections every two days for three weeks, while receiving 80% or 60% of normal daily feed intake.

5.3.10 Distribution of follicle sizes

The numbers of follicles in each size class for ovaries weighing less than 5.00 g were similar in long day control birds and birds that received PMSG treatment (Figure 5.16, see Table 5.17). However, follicles measuring 3-5 mm were present in ovaries weighing 0.16 – 0.50 g from long day birds, but were not present in PMSG treated birds. Also, in ovaries weighing 0.51 – 1.00 g there where some follicles measuring 5 – 10 mm in birds held on long days, but these were not present in the ovaries of any PMSG treated birds.

Table 5.17. Summary of two sample Student's t-tests comparing the numbers of follicles in ovarian size classes 0.16-0.50 g, 0.51–1.00 g and 1.01–5.00 g between birds held on long days and birds treated with PMSG while receiving 80% or 60% of normal daily feed intake. Tests could not be performed when one or both groups had no follicles in a particular size class (indicated by "No test").

Ovary size (g)	Follicle diameter (mm)	t	df	*p*
0.16 – 0.50	1 – 3	0.510	14	0.618
0.16 – 0.50	3 – 5		No test	
0.51 – 1.00	1 – 3	-0.325	11	0.751
0.51 – 1.00	3 – 5	-0.224	11	0.827
0.51 – 1.00	5 – 10		No test	
1.01 – 5.00	1 – 3	0.108	15	0.916
1.01 – 5.00	3 – 5	-1.189	15	0.253
1.01 – 5.00	5 – 10	-1.075	15	0.299
1.01 – 5.00	10 – 12	0.083	15	0.935
1.01 – 5.00	12 – 14	0.716	15	0.485
1.01 – 5.00	14 – 16	0.523	15	0.609

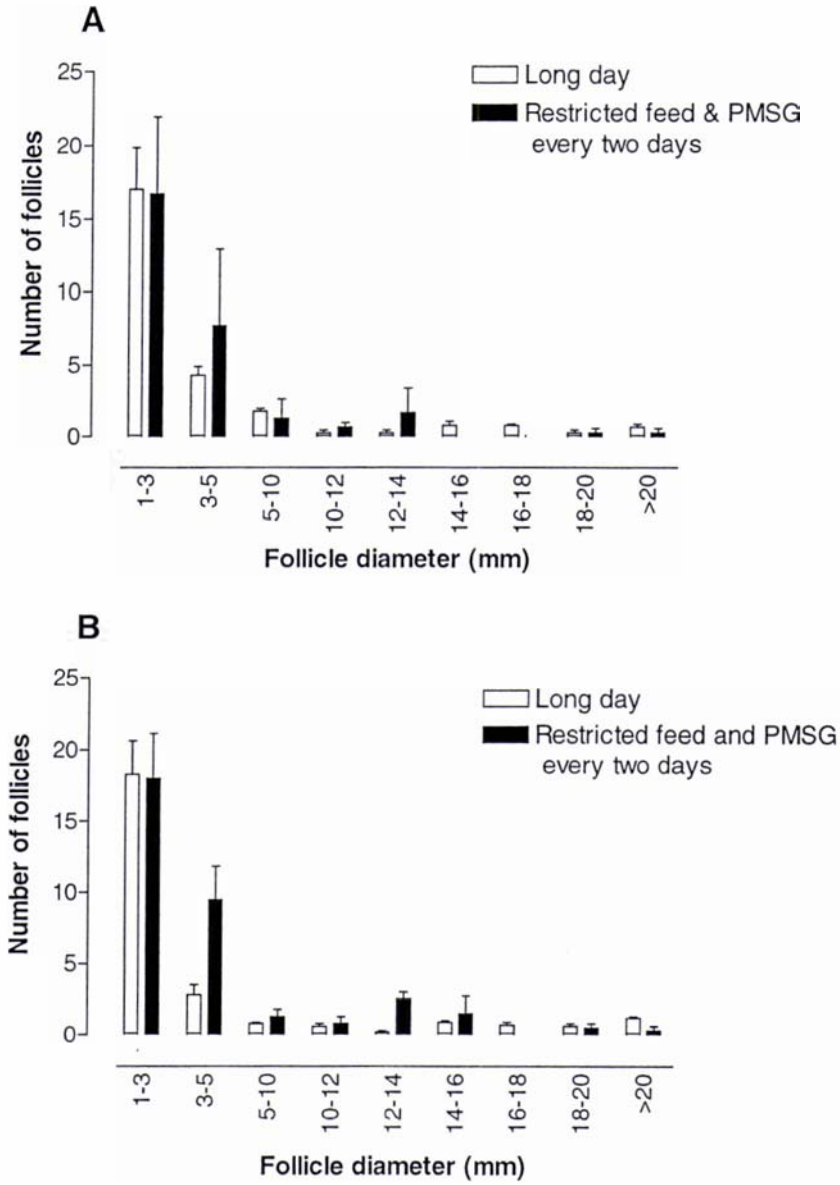

Figure 5.17. Distribution of follicle sizes in 5.01 – 9.00 g (A) and 9.01– 13.00 g (B) ovaries of female Japanese quail held on long days for three weeks, or given 20 IU or 40 IU PMSG injections every two days for three weeks, while receiving 80% or 60% of normal daily feed intake.

In ovaries weighing 5.01 - 9.00 g there were similar numbers of follicles in long day control birds and PMSG treated birds for each follicle size class except for follicles 14-18 mm in diameter which were not present in PMSG treated birds (Figure 5.17, see Table 5.18 for statistics). There were similar numbers of follicles in long day birds and PMSG treated birds in some size classes for ovaries that weighed 9.01 - 13.00 g, with differences for follicles 3-5 mm, 12-14 mm, 16-18 mm and >20 mm in diameter.

Table 5.18. Summary of two sample Student's t-tests comparing the numbers of follicles in ovarian size classes 5.01–9.00 g and 9.01 – 13.00 g between birds held on long days and birds treated with PMSG while receiving 80% or 60% of normal daily feed intake. Tests could not be performed when one or both groups had no follicles in a particular size class.

Ovary size (g)	Follicle diameter (mm)	t	df	*p*
5.01 – 9.00	1 – 3	0.058	11	0.955
5.01 – 9.00	3 – 5	-1.220	11	0.247
5.01 – 9.00	5 – 10	0.622	11	0.546
5.01 – 9.00	10 – 12	-1.111	11	0.290
5.01 – 9.00	12 – 14	-1.589	11	0.140
5.01 – 9.00	14 – 16		No test	
5.01 – 9.00	16 – 18		No test	
5.01 – 9.00	18 – 20	-0.101	11	0.921
5.01 – 9.00	> 20	0.846	11	0.415
9.01 – 13.00	1 – 3	0.072	12	0.944
9.01 – 13.00	3 – 5	-3.873	12	0.002
9.01 – 13.00	5 – 10	-1.263	12	0.230
9.01 – 13.00	10 – 12	-0.387	12	0.706
9.01 – 13.00	12 – 14	-6.279	12	0.000
9.01 – 13.00	14 – 16	-0.830	12	0.423
9.01 – 13.00	16 – 18		No test	
9.01 – 13.00	18 – 20	0.318	12	0.756
9.01 – 13.00	> 20	0.629	12	0.003

5.4 Discussion

Restricting the feed intake of female Japanese quail had a significant effect on body weight, peritoneal fat weight and pectoral muscle weight in long day controls and PMSG treated birds. Leeson *et al.* (1992) also found a significant correlation between degree of food restriction and body weight, pectoral muscle weight, and weight of the abdominal fat pad in male broiler chickens. In this experiment, there was no relationship between level of feed restriction and ovary weight or egg production in PMSG treated birds, even though peritoneal fat weight was inversely related to ovary weight. There was a considerable effect of feed restriction on ovary and oviduct weight and egg laying in long day birds and this has not been shown previously in Japanese quail.

Jaap and Muir (1968) showed a strong negative correlation between body weight and reproductive performance in broiler chickens, and this is generally the case for broiler breeders that are given restricted feeding regimens. In broiler chickens there are significantly more large yellow follicles in the ovaries of hens selected for low body fat than in the ovaries of fat line hens. Hocking and Whitehead (1990) showed that restricted feed decreased the number of yellow follicles in the ovary of birds selected for low body fat, but had no effect on fat line birds. It has also been suggested that restricted feed may become less effective at controlling multiple ovulations as broiler breeders become larger as a consequence of selection for higher juvenile growth rates (Hocking and Robertson, 2000). The selection for traits in the Japanese quail used in this experiment was not particularly rigorous, and selection was primarily based on growth rate and body weight. Therefore, our quail are likely to be similar to fat line chickens, which may explain why restricting the feed intake did not affect follicular development in PMSG treated birds.

The period of time during which a restricted feeding regimen was given to quail in this experiment may have been insufficient to have an effect on ovarian development in PMSG treated birds. Feed restriction in broiler chickens is recommended from 2 weeks of age

during the rapid growth phase to counteract the effect of excessive food intake that may cause excessive fatness and impair egg production (Costa, 1981; Yu *et al.*, 1992b). Hocking *et al.* (1989) found that the best response to restricted feed came when birds were given restricted feed from four days of age up until the onset of lay, but suggested that feed intake should be increased in relation to the rise in egg production. The response to restricted feed in PMSG treated quail might be enhanced if birds were restricted fed from a younger age up until sexual maturity.

The number of follicles is each size class was generally similar between feed restricted birds treated with PMSG and long day *ad lib* fed controls. In previous experiments, there were often significant differences in the number of follicles in the smaller size classes between long day and PMSG treated birds, particularly in ovaries weighing less than 5 g. In this experiment, only the ovaries of PMSG treated birds weighing more than 9 g had significantly greater numbers of 3-5 mm follicles than long day controls. Hocking and Robertson (2000) found that restricted feed decreased the number of white follicles in chickens. Feed restriction may have had a similar effect on numbers of white follicles in PMSG treated quail.

There was no effect of restricted feeding regimen on plasma corticosterone concentrations in long day controls and PMSG treated birds, whereas complete food withdrawal increases corticosterone in chickens. Nir *et al.* (1975) reported a three fold increase in plasma corticosterone concentrations in chickens after one or three days of starvation. Freeman *et al.* (1980) showed that plasma corticosterone concentrations in chickens were significantly elevated as early as 4 hours after food withdrawal, and levels remained elevated through the 24 hour sampling period. Scott *et al.* (1983) showed the plasma corticosterone concentrations rise significantly after only two hours of feed and water deprivation in chickens. Treating birds with PMSG may have had a more marked effect on corticosterone synthesis than a restricted feeding regimen.

Plasma progesterone concentrations were significantly higher in birds treated with 80 IU PMSG than in birds treated with 40 IU PMSG or long day controls. This is likely to be due

to the fact that treatment with the higher dose of PMSG stimulated development of more large yellow follicles, many of which may be producing significant amounts of progesterone. Plasma progesterone concentrations did not differ significantly between the three feeding regimens, although in the long day control groups, *ad lib* feed birds had a higher mean plasma progesterone concentration than feed restricted birds and this is also likely to be due to progesterone synthesis by F1 follicles in laying birds. Production of progesterone by F2 to F5 follicles is suppressed by the paracrine action of androgens and estrogens (Johnson *et al.*, 1988; Yu *et al.*, 1992a). The control of progesterone release into general circulation and its interaction with LH is critical in the timing of ovulation (Yu *et al.*, 1992c). Yu *et al.*, (1992c) showed that in *ad lib* fed broilers there are often two follicles with the endocrine profile of a F1 follicle and both follilces produce large amounts of progesterone. The preovulatory surge of LH is therefore likely to induce ovulation of both of these follicles.

Decuypere *et al.* (1993) showed that plasma progesterone production by granulosa cells of hierarchical follicles was significantly higher in chickens selected for food conversion compared to birds selected for body weight. However, in response to LH and FSH, granulosa cells from F1, F2 and F3 follicles of *ad lib* fed chickens selected for body weight or feed conversion produced similar amounts of progesterone (Onagbesan *et al.*, 1999a). Feed restriction in both lines led to a strict hierarchical order in granulosa cell responsiveness to LH, with F1 follicles producing more progesterone than the other large yellow follicles (Onagbesan *et al.*, 1999a). FSH also decreased progesterone production by the large yellow follicles in restricted fed birds selected for body weight. In contrast, the largest ovarian follicles from restricted fed hens selected for feed conversion efficiency produced significantly more progesterone in response to FSH stimulation (Onagbesan *et al.* 1999a). The results from the current experiment suggest that feed restriction does not affect progesterone production by large yellow follicles in response to PMSG, as mean plasma progesterone concentrations were similar for *ad lib*, 80% and 60% feed groups. However, progesterone production by individual follicles was not measured, and it may be that feed restriction did in fact lead to a decrease in progesterone production in response to

PMSG. A restricted feeding regimen changes the responsiveness of granulosa cells to LH and FSH in broiler breeders, but the mechanisms controlling this action are not clear.

In Chapter three, the idea that selection for growth rate may have favoured genes for growth factors or their receptors was discussed. Hocking *et al.* (1994) suggested that differences in egg production between *ad lib* and feed restricted broilers could be related to changes in the pattern of growth hormone (GH) or insulin-like growth factor-I (IGF-I) secretion. Growth hormone concentrations are higher in feed restricted birds during rearing than in *ad lib* fed chickens (Bruggeman *et al.*, 1997). There is also increased amplitude and frequency of GH pulses in feed restricted male broiler chickens (Buyse *et al.*, 2000) and turkeys (Anthony *et al.*, 1990). In contrast, chickens consuming only 50% of their normal daily protein requirement or fasting had plasma IGF-I concentrations which were approximately half those of birds receiving a normal or high protein diet (John Ballard *et al.*, 1990; Caperna *et al.*, 1999; Buyse *et al.*, 2000). Low IGF-I concentrations and high GH concentrations in feed restricted birds seems to be at odds with the precept that IGF-I production is stimulated by GH. Feed restricted chickens had lower numbers of GH receptors than *ad lib* fed birds (Bruggemann *et al.*, 1997), so it is therefore possible that GH receptor downregulation leads to reduced IGF-I production.

IGF-I significantly enhanced LH- or FSH-stimulated progesterone production by the granulosa cells in feed restricted chickens selected for rapid growth rate and birds selected for feed conversion efficiency (Onagbesan *et al.*, 1999a). There was no effect of IGF-I in conjunction with LH or FSH on responsiveness of granulosa cells in *ad lib* fed birds (Onagbesan *et al.*, 1999a). The lack of response in *ad lib* fed birds suggests that there is no interaction between gonadotropins and IGF-I under an *ad lib* feeding regimen. Granulosa proliferation in the presence of IGF-I is greater in *ad lib* fed birds than in restricted feed birds. Selection for growth combined with an *ad lib* feeding regimen appears to favour IGF-I actions, resulting in rapid follicular growth and breakdown of the follicular hierarchy (Onagbesan *et al.*, 1999b). Therefore, it is likely, that the mechanism by which feed restriction maintains hierarchical order involves the IGF system.

IGF is also likely to be involved in development and maintenance of the follicular hierarchy in non-broiler breeders. Treatment with PMSG often leads to breakdown of the follicular hierarchy in Japanese quail, and this provides a good opportunity to study the role of IGF in development of the hierarchy. Comparisons could be made between PMSG treated birds with excessive follicular development and normal laying birds. This could include using radioimmunoassay to determine IGF secretion, measuring the expression of IGF mRNA, and examining the numbers and affinity of IGF receptors within the granulosa and theca cells of the ovary.

The most notable result in this experiment was the large number of eggs laid by PMSG treated birds. A total of four eggs have been laid by PMSG treated quail in all of the previous experiments combined, less than 12% of the total number of eggs laid by PMSG treated birds in this experiment. It is difficult to attribute this to a restricted feeding regimen, as almost 50% of the eggs laid by PMSG treated quail were from *ad lib* feed birds. The only other difference between this and previous experiments was the age of the birds when PMSG treatment started. In previous experiments, PMSG treatment was started when the birds were six weeks of age. However, in this experiment birds were four to five weeks older at ten or eleven weeks of age. Williams and Sharp (1978) found that as birds age follicles in the follicular hierarchy mature at a slower rate leading to a decrease in the number of eggs in each sequence. There is also a decrease in the number of small follicles, and an increase in the number of small atretic follicles. FSH is believed to be responsible for follicular recruitment and growth, and small follicles have more binding sites for FSH than larger follicles (Masuda *et al.*, 1984). There may be some small changes in white follicle numbers or in growth rate of follicles from six to eleven weeks of age. This may have partly counteracted the excessive follicular development that occurs in response to PMSG in some birds, leading to an increase in the ovulation rate.

Although the number or eggs laid and the days on which eggs were laid were recorded, potentially useful information such as individual egg weights and egg shell thickness were not measured during the experiment. However, it was noted that the egg shell quality of many of the eggs laid by PMSG treated birds was very poor. Several eggs had very soft

shells, whilst others were incompletely pigmented or had abnormal colouration. Poor egg shell quality is usually due to inappropriate nutrient levels, particularly dietary calcium and phosphorus, in pre-laying and layer feeds (Roland, 1986). Eggs laid by birds on long days and 80% feed laid eggs that appeared normal, whereas unusual eggs were laid by birds treated with PMSG on *ad lib*, 80% and 60% feed. The poor shell quality in some eggs from PMSG treated birds is therefore unlikely to be due to restricted feeding. A layer dietary calcium level of 3.0% is optimum for maintaining optimal egg shell quality (shell weight, shell thickness, shape index and specific gravity) in Japanese quail (Philomina and Pillai, 2000). Egg shell quality was significantly poorer in pullets given a diet containing 2.5% calcium (Brahma and Ramakrishnan, 1989). The feed given to our quail contains 2.5% calcium, and although this seems to be sufficient for good quality shell development in ad lib feed birds, the decrease in calcium intake in feed restricted birds may lead to poor shell quality. In contrast to this idea, Elwinger (1982) stated that "feed restriction had no obvious effect on shell quality traits", and other researchers have shown that egg shell strength and thickness were improved in birds fasted for six hours of each day (Altan *et al.*, 2000) or five consecutive days in 10-20 weeks (Osuka and Nagata, 1998) compared to *ad lib* fed chickens.

The organic matrix of the egg shell is made up of highly sulfated glycoproteins. The synthesis of soluble precursors of these proteins occurs in the liver, and estrogens are involved in the control of this process (Wieser and Krampitz, 1987). Administration of antiestrogens such as tamoxifen results in the reduction of expression of shell protein-genes in the liver of laying hens. Administration of estrogen can trigger expression of shell protein-genes in juvenile birds in which little or no ovarian development has occurred. In contrast, in chickens with a high laying rate, exogenous estrogens cause the expression of shell protein-genes to cease (Wieser and Krampitz, 1987). The reasons for this are not clear, but birds with a high laying rate may have high estradiol concentrations, possibly leading to a negative feedback on endogenous estradiol production when birds are treated with exogenous estrogens. This, in turn, could lead to a cessation in expression of egg shell protein-genes. This may also be the case in PMSG treated birds, in which plasma estradiol concentrations were generally higher than long day controls. Unfortunately, because the

mechanisms controlling expression of shell protein-genes are not clear and there is no record of which birds laid poor quality eggs in this experiment, the information cannot be easily interpreted.

Hughes *et al.* (1986) showed that abnormal eggs were often produced in response to a disturbance or stress. Translocation of hens or exclusion from nest boxes resulted in a higher proportion of poor quality eggs being laid. Administration of 0.1, 0.25 or 1.0 mg of adrenaline resulted in retention of eggs currently in the hen's shell glands and in an increase proportion of eggs with abnormal egg shells being laid during the following 10 days. The nature of the abnormality was dependent on the stage of egg formation at which the disturbance occurred. Disturbances when eggs were only lightly calcified resulted in misshapen eggs, and when ovulation was imminent, chalky eggs were produced (Hughes *et al.*, 1986). Corticosterone is involved in the ovulation process (Etches, 1996) and in the current experiment, long day birds that were laying had higher plasma corticosterone concentrations that non-laying birds. However, many of the PMSG treated birds that laid eggs had higher plasma corticosterone concentrations than laying long day birds at the end of the experiment. The significant positive relationship between ovary weight and plasma corticosterone concentration suggests that the large number of yolky follicles in some ovaries could be a stressful physiological condition for the birds. This physiological stressor may have lead to production of poor quality eggs. Interestingly, the five PMSG treated birds that had not previously laid, but had an egg in the oviduct on dissection had the highest plasma corticosterone concentrations of all the birds in the same treatment group, suggesting that these eggs may have been retained in the oviduct due to high corticosterone concentrations.

There was considerable variation in ovarian response between quail receiving the same treatment regimen, as there has been in previous experiments. It would be useful to establish a relatively accurate way of predicting a birds response to PMSG treatment in future experiments. Plasma estradiol concentrations following a single injection of PMSG may correlate with the ovarian response to a longer period of treatment with PMSG. It has also been suggested that the avian ovarian response to exogenous gonadotropins may be

related to plasma estradiol, progesterone or corticosterone concentrations at hatch or during early growth (Ishii, pers. comm.). To test these ideas, blood samples could be taken at hatch or later, or following a single PMSG injection, and plasma estradiol, progesterone or corticosterone concentrations measured. Birds could then be treated with regular injections of PMSG for three weeks to stimulate ovarian development. Statistical analysis could then determine if ovarian weight or numbers of large yellow follicles correlate with plasma steroid concentrations from juvenile birds, or steroid concentrations produced in response to a single PMSG injection. If there was a significant correlation, in future experiments steroid concentrations could be measured prior to treatment and a PMSG treatment regimen designed for each individual bird based on the results.

Long day length (16L:8D) and warm temperatures (20°C) stimulated ovarian development, ovulation and oviposition in some of the *ad lib* fed birds. However, none of the birds on long days receiving 60% feed laid eggs during the experiment. This indicates that an adequate food supply is an important factor for the photostimulatory effect of long photoperiods to be expressed in Japanese quail. In future experiments, quail could be transferred to long days and given restricted feed to maintain the ovary in a regressed state, rather than being kept on short days at 10°C with *ad lib* feed. This would better mimic the situation that occurs each breeding season for free-living birds such as the kakapo. Kakapo are exposed to long daylengths every year, but breed only in the years when the availability of some food plants is greater than usual. The sensitivity of the ovary to gonadotropins may be modified by limited food availability as discussed previously. Treating Japanese quail held on long days receiving restricted feed with PMSG may lead to a different ovarian response than treating short day birds given *ad lib* feed. PMSG treatment may lead to development of a normal follicular hierarchy in restricted fed birds held on long days.

In summary, restricting the feed intake of quail does not appear to improve their response to PMSG treatment. However, numbers of small white follicles were reduced and egg production was increased compared to previous experiments in which birds were given *ad lib* feed. In addition, 60% feed restriction effectively stopped ovarian growth in quail transferred to long days. The use of 60% feed restriction is thus an interesting model in

which to investigate the effects of food restriction on photo-induced gonadal growth in quail. This model could also be used in future studies of PMSG treatment of quail.

6 General Discussion

Treatment with exogenous hormones has been used successfully in mammals to stimulate ovarian follicular development and induce ovulation, but until recently this was not the case for avian species. Exogenous reproductive hormones have been used in the studies of ovarian steroidogenesis, pituitary and hypothalamic function, and the mechanisms controlling the follicular hierarchy in birds. However, very few experiments have been designed specifically to stimulate ovarian development and increase reproductive output.

Treatment with exogenous mammalian gonadotropins can stimulate ovarian development and ovulation in chickens (Phillips, 1943; Das and Nalbandov 1961a; Imai 1972; Palmer and Bahr, 1992) and quail (Wakabayashi *et al.*, 1996). However, there is considerable variation in ovarian response between birds, with the ovaries of many birds showing excessive follicular development and breakdown of the follicular hierarchy (Phillips, 1943; Opel and Nalbandov, 1961a; Zadworny and Etches, 1988; Palmer and Bahr, 1992, Wakabayashi *et al.*, 1996). The development and maintenance of a follicular hierarchy in response to PMSG treatment has been reported once in chickens (Petitte and Etches, 1989), but this result could not be replicated in this series of experiments with quail.

Eggs were laid by PMSG treated quail, with up to 25% of treated birds producing eggs in the final experiment. However, eggs were variable in quality, with some being undersized, incompletely shelled or incompletely pigmented. The nutrients available in the quail feed pellets are sufficient for normal eggshell development, and poor quality eggs may be the result of a rise in plasma corticosterone concentrations in response to excessive follicular development.

Doses of 20-80 IU PMSG per day were the most appropriate for stimulating ovarian development in Japanese quail. Similar doses have been used in chickens to induce

ovarian growth (Petitte and Etches, 1989; Palmer and Bahr, 1992). Doses lower than 20 IU PMSG stimulated little or no ovarian development in most birds, and doses higher than 80 IU PMSG led to overstimulation of follicular development in most birds.

Continuous delivery of PMSG by osmotic pumps and daily treatment using injections were equally effective in stimulating ovarian development in Japanese quail. The use of daily injections is a more practical method of delivering PMSG to birds, as it does not involve surgery and allows more control over dosage and timing of treatment, although it does involve regular handling of the bird.

Treating birds with injections of PMSG every two days rather than daily led to a rate of ovarian growth similar to that of long day birds. Zadworny and Etches (1988) also treated turkeys with injections of PMSG every two days to stimulate ovarian development, although follicles were not arranged in a hierarchy. Treatment every three days with PMSG led to little ovarian growth, whilst treatment every four days was not sufficient to stimulate ovarian development in Japanese quail.

Treating avian species with gonadotropins to stimulate ovarian growth, ovulation and oviposition often leads to excessive follicular development (Imai, 1972; Imai *et al.*, 1972; Zadworny and Etches, 1988; Palmer and Bahr, 1992; Hocking and McCormack, 1995; Wakabayashi *et al.*, 1992, 1996). Similarly, current techniques used to stimulate reproduction in mammals using gonadotropins also lead to the growth and ovulation of several follicles at one time instead of ovulation of an individual follicle. Despite this, treatment with gonadotropins is used successfully to increase reproductive output in commercially important species such as cattle, sheep, pigs and horses (Alfuraiji *et al.*, 1993; González *et al.*, 1994b; Duanyai and Srikandakumar, 1998; Naqvi and Gulyani, 1998; Martinez *et al.*, 2000; Barros *et al.*, 2000; for review see Yavas and Walton, 2000) and has also been successful in non-domesticated species (Sawyer Steffan *et al.*, 1983; Karesh *et al.*, 1985; Waldham *et al.*, 1989; Howard *et al.*, 1996). If treatment with mammalian gonadotropins can lead to ovulation and egg-laying in avian species, resulting in the hatching of fertile young, then overcoming the problem of excessive follicular development may not be so important. Further research would be required to ensure that the ovary regresses normally after gonadotropin treatment has been stopped,

and that the bird can respond to future treatments or to normal stimulatory conditions. It would need to be shown that successive treatments did not compromise future reproduction in any way. Stimulating reproduction in birds using gonadotropins would only be useful in endangered species if it can be used in successive breeding seasons in as many individuals as possible, and if the percentage of birds which respond to treatment is high.

Although some PMSG treated birds laid eggs in this series of experiments, many birds developed large yellow follicles but did not lay any eggs. Birds were treated with hCG or chicken pituitary extract in the first experiments to stimulate ovulation, but this treatment was not continued in other experiments in order to concentrate on overcoming the problem of excessive follicular development in response to PMSG treatment. Although birds did lay eggs in response to PMSG treatment alone, the number of eggs produced by PMSG treated birds may be increased if an additional treatment was given to stimulate ovulation. Ultrasound proved to be a very useful tool for visualising ovarian follicles in Japanese quail to determine whether or not a follicle of F1 size was present, and could be used in future experiments.

The low number of eggs that were laid by PMSG treated Japanese quail in this series of experiments may be due, in part, to the variation in ovarian response to PMSG between birds. Numerous birds showed little or no ovarian follicular development in response to PMSG treatment. In order to maximize the number of birds that lay eggs in response to PMSG treatment, the variation in response that occurs between birds needs to be minimized. This may be accomplished by testing birds before treatment and then designing a specific treatment for each individual bird. There may be several ways in which birds could be tested in order to predict their responsiveness to PMSG treatment. It has been suggested that plasma progesterone, estradiol or corticosterone concentrations in juvenile birds may correlate with responsiveness to PMSG when the bird matures (Ishii, pers. comm.). It may also be possible to predict how a bird will respond to a particular dose of PMSG by measuring its estradiol output in response to a single injection of PMSG. These ideas would need to be tested with a number of experiments, but if proved to be a useful way of predicting a bird's response to PMSG treatment this would be a quick and simple way of determining appropriate treatment

regimens for each individual bird. For these tests to be useful, the repeatability of responsiveness would also need to be determined. There is also variation in response to normal stimulatory conditions in Japanese quail in that some quail will show ovarian growth after only two weeks under a long day photoperiod, whereas others may show no response after six weeks on long days. It has not yet been determined whether a bird that responds quickly to a stimulatory photoperiod will always respond quickly, or whether response time varies from one period of long daylength to the next. If the response to long days is similar in an individual bird each time it is exposed to a stimulatory photoperiod, it may be that response to PMSG is similar between different treatment periods. However, if there is variation in response to long days in an individual bird, it is likely that responsiveness to PMSG will also vary between treatment periods. If this is the case, it will be difficult to find a way of predicting the ovarian response to PMSG.

It is possible that other hormones are required in conjunction with PMSG treatment to stimulate normal ovarian development and ovulation in birds. Treatment with PMSG may need to be preceded, accompanied, or followed by treatment with either estradiol or progesterone in order to mimic the natural physiological state of a laying bird. Crude pituitary extracts used by other researchers have contained hormones other than FSH or LH. The avian pituitary preparation used by Mitchell (1970), and the glycoprotein extract used by Wakabayashi *et al.* (1992) also contained TSH. The extract of chicken anterior pituitaries prepared by Taber *et al.* (1958) contained both ACTH and TSH. These hormones may play a role in ovarian development, and the possibility of synergistic effect between these hormones and LH or FSH cannot be excluded.

Although restricting the feed intake of quail did not have an effect on the response to PMSG treatment, feeding birds only 60% of their normal daily feed intake prevented ovarian development in birds held on long days. As suggested in Chapter 5, this model could be used as an alternative to maintaining birds under a short day photoperiod to prevent development of the reproductive tract. This would be better mimic of the conditions that kakapo face each breeding season, and the sensitivity of the ovary to gonadotropins may be modified by limited food availability.

Although the PMSG treatment schedules used in the experiments conducted so far have not elucidated a suitable technique for stimulating egg production in endangered species, they do have considerable potential as a means of studying the factors controlling development of the follicular hierarchy. An ovarian follicular hierarchy is not established in Japanese quail treated with PMSG, so comparisons could be made between PMSG treated birds and normal laying birds. This could include examination of gonadotropin and steroid receptor numbers and affinity within numerous tissues of the hypothalamo-pituitary-gonadal axis. Other factors such as IGF, GH, tri-iodothyronine, thyroxine, ACTH, activin, inhibin, VIP and urokinase may be associated with development and maintenance of the avian follicular hierarchy. The production, secretion and receptor functioning of these hormones and metabolites could be compared in PMSG treated and laying Japanese quail. Growth factors are of particular interest as studies in broiler chickens, which show excessive follicular development when given *ad lib* feed, have suggested that IGF-I is involved in development and maintenance of the follicular hierarchy.

Once a suitable technique for stimulating ovarian development, ovulation and egg-laying has been established in Japanese quail, the technique would need to be tested in other bird species, particularly parrots, before it could be used in the kakapo. Other species that could be used for experiments include cockatiels and budgerigars, as these are readily available and relatively easy to maintain. Budgerigars are also more closely related to kakapo than other bird species and would therefore make an ideal model species for this research.

In order to establish a suitable technique for stimulating reproduction in birds, the practicalities of treating an endangered bird such as the kakapo using the current protocol must also be considered. Kakapo are currently free-living, but if they were to be treated with gonadotropins to stimulate ovarian development, they would need to be held in captivity during the treatment period. Regular handling would be required to give injections to the birds, measure the size of the cloacal opening and visualise the ovary using ultrasound. To ensure that any eggs that are produced are fertile, female kakapo would need to be artificially inseminated, as birds are unlikely to mate in a captive situation. Therefore a method of collecting and preserving semen from male

kakapo would also need to be established. Many of the kakapo are more than 30 years old and may not have the same reproductive capacity as younger birds. Ottinger (1992), Ottinger and Balthazart (1986) and Ottinger *et al.* (1983) have shown that there is an age-related decline in reproductive activity in Japanese quail. Therefore, the ovarian response to PMSG or other gonadotropins maybe different in old birds compared to young birds and the age of the birds must be considered when establishing a technique for stimulating reproduction using exogenous hormones.

7 Reference List

Adachi, T. and Ishii, S. (1977). Binding of rat follicle-stimulating hormone to the turtle ovary *in vitro* and inhibition of the binding by gonadotropin preparations of various vertebrates. *General and Comparative Endocrinology*, **33**:1-7.

Adashi, E. Y. and Roban, R. M. (1992). Intraovarian regulation: peptidergic signalling systems. *Trends in Endocrinology and Metabolism*, **3**:243-248.

Akazome, Y., Park, M. K., Mori, T. and Kawashima, S. (1994). Characterisation of cDNA-encoding N-terminal region of the quail lutropin receptor. *General and Comparative Endocrinology*, **95**:222-231.

Akazome, Y., Shimizu, F., Park, M. K., Mori, T. and Kawashima, S. (1996). Molecular characteristics of the N-terminal region of the quail follitropin receptor. *In Vivo*, **10**:345-350.

Alfuraiji, M. M., Atkinson, T., Broadbent, P. J. and Hutchinson, J. S. M. (1993). Superovulation in cattle using PMSG followed by PMSG-monoclonal antibodies. *Animal Reproduction Science,* **33**:99-109.

Allen, W. R and Stewart, F. (1992). Equine Chorionic Gonadotropin. In *Equine Reproduction,* McKinnon, A. O. and Voss, J. L. eds, Lea and Febiger, Philadelphia, USA, pp. 81-96.

Altan, O., Ozkan, S., Altan, A., Akbas, Y., Ayhan, V. and Ozkan, K. (2000). Effects of short-term fasting and midnight lighting on egg production traits of laying hens during summer season. *Archiv Fur Gefluegelkunde*, **64**(2):85-89.

Amin, S. O. and Gilbert, A. B. (1970). Cellular changes in the anterior pituitary of the domestic fowl during growth, sexual maturity and laying. *British Poultry Science,* **11**:451-458.

Ando, H. and Ishii, S. (1994). Molecular cloning of complementary deoxyribonucleic acids for the pituitary glycoprotein hormone α-subunit and luteinising hormone β-subunit precursor molecules of Japanese quail (*Coturnix coturnix japonica*). *General and Comparative Endocrinology*, **93**:357-368.

Anthony, N. B., Vasilatos-Younken, R., Bacon, W. L. and Lilburn, M. S. (1990). Secretory patterns of growth hormone, insulin and related metabolites in growing male turkeys: effects of overnight fasting and refeeding. *Poultry Science*, **69**:801-811.

Armstrong, D. G. (1994). The effect of LH, FSH and pregnant mare's serum gonadotrophin on ornithine decarboxylase activity in thecal and granulosa tissue during follicular growth and atresia in laying hens (*Gallus domesticus*). *Journal*

of Reproduction and Fertility, **100**:273-278.

Armstrong, D. G. and Hogg, C. O. (1996). Insulin-like growth factor (IGF-I), IGF-II and type-I IGF receptor gene expression in the ovary of the laying hen. *Journal of Reproduction and Fertility*, **106**:101-106.

Asem, E. K., Lintner, F., Biellier, H. V., Burke, W. H. and Hertelendy, F. (1983). Comparison of turkey luteinising hormone (LH)- and ovine LH-induced progesterone production in granulosa cells of the turkey (*Meleagris gallopavo*) and of the domestic fowl (*Gallus domesticus*). *General and Comparative Endocrinology*, **52**(3):445-451.

Asem, E., Marrone, B. L. and Hertelendy, F. (1985). Steroidogenesis in ovarian cells of the Japanese quail (*Coturnix coturnix japonica*). *General and Comparative Endocrinology*, **60**:353-360.

Barros, C. M., Moreira, M. B., Figueiredo, R. A., Teixeira, A. B. and Trinca, L. A. (2000). Synchronization of ovulation in beef cows (*Bos indicus*) using GnRH, $PGF_{2\alpha}$ and estradiol benzoate. *Theriogenology*, **53**(5):1121-1134.

Bielinska, M. and Biome, I. (1989) Identification of the combination domain of the human chorionic alpha subunit by site-directed mutagenesis. *Journal of Cell Biology*, **109**:13a.

Bolamba, D., Matton, P., Estrada, R. and Dufour, J. J. (1992). Effects of pregnant mare's serum gonadotropin treatment on follicular populations and ovulation rates in prepuberal gilts with two morphologically different ovarian types. *Journal of Animal Science*, **70**:1916-1922.

Bona Gallo, A. and Licht, P. (1979). Differences in the properties of FSH and LH binding sites in the avian gonad revealed by homologous radioligands. *General and Comparative Endocrinology*, **37**:521-532.

Bonney, R. C., Cunningham, F. J. and Furr, B. J. A. (1974). Effect of synthetic luteinising hormone releasing hormone on plasma luteinising hormone in the female domestic fowl, *Gallus domesticus*. *Journal of Endocrinology*, **63**:539-547.

Bonney, R. C. and Cunningham, F. J. (1977). Stimulation of luteinising hormone release from chicken pituitary cells by analogues of luteinising hormone-releasing hormone. *General and Comparative Endocrinology*, **32**(2):205-207.

Bousfield, G. R., Butnev, V. Y., Gotschall, R. R., Baker, V. L. and Moore, W. T. (1996). Structural features of mammalian gonadotropins. *Molecular and Cellular Endocrinology*, **125**:3-19.

Bousfield, G. R, Liu, W. K., Sugino, H. and Ward, D. N. (1987). Structural studies on equine glycoprotein hormones. *Journal of Biological Chemistry*, **262**:8610-8620.

Bousfield, G. R., Michale Perry, W. and Ward, D. N. (1994). Gonadotropins: Chemistry and Biosynthesis. In *The Physiology of Reproduction*, Knobil, E and Neill, J. D.

eds, Raven Press Ltd., New York, pp. 1749-1792.

Brahma, T. C. and Ramakrishnan, A. (1989). Calcium and phosphorus requirements of caged layers. *Kerala Journal of Veterinary Science*, **20**(1):1-10.

Bronson, F. H. (1986). Food-restricted, prepubertal, female rats: Rapid recovery of luteinising hormone pulsing with excess food, and full recovery of pubertal development with gonadotropin-releasing hormone. *Endocrinology*, **17**:215-223.

Bruggeman, V., Vanmontfort, D., Renaville, R., Portetelle, D. and Decuypere, E. (1997). The effect of food intake from two weeks of age to sexual maturity on plasma growth hormone, insulin-like growth factor-1, insulin-like growth factor-binding proteins, and thyroid hormones in female broiler breeder chickens. *General and Comparative Endocrinology*, **107**:212-220.

Bruggeman, V., D'Hondt, E., Berghman, L., Onagbesan, O. M., Vanmontfort, D., Vandesande, F. and Decuypere, E. (1998a). The effect of food intake from 2 to 24 weeks of age on LHRH-I content in the median eminence and gonadotropin levels in pituitary and plasma in female broiler breeder chickens. *General and Comparative Endocrinology*, **112**:200-209.

Bruggeman, V., Onagbesan, O. M., Vanmontfort, D., Berghman, L., Verhoeven, G. and Decuypere, E. (1998b). Effect of long-term food restriction on pituitary sensitivity to cLHRH-I in broiler breeder females. *Journal of Reproduction and Fertility*, **114**:267-276.

Buonomo, F. C., Griniger, P. and Scanes, C. G. (1982). Effects of gradation in protein-calorie restriction on the hypothalamo-pituitary-gonadal axis in young domestic fowl. *Poultry Science*, **61**:800-803.

Burke, W. H. and Cogger, E. A. (1977). The effect of luteinising hormone releasing hormone (LH-RH) on serum LH and ovarian growth in turkeys. *Poultry Science*, **56**:234-242.

Buyse, J., Decuypere, E., Darras, V. M., Vleurick, L. M., Kühn, E. R. and Veldhuis, J. D. (2000). Food deprivation and feeding of broiler chickens is associated with rapid and interdependent changes in the somatotrophic and thyrotrophic axes. *British Poultry Science*, **41**:107-116.

Caperna, T. J., Rosebrough, R. W., McMurtry, J. P. and Vasilatos-Younken, R. (1999). Influence of dietary protein on insulin-like growth factor binding proteins in the chicken. *Comparative Biochemistry and Physiology C*, **124**(4):417-421.

Cockrem, J. F. (1999). Saving the kakapo. In *The use of wildlife in research*, Mellor, D. and Monomay, V. eds, Australia and New Zealand Council for the Care of Animals in Research and Teaching, Glen Osmond, Australia, pp. 60-66.

Cole, H. H., Bigelow, M., Finkel, J. and Rupp, G. R. (1967). Biological half-life of endogenous PMS following hypophysectomy and studies on losses in urine and milk, *Endocrinology*. **81**: 927-930.

Combarnous, Y., Guillou, F. and Martinat, N. (1984). Comparison of *in vitro* follicle

stimulating hormone (FSH) activity of equine gonadotropins (luteinising hormone, FSH and chorionic gonadotropin) in male and female rats. *Endocrinology*, **115**:1821-1827.

Conrad, R. M. and Warren, D. C. (1940). The production of double yolked eggs in the fowl. *Poultry Science*, **19**:9-17.

Costa, M. S. (1981). Fundamental principles of broiler breeders nutrition and the design of breeding programmes. *World's Poultry Science Journal*, **37**:177-192.

Das, B. C. and Nalbandov, A. V. (1955). Responses of ovaries of immature chickens to avian and mammalian gonadotropins. *Endocrinology*, **57**:705-710.

Davies, D. T., Bayle, J. D., Bicknell, R. J. and Ashton, S. A. (1976). The half-life of luteinizing hormone in intact and castrated quail during photoperiodic stimulation. *Endocrine Research Communications*, **3**(3-4):243-256.

Davies, D. T. and Collins, J. (1979). Possible priming effect of luteinising hormone releasing hormone on the anterior pituitary gland in the Japanese quail and the stimulation of secretion of follicle-stimulating hormone. *Journal of Endocrinology*, **82**:71-75.

Delobelle, K., Decuypere, E., Vanmontfort, D., Milton, R. and Millar, R. (1995). LH-releasing activity of novel GnRH analogues in laying hens: insights into favourable substitutions for the design of superactive analogues. *Experimental Clinical Endocrinology*, **14**:17-25.

Decuypere, E., Leenstra, F., Huybrechts, L. M., Feng, P. Y., Arnouts, S., Herremans, M. and Nys, M. (1993). Selection for weight gain or food conversion in broilers affects the progesterone production capacity of large follicles in the reproductive adult. *British Poultry Science*, **34**:543-552.

Dieleman, S. J., Bevers, M. M., Vos, P. L. A. M. and de Loos F.A.M. (1993). PMSG/anti-PMSG in cattle: a simple and efficient superovulatory treatment. *Theriogenology*, **39**:25-41.

Doi, O., Takai, T., Nakamura, T. and Tanabe, Y. (1980). Changes in the pituitary and plasma LH, plasma and follicular progesterone and estradiol, and plasma testosterone and estrone concentrations during the ovulatory cycle of the quail (*Coturnix coturnix japonica*). *General and Comparative Endocrinology*, **41**:156-163.

Duanyai, S. and Srikandakumar, A. (1998). Premature elevation of progesterone shortens duration of ovulation in PMSG/hCG-treated prepubertal gilts. *Theriogenology*, **50**(3):433-443.

Dunn, I. C., Sharp, P. J. and Hocking, P. M. (1990). Effects of interactions between photostimulation, dietary restriction and dietary maize oil dilution on plasma LH and ovarian and oviduct weights in broiler breeder females during rearing. *British Poultry Science*, **31**:415-427.

Elwinger, K. (1982). Different energy levels and restricted feeding to three strains of single comb white leghorn hybrids 2. Effects on egg shell quality and egg composition. *Swedish Journal of Agricultural Research*, **12**(4):177-184.

Etches, R. J. and Cunningham, F. J. (1976). The effect of pregnenolone, progesterone, deoxycorticosterone or corticosterone on the time of ovulation and oviposition in the hen. *British Poultry Science*, **17**:637-642.

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

Etches, R. J., McCutcheon, J., McBride-Warren, P. A. and Brown, R. G. (1982). The ovulation-inducing activity of highly purified ACTH in the hen (*Gallus domesticus*). *Comparative Biochemistry and Physiology*, **72A**(1):195-198.

Etches, R. J. and Croze, F. (1983). Plasma concentrations of LH, progesterone, and corticosterone during ACTH- and corticosterone-induced ovulation in the hen (*Gallus domesticus*). *General and Comparative Endocrinology*, **50**:359-365.

Etches, R. J. (1990). The ovulatory cycle of the hen. *Critical Reviews in Poultry Biology*, **2**(4):293-318.

Etches, R. J. (1993). Reproduction in poultry. In *Reproduction in Domestic Animals*, Inking, G. J ed, Elsevier, Amsterdam, pp. 493-530.

Etches, R. J. (1996). *Reproduction in Poultry*. CAB International, Wallingford, U.K., pp. 125-166.

Fiddes, J. C. and Goodman, H. M. (1979). Isolation, cloning and sequence analysis of the cDNA for the α-subunit of human chorionic gonadotropin. *Nature*, **281**:351-356.

Follett, B. K. (1976). Plasma follicle-stimulating hormone during photoperiodically induced sexual maturation in the male Japanese quail. *Journal of Endocrinology*, **69**:117-126.

Follett, B. K. (1984) Birds. In *Marshall's Physiology of Reproduction: Reproductive cycles of Vertebrates*, Lamming, G. E. ed, Churchill Livingston, New York, pp. 283-350.

Foster, D. N., Galehouse, D., Giordano, T., Min, B., Lamb, I. C., Porter, D. A., Intehar, K. J. and Bacon, W. L. (1992). Nucleotide sequence of the cDNA encoding the common α-subunit of the chicken pituitary glycoprotein hormones. *Journal of Molecular Endocrinology*, **8**:21-27.

Fraps, R. M. and Riley, G. M. (1942). Hormone-induced ovulation in domestic fowl. *Proceedings of the Society For Experimental Biology and Medicine*, **49**:253-257.

Fraps, R. M., Olsen, M. W. and Neher, B. H. (1942a). Forced ovulation of normal

ovarian follicles in the domestic fowl. *Proceedings of the Society for Experimental Biology and Medicine*, **50**:308-312.

Fraps, R. M., Riley, G. M, and Olsen, M. W. (1942b). Time required for induction of ovulation following intravenous injection of hormone precipitations in fowl. *Proceedings of the Society for Experimental Biology and Medicine*, **50**:313-317.

Fraps, R. M. and Dury, A. (1943). Occurrence of premature ovulation in the domestic fowl following administration of progesterone. *Proceedings of the Society for Experimental Biology and Medicine*, **52**:346-349.

Fraps, R. M., Fevold, H. L. and Neher, B. H. (1947). Ovulatory response of the hen to presumptive luteinising and other fractions from the fowl anterior pituitary tissue. *Anatomical Record*, **99**:571-572.

Freeman, B. M., Manning, A. C. C. and Flack, I. H. (1980). Short-term stressor effects of food withdrawal on the immature fowl. *Comparative Biochemistry and Physiology*, **67A**:569-571.

Fricke, P. M., Al-Hassan, M. J., Roberts, A. J., Reynolds, L. P., Redmer, D. A. and Ford, J. J. (1997). Effect of gonadotropin treatment on size, number, and cell proliferation of antral follicles in cows. *Domestic Animal Endocrinology*, **14**(3):171-180.

Fry, R. C., Cahill, L. P., Cummins, J. P., Bindon, B. M., Piper, L. R. and Clarke, I. J. (1987). The half-life of follicle-stimulating hormone in ovary-intact and ovariectomised booroola and control merino ewes. *Journal of Reproduction & Fertility*, **81**(2):611-615.

Gilbert, A. B. (1971). The ovary. In *Physiology and Biochemistry of the Domestic Fowl*, Bell, D. J. and Freeman, B. M. eds, Academic Press, London, pp. 1163-1208.

Gilbert, A. B. (1979). Female genital organs. In *Form and Function in Birds*, King, A. S. and McLelland, J. eds, Academic Press, New York, pp. 237-360.

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

Glazier, A. M. (1998). Seasonal variation in ovarian response to pregnant mares serum gonadotropin in the brushtail possum (*Trichosurus vulpecula*). *Reproduction, Fertility and Development*, **10**(6):499-503.

González, A., Wang, H., Carruthers, T. D., Murphy, B. D. and Mapletoft, R. J. (1994a). Increased ovulation rates in PMSG-stimulated beef heifers treated with a monoclonal PMSG antibody. *Theriogenology*, **41**:1631-1642.

González, A., Wang, H., Carruthers, T. D., Murphy, B. D. and Mapletoft, R. J. (1994b). Superovulation in the cow with pregnant mare serum gonadotrophin: effects of

dose and antipregnant mare serum gonadotropin serum. *Canadian Veterinary Journal*, **35**:158-162.

Gordon, W. L., Bousfield, G. R. and Ward, D. N. (1989). Comparative binding of FSH to chicken and rat testis. *Journal of Endocrinological Investigation*, **12**:383-392.

Gowe, R. S., Johnson, A. S., Crawford, R. D., Downs, J. H., Hill, A. T., Mountain, W. F., Pelletier, J. R. and Strain, J. H. (1960) Restricted versus full-feeding during the growing period for egg production stock. *British Poultry Science*, **1**:37-58.

Gulati, D. P., Nakamura, T. and Tanabe, Y. Diurnal variations in plasma LH, progesterone, testosterone, estradiol, and estrone in the Japanese quail. *Poultry Science*, **60**:668-673.

Guyomarc'h, C., Guyomarc'h, J.-C. and Garnier, D.-H. (1980). Effect of male vocalisations on reproduction in *Coturnix coturnix japonica* females. *Biology of Behaviour*, **6**(2):167-182.

Hamilton, T. D., Vizcarra, J. A., Wettemann, R. P., Keefer, B. E. and Spicer, L. J. (1999). Ovarian function in nutritionally induced anoestrous cows: effect of exogenous gonadotropin-releasing hormone *in vivo* and effect of insulin and insulin-like growth factor I *in vitro*. *Journal of Reproduction and Fertility*, **117**(1):179-187.

Hammond, R. W., Todd, H. and Hertelendy, F. (1980). Effects of mammalian gonadotropins on progesterone release and cyclic nucleotide production by isolated avian granulosa cells. *General and Comparative Endocrinology*, **41**(4):467-476.

Hammond, R. W., Burke, W. H. and Hertelendy, F. (1981) Influence of follicular maturation on progesterone release in chicken granulosa cells in response to turkey and ovine gonadotropins. *Biology of Reproduction*, **24**(5):1048-1055.

Hassin, S., De Monbrison, D., Hanin, Y., Elizur, A., Zohar, Y. and Popper, D. M. (1997). Domestication of the white grouper, *Epinephelus aeneus*: 1. Growth and reproduction. *Aquaculture*, **156**(3-4):305-316.

Hattori, A. and Wakabayashi, S. (1979). Isoelectric focusing and gel filtration studies on the heterogeneity of avian pituitary luteinising hormone. *General and Comparative Endocrinology*, **39**:215-221.

Hattori, A., Ishii, S. and Wada, M. (1986). Effects of two kinds of chicken luteinising hormone releasing hormone (LH-RH), mammalian LH-RH and its analogues on the release of LH and FSH in Japanese quail and chicken. *General and Comparative Endocrinology*, **64**:446-455.

Hayashi, T., Hanaoka, Y. and Hayashi, H. (1992). The complete amino acid sequence of the follotropin beta-subunit of the bullfrog *Rana catesbeiana*. *General and Comparative Endocrinology*, **88**:144-150.

Hildebrandt, T. B., Pitra, C. and Thielebein, J. (1994). Transintestinale ultraschalluntersuchung bei nutzgeflügel. *Monatshefte Fur Veterinarimedizin,*

49(7):337-343.

Hildebrandt, T. B., Pitra, C.; Sömmer, P. and Pinkowski, M. (1995). Sex identification in birds of prey by ultrasonography. *Journal of Zoo and Wildlife Medicine*, **26**(3):367-376.

Hildebrandt, T. B., Göritz, F., Bosch, H., Seidel, B. and Pitra, C. (1996). Ultrasonographic sexing and reproductive assessment of penguins. *Penguin Conservation*, **4**:6-12.

Hildebrandt, T. B., Göritz, F., Stetter, M. D., Hermes, R. and Hofmann, R. R. (1998). Applications of sonography in vertebrates. *Zoology*, **101**:200-209.

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

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

Hocking, P. M. and Whitehead, C. C. (1990) Relationships between body fatness, ovarian structure and reproduction in mature females from lines of genetically lean or fat broilers given different food allowances. *British Poultry Science*, **31**:319-330.

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

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

Hocking, P. M. and McCormack, H. A. (1995). Differential sensitivity of ovarian follicles to gonadotropin stimulation in broiler and layer lines of domestic fowl. *Journal of Reproduction and Fertility*, **105**:49-55.

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

Hocking, P. M. and Robertson, G. W. (2000). Ovarian follicular dynamics in selected and control (relaxed selection) male- and female-lines of broiler breeders fed *ad libitum* or on restricted allocations of food. *British Poultry Science*, **41**:229-234.

Hodson, R. G. and Sullivan, C. V. (1993). Induced maturation and spawn in of domestic and wild striped bass, Morone saxatilis (Walbaum), brookstock with implanted GnRH analogue and injected hCG. *Aquaculture & Fisheries Management*, **24**(3):389-398.

Hoppen, H. O. (1994) The equine placenta and equine chorionic gonadotropin - an overview. *Experimental and Clinical Endocrinology*, **102**:235-243.

Hosoda, T., Kaneko, T., Mogi, K. and Abe, T. (1955). Effect of gonadotropic hormone on ovarian follicles and serum vitellin of fasting hens. *Proceedings of the Society for Experimental Biology and Medicine*, **88**:502-504.

Hosoda, T., Kaneko, T., Mogi, K. and Abe, T. (1956). Forced ovulation in gonadotrophin-treated fasting hens. *Proceedings of the Society for Experimental Biology and Medicine*, **92**:360-362.

Howard, J., Byers, A. P., Brown, J. L., Barrett, S. J., Evans, M. Z., Schwartz, R. J. and Wildt, D. E. (1996). Successful ovulation induction and laparoscopic intrauterine artificial insemination in the clouded leopard (*Neofelis nebulosa*). *Zoo Biology*, **15**(1):55-69.

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

Hughes, B. O., Gilbert, A. B. and Brown, M. F. (1986). Catergorisation and causes of abnormal egg shells relationship with stress. *British Poultry Science*, **27**(2):325-338.

Imai, K. (1972). Effects of avian and mammalian pituitary preparations on follicular growth in hens treated with methallibure or fasting. *Journal of Reproduction and Fertility*, **31**:387-397.

Imai, K., Tanaka, M. and Nakajo, S. (1972). Gonadotrophic activities of anterior pituitary and of blood plasma and ovarian response to exogenous gonadotrophin in moulting hens. *Journal of Reproduction and Fertility*, **30**:433-443.

Ishii, S. and Farner, D. S. (1976). Binding of follicle-stimulating hormone by homogenates of testes of photostimulated white-crowned sparrows, *Zonotrichia leucphrys gambelii*. *General and Comparative Endocrinology*, **30**:443-450.

Ishii, S. and Adachi, T. (1977). Binding of avian testicular homogenate with rat follicle stimulating hormone and inhibition of the binding by hypophyseal extracts of lower vertebrates. *General and Comparative Endocrinology*, **31**:287-294.

Ishii, S. and Kubokawa, K. (1984). Avian gonadotropins receptors: a comparative view. *Journal of Experimental Zoology*, **232**:431-434.

Ishii, S. (1988). Evolution of gonadotropin receptors. In *Progress in Endocrinology*, Imura, H., Shizume, K. and Yoshida, S. eds, Elsevier Science Publishers, New York/Oxford, pp. 233-238.

Ishii, S. (1993). The molecular biology of avian gonadotropin. *Poultry Science*, **72**:856-866.

Ishii, S., Ando, H., Wako, H. and Kubota, K. (1993). Molecular biology of gonadotropins. In *Avian Endocrinology*, Sharp, P. J. ed, Journal of Endocrinology Ltd., Bristol, pp. 124-133.

Ishii, S., Wada, M., Wakabayashi, S., Sakai, H., Kubodera, Y., Yamaguchi, N. and Kikuchi, M. (1994). Endocrinological studies for the artificial breeding of the Japanese ibis, *Nipponia nippon*, an endangered avian species in Asia. *Journal of Bioscience*, **19**(4):491-502.

Jaap, R. G. and Muir, F. V. (1968). Erratic oviposition and egg defects in broiler-type pullets. *Poultry Science*, **47**:417-423.

Janne, J., Poso, H. and Raina, A. (1978). Polyamines in rapid growth and cancer. *Biochimica Et Biophysica*, **463**:241-293.

John Ballard, F., Johnson, R. J., Owens, P. C., Francis, G. L., Upton, F. M., McMurtry, J. P. and Wallace, J. C. (1990). Chicken insulin-like growth factor-1: amino acid sequence, radioimmunoassay, and plasma levels between strains and during growth. *General and Comparative Endocrinology*, **79**:459-468.

Johnson, A. L. and van Teinhoven, A. (1981). Hypothalamo-hypophyseal sensitivity to hormones in the hen. II. Plasma concentrations of LH, progesterone, and testosterone in response to peripheral and central injections of LHRH or testosterone. *Biology of Reproduction*, **25**:153-161.

Johnson, A. L. (1983). Effects of ovine LH on steroid secretion and ovulation relative to stage of the sequence, and in PMSG-primed hens. *Poultry Science*, **62**:1444.

Johnson, A. L., Dickerman, R. W. and Advis, J. P. (1984a). Comparative ability of chicken and mammalian LHRH to release LH from rooster pituitary cells *in vitro*. *Life Sciences*, **34**(19):1847-1851.

Johnson, A. L., Johnson, P. A. and van Tienhoven A. (1984b). Ovulatory response, and plasma concentrations of luteinising hormone and progesterone following administration of synthetic mammalian or chicken luteinising hormone-releasing hormone relative to the first or second ovulation in the sequence of the domestic hen. *Biology of Reproduction*, **31**:646-655.

Johnson, A. L. and Leone, E. W. (1985). Ovine luteinising hormone-induced steroid and luteinising hormone secretion, and ovulation in intact and pregnant mare serum gonadotropin-primed hens. *Poultry Science*, **64**:2171-2179.

Johnson, P. A., Green, C., Lee, H. T. and Bahr, J. M. (1988). Inhibition of progesterone secretion from granulosa cells by estradiol and androgens in the domestic hen. *Endocrinology*, **123**:473-477.

Johnson, A. L. (1996). The avian ovarian hierarchy: a balance between follicle differentiation and atresia. *Poultry and Avian Biology Reviews*, **7**(2/3):99-110.

Johnson, A. L., Bridgham, J. T. and Wagner, B. (1996). Characterisation of a chicken luteinising hormone receptor (cLH-R) complementary deoxyribonucleic acid, and expression of cLH-R messenger ribonucleic acid in the ovary. *Biology of Reproduction*, **55**:304-309.

Kamiyoshi, M. and Tanaka, K. (1972). Augmentative effect of FSH on LH-induced ovulation in the hen. *Journal of Reproduction and Fertility*, **29**:141-143.

Karesh, W. B., Willis, M. S., Czekala, N. M. and Lasley, B. L. (1985). Induction of a fertile mating in a red ruffed lemur *Varecia variegata rubra* using pregnant mare serum gonadotropin. *Zoo Biology*, **4**(2):147-152.

Kikuchi, M. and Ishii, S. (1989). Radioiodination of chicken luteinising hormone without affecting receptor binding potency. *Biology of Reproduction*, **41**:1047-1054.

Kikuchi, M. and Ishii, S. (1992). Changes in luteinising hormone receptors in the granulosa and theca layers of the ovarian follicle during follicular maturation in the Japanese quail. *General and Comparative Endocrinology*, **85**:124-137.

Kikuchi, M., Kobayashi, M., Ito, T., Kato, Y. and Ishii, S. (1998). Cloning of complementary deoxyribonucleic acid for the follicle-stimulating hormone α-subunit in the Japanese quail. *General and Comparative Endocrinology*, **111**:376-385.

Knight, P. G., Gladwell, R. T. and Cunningham, F. J. (1985). Release of LHRH *in vitro* and anterior pituitary responsiveness to LHRH *in vivo* during sexual maturation in pullets (*Gallus domesticus*). *Journal of Reproduction and Fertility*, **74**:145-151.

Koide, Y., Papkoff, H. and Kawauchi, H. (1996). Complete amino acid sequences of follutropin and lutropin in the ostrich, *Struthio camelus*. *European Journal of Biochemistry*, **240**:262-267.

Kowalski, K. I., Tilly, J. L. and Johnson, A. L. (1991). Cytochrome P450 side-chain cleavage (P450scc) in the hen ovary. I. Regulation of P450scc messenger RNA levels and steroidogenesis in theca cells of developing follicles. *Biology of Reproduction*, **45**:955-966.

Kuenzel, W. J., McCune, S. K., Talbot, R. T., Sharp, P. J. and Hill, J. M. (1997). Sites of gene expression for vasoactive intestinal polypeptide throughout the brain of the chick (*Gallus domesticus*). *Journal of Comparative Neurology*, **381**:101-118.

Landefeld, T. D., Ebling, F. J. P., Suttie, J. M., Vannerson, L. A., Padmanabhan, V., Beitins, I. Z. and Foster, D. L. (1989). Metabolic interfaces between growth and reproduction. II. Characterisation of changes in messenger ribonucleic acid concentrations of gonadotropin subunits, growth hormone and prolactin in nutritionally growth limited lambs and the differential effects of increased nutrition. *Endocrinology*, **125**:351-356.

Lasserre, A., Espinosa, M. B. and Vitullo, A. (1998). Superovulation in the vesper mouse *Calomys musculinus* (Rodentia-Sigmodontinae). *Biocell*, **22**(3):157-162.

Leeson, S., Summers, J. D. and Caston, L. J. (1992). Response of broilers to feed restriction or diet dilution in the finisher period. *Poultry Science*, **71**:2056-2064.

Leyva, V., Buckrell, B. C, and Walton, J. S. (1998). Follicular activity and ovulation

regulated by exogenous progestagen and PMSG in anestrous ewes. *Theriogenology*, **50**(3):377-393.

Li, Z. D., Koga, O., Tanaka, K. and Fujihara, N. (1993). Effects of gonadotropins on follicular steroid hormone production in hypophysectomised hens (*Gallus domesticus*). *Asian-Australian Journal of Animal Sciences*, **6**(3):411-416.

Li, Z. Z. and Burke, W. H. (1987). Influence of 12 hours of sound stimuli on gonad development and plasma luteinising hormone in Japanese quail (*Coturnix coturnix japonica*) exposed to 6 hours of daily light. *Poultry Science*, **66**:1045-1052.

Licht, P. and Midgley, A. R. (1976a). *In vitro* binding of radioiodinated human follicle-stimulating hormone to reptilian and avian gonads: radioligand studies with mammalian hormones. *Biology of Reproduction*, **15**:195-205.

Licht, P. and Midgley, A. R. (1976b). Competition for the *in vitro* binding of radioiodinated human follicle-stimulating hormone in reptilian, avian, and mammalian gonads by nonmammalian gonadotropins. *General and Comparative Endocrinology*, **30**:364-371.

Licht, P., Papkoff, H., Farmer, S. W., Muller, C. H., Tsui, H. W. and Crews, D. (1977). Evolution in gonadotropin structure and function. *Recent Progress in Hormone Research*, **33**:169-248.

Maney, D. L., Richardson, R. D. and Wingfield, J. C. (1997). Central administration of chicken gonadotropin-releasing hormone-II enhances courtship behaviour in a female sparrow. *Hormones and Behaviour*, **32**:11-18.

Martinez, M. F., Adams, G. P., Bergfelt, D. R., Kastelic, J. P. and Mapletoft, R. J. (1999). Effect of LH or GnRH on the dominant follicle of the first follicular wave in beef heifers. *Animal Reproduction Science*, **57**(1-2):23-33.

Martinez, M. F., Adams, G. P., Kastelic, J. P., Bergfel, D. R. and Mapletoft, R. J. (2000). Induction of follicular wave emergence for estrus synchronization and artificial insemination in heifers. *Theriogenology*, **54**(5):757-769.

Masuda, S., Wakabayashi, K. and Koga, O. (1984). Change of the properties of follicle stimulating hormone like gonadotropin binding sites in the follicles of the hen during follicular growth. *Japanese Journal of Zootechnology*, **55**:399-408.

Matsuzaki, T., Matsuzaki, K., Yokoyama, M., Yamada, S. and Saito, M. (1997). Superovulation induction in the house musk shrew (*Suncus murinus*). *Experimental Animals*, **46**(3):183-189.

McIntosh, J. E. A., Moor, R. M. and Allen, W. R. (1975). Pregnant mare serum gonadotropin: rate of clearance from the circulation of sheep. *Journal of Reproduction and Fertility*, **44**:95-100.

McNatty, K. P., Hudson, N. L., Heath, D. A., Shaw, L., Blay, L., Berry, L. and Lun, S. (1993). Effect of chronic FSH administration on ovarian follicular development, ovulation rate and corpora lutea formation in sheep. *Journal of Endocrinology*,

138(2):315-25.

Means, A. R. and Vaitukaitis, J. (1974). Peptide hormone "receptors": specific binding of ^{3}H-FSH to testes. *Endocrinology*, **90**:39-46.

Merton, D. V., Morris, R. B. and Atkinson, I. A. E. (1984). Lek behaviour in a parrot: the kakapo *Strigops habroptilus* of New Zealand. *Ibis*, **126**:227-283.

Millam, J. R., El Halawani, M. E. and Burke, W. H. (1985). Effect of cyclic sound cues on sexual development in nonphotostimulated Japanese quail. *Poultry Science*, **64**:169-175.

Millar, R. P. and King, J. A. (1984). Structure-activity relations of LHRH in birds. *Journal of Experimental Zoology*. **232**(3):425-430.

Minegishi, T., Delgado, C. and Dufau, M. L. (1989). Phosphorylation and glycosylation of the luteinising hormone receptor. *Proceedings of the National Academy of Sciences*, **86**:1470-1474.

Mitchell, M. E. (1966). Effect of fowl gonadotropin following treatment with a pituitary inhibitor. In *Physiology of the Domestic Fowl*, Horton-Smith, C. and Amoroso, E. C. eds, Oliver and Boyd Ltd., Edinburgh, pp. 14-22.

Mitchell, M. E. (1967a). Stimulation of the ovary in hypophysectomised hens by an avian pitiutary preparation. *Journal of Reproduction and Fertility*, **14**:249-256.

Mitchell, M. E. (1967b). The effects of avian gonadotropin precipitate on pituitary-deficient hens. *Journal of Reproduction and Fertility*, **14**:257-263.

Mitchell, M. E. (1970). Treatment of hypophysectomised hens with partially purified avian FSH. *Journal of Reproduction and Fertility*, **22**:233-241.

Miyamoto, K., Hasegawa, Y., Namura, M., Igarashi, M., Kanagawa, K. and Matsuo, H. (1984). Identification of the second gonadotropin releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin releasing hormones in avian species. *Proceedings of the National Academy of Science*, **81**:3874-3878.

Mizutani, T., Minegishi, T., Nonobe, Y., Abe, Y., Hasegawa, Y., Wakabayashi, K., Kamiyoshi, M. and Miyamoto, K. (1998). Molecular cloning and functional expression of chicken luteinising hormone receptor. *Biochimica Et Biophysica Acta*, **1397**:1-8.

Mori, M., Aoki, F., Kohmoto, K. and Shoda, Y. (1985). Metabolism of steroid hormones *in vitro* by follicular tissues of the Japanese quail. *Biology of Reproduction*, **33**:11-20.

Mori, M. and Kantou, T. (1987). Changes in progesterone production in granulosa cells during the ovulatory cycle of the Japanese quail (*Coturnix coturnix japonica*). *General and Comparative Endocrinology*, **68**:57-63.

Moyle, W. R., Cambell, R. K., Myers, R. V., Bernard, M. P., Han, Y. and Wang, X. Co-

evolution of ligand-receptor pairs. *Nature*, **3368**:255-261.

Nakada, T., Koja, Z. and Tanaka, K. (1994). Effect of progesterone on ovulation in the hypophysectomised hen. *British Poultry Science*, **35**:153-156.

Naqvi, S. M. and Gulyani, R. (1998). The effect of gonadotropin releasing hormone and follicle stimulating hormone in conjunction with pregnant mare serum gonadotropin on the superovulatory response in crossbred sheep in India. *Tropical Animal Health & Production*, **30**(6):369-76.

Nir, I., Yam, D. and Perek, M. (1975). Effects of stress on the corticosterone content of the blood plasma and adrenal gland of intact and bursectomised *Gallus domesticus*. *Poultry Science*, **54**:2101-2110.

Noce, T., Ando, H., Ueda, T., Kubokawa, K., Higashinakagawa, T. and Ishii, S. (1989). Molecular cloning and nucleotide sequence analysis of the putative cDNA for the precursor molecule of the chicken LH-β subunit. *Journal of Molecular Endocrinology*, **3**:129-137.

Ogawa, K., Goto, K. and Tokunaga, T. (1985). Effects of gonadotropins on progesterone production by isolated granulosa cells of the adenohypophysectomized domestic fowl (*Gallus domesticus*). *Journal of Reproduction and Fertility*, **73**:79-84.

Onagbesan, O. M. and Peddie, M. J. (1988a). Induction of ovulation and oviposition in female quail with luteinising hormone, luteinising hormone releasing hormone, or progesterone. *General and Comparative Endocrinology*, **71**:124-131.

Onagbesan, O. M. and Peddie, M. J. (1988b) Steroid secretion by ovarian cells of the Japanese quail (*Coturnix coturnix japonica*). *General and Comparative Endocrinology*, **72**:272-281.

Onagbesan, O. M., Peddie, M. J. and Williams, L. (1994). Regulation of cell proliferation and estrogen synthesis by ovine LH, IGF-1, and EGF in thecal interstitial cells of the domestic hen cultured in defined media. *General and Comparative Endocrinology*, **94**:261-272.

Onagbesan, O. M., Decuypere, E., Leenstra, F. and Ehlhardt, D. A. (1999a). Differential effects of amount of feeding on cell proliferation and progesterone production in response to gonadotrophins and insulin-like growth factor I by ovarian granulosa cells of broiler breeder chickens selected for fatness or leaness. *Journal of Reproduction and Fertility*, **116**:73-85.

Onagbesan, O. M., Vleugels, B., Buys, N., Bruggeman, V., Safi, M. and Decuypere, E. (1999b). Insulin-like growth factors in the regulation of avian ovarian functions. *Domestic Animal Endocrinology*, **17**:299-313.

Opel, H. and Nalbandov, A. V. (1961a). Follicular growth and ovulation in hypophysectomised hens. *Endocrinology*, **69**:1016-1028.

Opel, H. and Nalbandov, A. V. (1961b). Ovulability of ovarian follicles in the hypophysectomised hen. *Endocrinology*, **69**:1029-1035.

Osuka, A. and Nagata, S. (1998). Effect of frequency of short-term fasting on egg shell qualities and egg production in hens at later laying stage. *Research Bulletin of the Aichi-Ken Agricultural Research Center*, **30**:329-332.

Ottinger, M. A., Duchala, C. S. and Masson, M. (1983). Age-related reproductive decline in the male Japanese quail. *Hormones and Behaviour*, **17**:197-207.

Ottinger, M. A. and Balthazart, J. (1986). Altered endocrine and behavioural responses with reproductive aging in the male Japanese quail. *Hormones and Behaviour*, **20**:83-94.

Ottinger, M. A. (1992). Altered neuroendocrine mechanisms during reproductive aging. *Poultry Science Review*, **4**:235-248.

Palmer, S. S. and Bahr, J. M. (1992). Follicle stimulating hormone increases serum oestradiol-17beta concentrations, number of growing follicles and yolk deposition in aging hens (*Gallus gallus domesticus*) with decreased egg production. *British Poultry Science*, **33**:403-414.

Parlow, A. F. and Ward, D. N. (1961). Rate of disappearance of LH, PMS and hCG from plasma. In *Human Pituitary Gonadotropins*, Albert, A., ed., Charles C. Thomas, Springfield, Illinois, pp. 204-209.

Peddie, M. J., Onagbesan, O. M. and Williams, J. (1994). Chicken granulosa cell proliferation and progesterone production in culture: effects of EGF and thecal secretions. *General and Comparative Endocrinology*, **94**:341-356.

Petitte, J. N. and Etches, R. J. (1989). The effect of corticosterone on the response of the ovary to pregnant mare's serum gonadotropin in sexually immature pullets. *General and Comparative Endocrinology*, **74**:377-384.

Phillips, R. E. (1943). Ovarian response of hens and pullets to injections of Ambinon. *Poultry Science*, **22**:368-373.

Phillips, D. J., Hudson, N. L., Lun, S., Condell, L. A. and McNatty, K. P. (1993). Biopotency in vitro and metabolic clearance rates of five pituitary preparations of follicle stimulating hormone. *Reproduction, Fertility, & Development*, **5**(2):181-190.

Philomina, P. T. and Pillai, R. (2000). Effect of dietary calcium and age on the egg shell quality in Japanese quails. *Indian Journal of Poultry Science*, **35**(1):62-65.

Pintado, B., Gutierrez-Adan, A. and Perez Llano, B. (1998). Superovulatory response of Murciana goats to treatments based on PMSG/anti-PMSG or combine FSH/PMSG administration. *Theriogenology*, **50**(3):357-364.

Porter, M. B., Cleaver, B. D., Peltier, M., Robinson, G., Thatcher, W. W. and Sharp, D. C. (1997). Comparative study between pony mares and ewes evaluating gonadotropic response to administration of gonadotropin-releasing hormone. *Journal of Reproduction and Fertility*, **110**(2):219-229.

Ralph, C. L. and Fraps, R. M. (1960). Induction of ovulation of progesterone into the

brain. *Endocrinology*, **66**:269-272.

Reeves, J. J., Harrison, P. C. and Casey, J. M. (1973). Ovarian development and ovulation in hens treated with synthetic (porcine) luteinising hormone releasing hormone/follicle-stimulating hormone releasing hormone (LHRH/FSHRH). *Poultry Science*, **52**:1883-1886.

Ritzhaupt, L. K. and Bahr, J. M. (1987). A decrease in FSH receptors of granulosa cells during follicular maturation in the domestic hen. *Journal of Endocrinology*, **115**:303-310.

Roberts, R. D., Sharp, P. J., Burt, D. W, and Goddard, C. (1994). Insulin-like growth factor-1 in the ovary of the laying hen: gene expression and biological actions on granulosa and thecal cells. *General and Comparative Endocrinology*, **93**:327-336.

Roland, D. A. (1986). Egg shell quality III: Calcium and phosphorus requirements of commercial leghorns. *World Poultry Science Journal*, **42**(2):154-165.

Romanoff, A. L. and Romanoff, A. J. (1949). *The Avian Egg*, John Wiley and Sons, New York.

Rothchild, I. and Fraps, R. M. (1944). On the function of the ruptured follicle of the domestic fowl. *Proceedings of the Society for Experimental Biology and Medicine*, **56**(2):79-82.

Rothchild, I. and Fraps, R. M. (1949). The induction of ovulating hormone release from the pituitary of the domestic hen by means of progesterone. *Endocrinology*, **44**:141-149.

Rowlands, I. W. (1965). General discussion on pregnant mare's serum gonadotropin. In *Gonadotropins: Physiochemical and Immunological Properties*, Wolstenholme, G. E. W. and Knight, J. eds, J. & A. Churchill Ltd., London, pp. 62-69.

Russell, D. and Snyder, S. H. (1968). Amine synthesis in rapidly growing tissues: ornithine decarboxylase activity in regenerating rat liver, chick embryo and various tumors. *Proceedings of the National Academy of Science*, **60**:1420-1427.

Ryan, J. P., Hunton, J. R, and Maxwell, W. M. C. (1991). Increased production of sheep embryos following superovulation of merino ewes with a combination of pregnant mare serum gonadotropin and follicle-stimulating hormone. *Reproduction, Fertility and Development*, **3**:551-560.

Saharrea, A., Valencia, J., Balcazar, A., Mejia, O., Cerbon, J. L., Caballero V. and Zarco, L. (1998). Premature luteal regression in goats superovulated with PMSG: effect of hCG or GnRH administration during the early luteal phase. *Theriogenology*, **50**(7):1039-1052.

Sasamoto, S., Sato, K. and Naito, H. (1972). Biological active life of PMSG in mice with special reference to follicular ability to ovulate. *Journal of Reproduction and Fertility*, **30**:371-379.

Sawyer Steffan, J. E., Kirby, V. L. and Gilmartin, W. G. (1983). Progesterone and estrogens in the pregnant and nonpregnant dolphin, Tursiops truncatus, and the effects of induced ovulation. *Biology of Reproduction*, **28**(4):897-901.

Scanes, C. G. and Follett, B. K. (1973). The half-life of luteinising hormone in the circulation of domestic fowl and Japanese quail. *Journal of Endocrinology*, **58**:125-126.

Scanes, C. G., Godden, P. M. M. and Sharp, P. J. (1977). A homologous radioimmunoassay for chicken follicle-stimulating hormone: Observations on the ovulatory cycle. *Journal of Endocrinology*, **73**:473-481.

Scanes, C. G. and Fagioli, J. H. (1980). Effects of mammalian and avian gonadotropins in *in vitro* progesterone production by avian ovarian granulosa cells. *General and Comparative Endocrinology*, **41**:1-7.

Scott, T. R., Satterlee, D. G. and Jacobs-Perry, L. A. (1983). Circulating corticosterone responses of feed and water deprived broilers and Japanese quail. *Poultry Science*, **62**:290-297.

Sechin, A., Deschamps, J. C., Lucia, T. Jr., Aleixo, J. A. and Bordignon, V. (1999). Effect of equine chorionic gonadotropin on weaning-to-first service interval and litter size of female swine. *Theriogenology*, **51**(6):1175-1182.

Sekiguchi, S. and Imai, K. (1987). Induction of ovulation by LH, progesterone and LHRH administered at various times during the ovulatory cycle in Japanese quail. *Japanese Poultry Science*, **24**:79-85.

Sharp, P. J. and Gow, C. B. (1983). Neuroendocrine control of reproduction in the cockerel. *Poultry Science*, **62**(8):1671-1675.

Sharp, P. J., Sterling, R. J., De Milton, R. C. and Millar, R. P. (1986). Effect of luteinising hormone releasing hormone and its analogues on plasma luteinising hormone concentrations in incubating bantam hens. *British Poultry Science*, **27**(1):129-135.

Sharp, P. J., Dunn, I. C. and Talbot, R. T. (1987). Sex differences in the LH response to chicken LHRH-I and -II in the domestic fowl. *Journal of Endocrinology*, **115**:323-331.

Sharp, P. J. (1996). Strategies in avian breeding cycles. *Animal Reproduction Science*, **42**:505-513.

Sterling, R. J. and Sharp, P. J. (1984). A comparison of the luteinising hormone-releasing activities of synthetic chicken luteinising hormone-releasing hormone (LH-RH), synthetic porcine LH-RH, and Buserelin, an LH-RH analogue, in the domestic fowl. *General and Comparative Endocrinology*, **55**:403-471.

Stewart, F., Allen, W. R. and Moor, R. M. (1976). Pregnant mare serum gonadotropin: ratio of follicle-stimulating hormone and luteinising hormone activities measured by radioreceptor assay. *Journal of Endocrinology*, **71**:371-382.

Stewart, F., Thomson, J. A., Leigh, S. E. A. and Warwick, J. M. (1987). Nucleotide (cDNA) sequence encoding the horse gonadotropin α-subunit. *Journal of Endocrinology*, **115**:341-346.

Sugino, H., Bousfield, G. R., Moore, W. T. and Ward, D. N. (1987). Structural studies on equine glycoprotein hormones. *Journal of Biological Chemistry*, **262**(18):8603-8609.

Taber, E. (1948). The relation between ovarian growth and sexual characters in brown leghorn chicks treated with gonadotropins. *Experimental Zoology*, **107**:65-107.

Taber, E., Claytor, M., Knight, J., Gambrell, D., Flowers, J. and Ayers, C. (1958). Ovarian stimulation in the immature fowl by desiccated avian pituitaries. *Endocrinology*, **62**:84-90.

Talmadge, K., Vamvakopoulos, N. C. and Fiddes, J. C. (1984). Evolution of the genes for the β-subunits of human chorionic gonadotropin and luteinising hormone. *Nature*, **307**:37-40.

Tanaka, K., Mather, F. B., Wilson, W. O. and McFarland, L. Z. (1965). Effect of photoperiod on early growth of gonads and on potency of gonadotropins of the anterior pituitary of *Coturnix*. *Poultry Science*, **44**:662-665.

Tanaka, K. and Kamiyoshi, M. (1976). Induction of ovulation in hens by an analogue of LH-RH. *Journal of Reproduction and Fertility*, **46**:243-244.

Taponen, J., Katila, T. and Rodriguez-Martinez, H. (1999). Induction of ovulation with gonadotropin-releasing hormone during proestrus in cattle: influence on subsequent follicular growth and luteal function. *Animal Reproduction Science*, **55**(2):91-105.

Tilly, J. L., Kowalski, K. I., Li, Z., Levorse, J. M. and Johnson, A. L. (1992). Plasminogen activator and DNA synthesis in avian granulosa cells during follicular development and the periovulatory period. *Biology of Reproduction*, **46**:195-200.

Tsutsui, K. and Ishii, S. (1978). Effects of follicle-stimulating hormone and testosterone on receptors of follicle-stimulating hormone in the testis of the immature Japanese quail. *General and Comparative Endocrinology*, **36**:297-305.

Urban, R. J., Padmanabhan, V., Beitins, I. and Veldhuis, J. D. (1991). Metabolic clearance of human follicle-stimulating hormone assessed by radioimmunoassay, immunoradiometric assay, and in vitro Sertoli cell bioassay. *Journal of Clinical Endocrinology & Metabolism*, **73**(4):818-823.

Van Tienhoven, A. and Schally, A. V. (1972). Mammalian luteinising hormone-releasing hormone induces ovulation in the domestic fowl. *General and Comparative Endocrinology*, **19**:594-595.

Vizcarra, J. A., Wettemann, R. P., Braden, T. D., Turzillo, A. M. and Nett, T. M. (1997). Effect of gonadotropin-releasing hormone (GnRH) pulse frequency on serum and pituitary concentrations of luteinising hormone and follicle-

stimulating hormone, GnRH receptors, and messenger ribonucleic acid for gonadotropin subunits in cows. *Endocrinology*, **138**(2):594-601.

Vizcarra, J. A., Wettemann, R. P. and Morgan, G. L. (1999). Influence of dose, frequency, and duration of infused gonadotropin-releasing hormone on secretion of luteinising hormone and follicle-stimulating hormone in nutritionally anestrous cows. *Domestic Animal Endocrinology*, **16**(3):171-181.

Wade, G. N., Schneider, J. E. and Li, H.-Y. (1996). Control of fertility by metabolic cues. *American Journal of Physiology*, **270**:E1-E19.

Wakabayashi, N., Suzuki, A., Hoshino, H., Nishimori, K. and Mizuno, S. (1997). The cDNA cloning and transient expression of a chicken gene encoding a follicle-stimulating hormone receptor. *Gene*, **197**:121-127.

Wakabayashi, S., Kikuchi, M., Wada, M., Sakai, H. and Ishii, S. (1992). Induction of ovarian growth and ovulation by administration of a chicken gonadotrophin preparation to Japanese quail kept under a short day regimen. *British Poultry Science*, **33**:847-858.

Wakabayashi, S., Kikuchi, M. and Ishii, S. (1996). Hormonal induction of ovulation and oviposition in Japanese quail kept under a short-day regimen. *Poultry and Avian Biology Reviews*, **7**(4):183-192.

Waldhalm, S. J., Jacobson, H. A., Dhungel, S. K. and Bearden, H. J. (1989). Embryo transfer in the white tailed deer: a reproductive model for endangered deer species of the world. *Theriogenology*, **31**(2):437-450.

Wang, S. Y. and Johnson, P. A. (1993). Increase in ovarian α-inhibin gene expression and plasma immunoreactive inhibin level is correlated with a decrease in ovulation rate in the domestic hen. *General and Comparative Endocrinology*, **91**:52-58.

Wells, J. W., Gilbert, A. B. and Culbert, J. (1980). Effect of luteinising hormone on progesterone secretion *in vitro* by the granulosa cells of the domestic fowl (*Gallus domesticus*). *Journal of Endocrinology*, **84**:249-254.

Wells, J. W., Walker, M. A., Gilbert, A. B. and Culbert, J. (1983). Effect of luteinising hormone on progesterone production by the follicular granulosa in the ovarian hierarchy of the domestic fowl (*Gallus domesticus*). *General and Comparative Endocrinology*, **52**(2):265-271.

Wells, J. W., Walker, M. A., Culbert, J. and Gilbert, A. B. (1985) Comparison of the response *in vivo* to luteinising hormone and follicle stimulating hormone of the granulosa of six follicles from the ovarian hierarchy in the chicken (*Gallus domesticus*). *General and Comparative Endocrinology*, **59**:369-374.

Wieser, D. and Krampitz, G. (1987). Estrogens activate egg shell protein-genes in the liver of laying hens. *Zuchthygiene*, **22**(6):267-274.

Williams, J. B. and Sharp, P. J. (1978). Ovarian morphology and rates of ovarian follicular development in laying broiler breeders and commercial egg-producing

hens. *British Poultry Science*, **19**:387-395.

Wilson, S. C. and Sharp, P. J. (1976). The effects of progesterone on oviposition and ovulation in the domestic fowl (*Gallus domesticus*). *British Poultry Science*, **17**:163-173.

Wilson, S. C., Cunningham, F. J., Chairil, R. A, and Gladwell, R. T. (1989). Maturational changes in the LH response of domestic fowl to synthetic chicken LHRH-I and -II. *Journal of Endocrinology*, **123**:311-318.

Witschi, E. and Keck, W. N. (1935). Differential effect of some gonadotropic substances on development of cyclical sex characters in the English sparrow. *Proceedings of the Society for Experimental Biology and Medicine*, **32**:598-603.

Witschi, E. and Johnson, D. C. (1960). Effect of repeated PMS injections in the Weaver finch. *Anatomical Record*, **137**:401.

Yavas, Y. and Walton, J. S. (2000). Induction of ovulation in postpartum suckled beef cows: a review. *Theriogenology*, **54**(1):1-23.

Yoshimura, Y., Tischkau, S. A. and Bahr, J. M. (1994). Destruction of the germinal disc region of an immature preovulatory follicle suppresses follicular maturation and ovulation. *Biology of Reproduction*, **51**:229-233.

You, S., Foster, L. K., Youngren, O. M., El Halawanai, M. E. and Foster, D. N. (1992). Turkey luteinising hormone beta-subunit complimentary DNA nucleotide sequence. *Poultry Science*, **71**(Suppl. 1):57.

You, S., Foster, L. K., Silsby, J. L., El Halawani, M. E. and Foster, D. N. (1995). Sequence analysis of the turkey LH β subunit and its regulation by gonadotropin-releasing hormone and prolactin in cultured pituitary cells. *Journal of Molecular Endocrinology*, **14**:117-129.

You, S., Bridgham, J. T., Foster, D. N. and Johnson, A. L. (1996). Characterisation of the chicken follicle-stimulating hormone receptor (cFSR-R) complementary deoxyribonucleic acid, and expression of cFSH-R messenger ribonucleic acid in the ovary. *Biology of Reproduction*, **55**:1055-1062.

Yu, M. W., Robinson, F. E. and Etches, R. J. (1992a). Quantification of ovarian steroidogenesis in the domestic fowl by incubation of large intact follicles. *Poultry Science*, **71**:346-351.

Yu, M. W., Robinson, F. E., Charles, R. G. and Weingardt, R. (1992b). Effect of feed allowance during rearing and breeding on female broiler breeders. 2. Ovarian morphology and production. *Poultry Science*, **71**:1750-1761.

Yu, M. W., Robinson, F. E. and Etches, R. J. (1992c). Effect of feed allowance during rearing and breeding on female broiler breeders. 3. Ovarian steroidogenesis. *Poultry Science*, **71**:1762-1767.

Zadworny, D. and Etches, R. J. (1988). Effect of pregnant mare serum gonadotropin on plasma prolactin, luteinising hormone, estradiol and ovarian growth in

incubating and out-of-lay turkeys. *Poultry Science*, **67**:319-326.

Zakár, T. and Hertelendy, F. (1980). Steroidogenesis in avian granulosa cells: Early and late kinetics of oLH- and dibutyryl cyclic AMP-promoted progesterone production. *Biology of Reproduction*, **23**:974-980.

Zhang, C., Shimada, K., Saito, N. and Kansaku, N. (1997). Expression of messenger ribonucleic acids of luteinising hormone and follicle-stimulating hormone receptors in granulosa and theca layers of chicken preovulatory follicles. *General and Comparative Endocrinology*, **105**:402-409.