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Immunocontraception in Japanese Quail Using Mammalian GnRH Compared to Chicken GnRH-I and Chicken GnRH-II

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Immuocontraception in Japanese Quail using Mammalian GnRH compared to Chicken GnRH-I and Chicken GnRH-II

by

Marianna Burks

A Thesis

Presented to the Faculty of
The Graduate College at the University of Nebraska
In Partial Fulfillment of the Requirements
For the Degree of Master of Science

Major: Animal Science

Under the Supervision of Professor Mary M. Beck

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Because of increasing numbers in wildlife, immunocontraceptives are being developed to decrease these animals and other birds. This study was conducted in conjunction with the United States Geological Survey Midcontinent Science Center in Fort Collins, Colorado (USGS), to determine whether mammalian GnRH (m-GnRH) can be used as a potential immunocontraceptive to decrease reproductive fertility in mature Japanese quail.

Eighty male and female Japanese quail were injected with either mGnRH, chicken GnRH-I (c-GnRH-I), chicken GnRH-II (c-GnRH-II), or vehicle (controls). Blood samples were analyzed for estrogen (E2) and testosterone (T) levels, in addition to antibody titer (Aby) development against the corresponding conjugated antigen. Testes were weighed and stored in -70° C until histological slides were made. The granulosa layer was isolated and stained for 3\(^{\beta}\)-Hydroxysteroid Dehydrogenase activity (3\(^{\beta}\)-HSD).

The data were analyzed using repeated measures analysis of variance (ANOVA) to determine antibody percentages, repeated measures analysis of covariance (ANCOVA) to determine hormone concentrations, and Pearson correlation coefficient to determine correlation between antibody and hormone concentrations. All analyses were conducted using Proc Mixed (SAS Institute Inc., 2001).
There was a significant difference in Aby concentrations between treatment groups (P=0.001) and the interaction of treatment by week was highly significant (P=0.0094). There was no difference in testes weight of males killed midway (WK 5) (P=0.2259) and those killed at the end of the study (P=0.9995). There were no apparent differences in gross morphology of testicular tissue. Overall, testosterone concentrations between treatment groups approached significance (P=0.1071), but there was no difference between week of treatment (P=0.3551) or between treatment by week (P=0.3876). Correlation between antibody percentages and hormones indicated that antibodies to both m-GnRH and c-GnRH-I were transiently inversely correlated with testosterone concentrations, but that antibodies to c-GnRH-II were not. Dispersed granulosa cells, stained for 3β-HSD, were found to have no differences in staining percentage between treatment groups (P=0.6822). Estradiol concentrations were found to have differences in estradiol concentrations between treatment groups (P=0.1071). The conclusion of this study determined that immunization of Japanese quail using m-GnRH, c-GnRH-I, and c-GnRH-II affected sex steroid hormones, but not to the point that fertility was affected.
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I. GENERAL INTRODUCTION

For decades, animal science management, United States Department of Agriculture (USDA), and other corresponding agencies have implemented ways to increase and protect reproduction among animals. Yet species of particular wildlife have substantially increased in numbers. Now some of these agencies, particularly government agencies, are looking to reduce these numbers by means of fertility control. Studies have been carried out in various research settings, including those within universities, to implement ways of controlling fertility.

Several contraceptives have been introduced thus far, these being oral, implanted, or injected intramuscularly. Many studies have focused on reducing numbers because conflicts have occurred between humans and particular wild species. For example, the USDA, Animal and Plant Health Inspection Service (APHIS), Wildlife Services (WS) program offers direction in managing issues between human activity and wildlife (Johnston et al., 2001).

Several indicators of conflicts are when birds damage food crops, and public health and interfere with aviation safety (Johnston et al., 2001). Wildlife in record numbers also spread infection in water supplies shared by other migratory animals. For example, one study was conducted in which avian cholera eradicated an estimated 70,000 – 100,000 waterfowl in the Nebraska’s Rainwater Basin (Price and Brand, 1983). Moreover, the control of reproduction in small mammals is potentially important in controlling diseases such as rabies or Lyme disease (Kirkpatrick et al., 1993).
With the arising developments and implementations of contraception for several species in the wild, an excessive surplus of animals may be prevented. In addition, turning to these new approaches could solve certain issues, which include disturbing animals by removal from their well-defined social groups and conflict with the public and certain wildlife interest groups. These ideas in reducing fertility could provide to signify an adequate method for management of excess animals and wildlife (Kirkpatrick et al., 1993).
II. Literature Review

A. General Introduction to the Japanese Quail

The Japanese quail has gained wide acceptance in the poultry industry in the United States for the easy manipulation of its reproduction system. The Japanese quail, also referred to as the *Coturnix coturnix japonica*, was projected by Wilson *et al.* (1959) and Padgett and Ivey (1959) as a proposed animal for avian research (Wilson, 1986). Since then, these quail have been utilized throughout the world for various studies, including increased fertility as well as nutrition. For example, Vohra (1971) examined research for the nutrition of Coturnix as this aids scientists and researchers in proper feeding regimens for use of these birds as an experimental animal.

The Coturnix Quail are small galliforms which generally live 2-3 years in their natural habitat (Ottinger, 2001). For several centuries in Japan, a semi-domesticated strain was introduced as a pet, for animal protein and eggs, and additionally as a singing bird (Fitzgerald, 1969). The Japanese quail that were cultivated and brought to the United States are considered to be post-World War II birds, in that their introduction to the United States was after this time (Crowford, 1990; Kovach, 1974; Wakasugi, 1984).

Japanese quail are considered to possess high fertility, egg production, and reproductive competence under a stimulated environment of continuous light (16L:8D) (Ottinger, 2001). On average Japanese quail reach puberty at about 6-8 weeks of age and arrive at full adult functions within another 2-3 weeks. Hsu *et al.* (1986) conducted a study to determine the effects of long-term testosterone replacement on copulatory activity in old male rats. They found that the hypothalamus continues a reaction to exogenous steroid hormones. This in turn aids in restoring reproductive activity in male
rats (Hsu et al., 1986). From the previous determination by Hsu et al. (1986), Ottinger (2001) was able to use these findings in restoring reproductive behavior in senescent (old) male Japanese quail.

B. Anatomy of the Reproduction System of the Male Quail

1. Male Japanese Quail

The male reproductive system consists of major structures such as the testes, epididymis, and seminal vesicles. In addition they possess an accessory organ, located at the distal end of each ductus deferens (Fitzgerald, 1969). The male reproductive system also consists of the papillae of the ductus deferens, which is located in the cloaca, and the rudimentary penis (Fitzgerald, 1969). All of these organs function together in addition to the endocrine system to deliver vital sperm to the exterior of the bird (Fig. 1).

Fig. 1. A diagram of the testis and epididymis of the bird showing the various types of ducts. Not drawn to scale. (Aire, 2000)
2. Testicular Physiology

The testicles are white in appearance, and have a shape similar to that of a kidney bean. They also have a vascular blood supply, to provide nourishment and oxygen. Their location is within the body cavity, caudal to the lungs and abdominal air sacs (Fitzgerald, 1969).

The histological composition of the testis of adult quail is comparable to that of the domestic rooster (Marvan, 1969; Lake, 1957). These testes appear very large, when compared to the quail’s body size, because they increase immensely during the breeding season (Fitzgerald, 1969). Inside the testis are a considerable number of cells. Histologically, the seminiferous tubules of galliforms are lined with stratified epithelium containing different types of germinal cells (Sturkie, 1967; Maruch et al., 1991). Sertoli cells are within the tubules, which are differentiated into spermatogenic cells. Beginning from the wall of the seminiferous tubules and moving to the seminiferous tubular lumen, the following structures are present: spermatogonia, primary spermatocytes (I), secondary spermatocytes (II), spermatids, and sperm ‘tufts’ (Nagano, 1962, McIntosh and Porter, 1967; Nicander, 1968; Maruch et al., 1991). In the testis of the quail, as in that of the rooster, the testicular stroma is fine, distinguished by a fine tunica albuginea, and by the lack of septa and mediastinum. A feature that is characteristic of birds is that the testes are located within the body cavity (intra-abdominally) (Lake, 1971). In between spermatozoa producing tubules is the interstitial space which contains different cells, known as Leydig cells. Leydig cells of the interstitium are responsible for testicular androgen production, such as testosterone (Kirby and Froman, 2000).
3. Accessory Ducts

Male Japanese quail also possess accessory ducts, one being the vas efferens, which is fairly short and positioned outside the testicles (Fitzgerald, 1969). The epididymis of birds is an involved organ, composed of various ducts such as the rete testis, efferent ducts, connecting ducts, and the epididymal duct (Lake, 1957; Tingari, 1971, 1972; Hess et al., 1976, 1977; Aire, 1979). The rete testis functions to drain spermatozoa to the epididymis, by way of the efferent ducts. It is now recognized with the maturational course of spermatozoa as they pass along the ducts, that by the time the spermatozoa reach the epididymis they have matured and gained the ability for both motility and fertility (Robaire and Hermo, 1988; Cooper et al., 1990). Following this is the ductus deferens, which occurs as a folded mucosa, and is known as the excurrent duct of the testis (Kirby and Froman, 2000). This duct ends with an expanded lumen for limited sperm storage. It particularly functions to carry sperm from the testes to the cloaca.

4. Accessory Organs

The accessory organs consist of the paracloacal bodies, which originate on the side of the deferent duct’s collecting end; dorsal proctodeal gland; and lymphatic folds (Fujihara, 1992). The vascular paracloacal bodies are necessary for lymphatic tissue production, for these are the sites where lymph is produced by filtration of the blood (Fujihara, 1992). The dorsal cloacal wall protrudes extensively during the breeding season and at this time a sizable amount of frothy white secretion is apparent (Wilson, 1986). In a study conducted by Siopes and Wilson (1975), they wanted to describe, in
Japanese quail, the quantitative relationship between the cloacal gland size and testis weight during the stages of initial growth, involution, and recrudescence (restarting of an activity, after being inactive). They determined that this gland is considered distinctive, because the production of a meringue-like foam and bulge is caused by the growth of glandular tissues lining the cloaca (Coil and Wetherbee, 1959) and the presence of this foam is a good indicator of spermatogenic activity in Japanese quail (Siopes and Wilson, 1975). Furthermore, studies were done by Nagra et al. (1959) determining cloacal gland response of sex steroids in the Japanese quail and by Sachs (1969), determining photoperiodic control of reproductive behavior and physiology of the Japanese quail. During these studies both researchers administered testosterone to coturnix in which the cloacal gland was removed. This proved to result in hypotrophy (reduction) of the glands and to induce the appearance of secretory material within the cloacal gland. It was also shown by Nagra et al. (1959) that males who were castrated of the cloacal gland had a decrease in the size of the cloacal wall, and showed few secondary folds. It was concluded that the cloacal gland of the coturnix is androgen dependent, and can be used as an indication of changes in the photoperiod as determined by Sach, 1967 and 1969).

5. Hormonal Control of Male Reproduction

The regulatory centers in the extra-hypothalamic areas of the brain are influenced by hypothalamic secretion of gonadotropin releasing hormone (Ottinger, 1983). Luteinizing hormone releasing hormone (LHRH) is synthesized and secreted from the hypothalamus and triggers the secretion of luteinizing hormone (LH) from the pituitary
gland in the brain. The LH that is secreted then stimulates androgen synthesis and secretion from the testes of males. Gonadotropins exert their effects on the testis by binding to specific cell-surface receptors on two distinct types of testicular cells: Leydig cells and Sertoli cells. Leydig cells contain the steroidegenic enzymes necessary for the production of androgens, and it is LH that specifically acts on these cells (Johnson, 1986). The major testicular androgen secreted is testosterone, which then affects male target tissues, such as the cloacal gland in the male quail or the comb and wattles in the rooster (Ottinger, 1983). Follicle stimulating hormone (FSH) influences the Sertoli cells and is controlled by testosterone (Tsutsui and Ishii, 1978). Brown and Follett (1977) conducted an experiment to determine the effects of androgens on the testes of intact and hypophysectomized Japanese quail and concluded that LH and FSH are important for spermatogenesis in galliform birds. This was evident in the inability of exogenous testosterone to maintain spermatogenesis in hypophysectomized quail. Spermiogenesis is defined as the transformation of spermatids into mature sperm cells without further cell division.

Testosterone reaches target areas in the brain and acts to regulate gonadotropin secretion and stimulate reproductive behavior by action on the specific areas of the brain. Testosterone in the mature male is needed for spermatogenesis, to maintain the excurrent ducts and secondary sexual characteristics of the male, the expression of certain male behavior, and determining the pattern of GnRH secretion.

C. Reproductive Anatomy of the Female Japanese Quail
Female Japanese quail possess a solitary left ovary and corresponding oviduct, but a rare functioning right ovary and oviduct may be present (Johnson, 2000). The always present left ovary is connected by the mesovarian ligament at the anterior end of the left kidney. For the duration of embryonic growth in birds, estradiol seems to be vital in the sexual differentiation of females in development of the ovary, oviduct, and accessory organs associated in a number of female species (Ottinger and Abdelnabi, 1997). Because of the differentiation during embryonic development, due to distribution of primordial germ cells to ovaries of the bird, very different reproductive organs evolved for females from that of males. In female Japanese quail, the reproductive tract is quite similar to that of all female galliform species, but with some small modifications.

1. The Ovary

The ovary of the female quail contains approximately 2,000 follicles visible to the naked eye, (Johnson, 2000) and of which vary in size in a distinct hierarchy based on their order of maturity (Fitzgerald, 1969). These ovarian follicles comprise most of the ovary’s tissue, but it also contains a restricted mass of stroma and smooth muscle (Fitzgerald, 1969). The prehierarchal and hierarchal follicles are similar to those of the hen, in that there are four to six large yolk-filled follicles followed by smaller follicles containing a white deposition (Johnson, 2000).

2. The Ovarian Follicle

The viable ovum is comprised of a nucleus and a thin circle of cytoplasm, with the follicular membranes providing a case for the yolk and yolk deposition (Fitzgerald, 1969).
The membranes surrounding the yolk are the vitelline membrane, the zona radiata, and the zona granulosa also known as the granulosa cell layer (Fitzgerald, 1969). The follicle is greatly vascularized, except for the stigma, which is the avascular zone around the circumference of the follicle and which is the site of membrane rupture right before the onset of ovulation (Nalbandov and James, 1949). The major arteries such as the caudal aorta and ovarian artery are intended for the immediate growing follicles which eventually branch into arterioles (Fitzgerald, 1969). These go through the theca and basal lamina and materialize into arterial capillaries (Dahl, 1970).

According to Bacon and Koontz (1971) “the sequence of events occurring during follicular growth and maturation in the Coturnix, is of shorter duration, yet quite similar to that found in chickens and turkeys.” As the follicle matures, the granulosa layer cells become squamous in shape and form a single layer (Johnson, 2000). Previous studies done by Huang et al. (1979) and Mori et al. (1984) demonstrated that the crucial supply of progesterone in the chicken and Japanese quail is the granulosa layer of the prevalent (F1) follicle. Important biochemical pathways occur in relation to follicular development, in that progesterone increases as a result of luteinizing hormone (LH) (Huang et al., 1979; Hammond et al., 1981; Marrone, 1989).

3. The Oviduct

The oviduct of the female Japanese quail is similar to that of the hen in that it is developed from the left Mullerian duct (Johnson, 2000). The oviduct is comprised of five distinctive areas: infundibulum, magnum, isthmus, shell gland, and vagina (Fig. 2). It is within the infundibulum that fertilization takes place. Birkhead et al. (1993, 1994)
determined a greater proportion of inseminated sperm reach this site in the female reproductive tract than in mammals.

4. Fertilization in Female Quail

Fertilization transpires at the top of the female tract, known as the infundibulum. The time of fertilization occurs within 15 minutes of the ovum being released from the ovary (Howarth, 1984; Perry, 1987; Birkhead et al., 1994). Spermatozoa are collected in the infundibulum during the course of the fertilization. Since sperm storage tubules are positioned at the utero-vaginal junction, this helps in the storage of sperm (Birkhead et al., 1994).

Once freed from the ovary, the ovum is enclosed by the inner perivitelline layer. During the acrosomal (actual fertilization of the ovum by spermatozoa) reaction, this layer is left with large holes, as a result of sperm penetration of the layer (Bakst and Howarth, 1977; Birkhead et al., 1994). This was important in understanding the changes associated with the hen’s perivitelline layer by cock sperm in vitro. After fertilization, the second layer, known as the outer perivitelline layer, is positioned around the ovum; confining sperm present within the infundibulum (Birkhead et al., 1994). This outer layer is considered to act as obstruction to remaining sperm, in addition to sustaining the
Fig. 2. Schematic drawing of an oviduct from a sexually mature bird showing the regions: infundibulum, magnum, isthmus, uterus (shell gland), utero-vaginal junction and vagina. (Holm and Ridderstrale, 2002)
permanence of the ovum already penetrated by the initial sperm (Bakst and Howarth, 1977; Howarth, 1984; Birkhead, 1994). This is important in that birds tend to have several spermatozoa penetrate the inner perivitelline layer of the ovum, but actually only one unites with the females pronucleus (Harper, 1904; Howarth, 1984; Perry 1987). The primary layers of albumen are then secreted around the ovum after fertilization (Birkhead, 1994). Continuation of development occurs in the egg as it moves down the reproductive tract (Birkhead et al., 1994; Johnson, 1986). From ovulation, until actual oviposition (egg lay) is 24 hours (Johnson, 1986).

5. Hormonal control of Female Reproduction

The steroid hormones LH, FSH, progesterone, and estrogens all act together to bring about ovulation of a mature follicle from the ovary in female birds. Usually, a preovulatory surge of LH in the domestic hen occurs 4-6 hr before actual ovulation, which allows for the release of the mature follicle from the ovary (Johnson and van Tienhoven, 1980). Furthermore, FSH functions to stimulate maturation of immature follicles to be eventually released from the ovary. Its primary role is also related to the granulosa cell differentiation and initiation of steroidogenesis (production of steroid hormones) in prehierarchal follicle granulosa cells. A study conducted by Scanes et al. (1977) to observe the ovulatory cycle and to determine a homologous radioimmunoassay for chicken follicle-stimulating hormone, found that a rise in plasma FSH occur 15 hrs before ovulation in the domestic hen.

Progesterone is important in that it has been shown to coincide with the preovulatory peak of LH, and this increase of LH is a result of progesterone secretion by
the largest preovulatory follicle (Etches, 1990). Therefore, progesterone allows the follicle to develop until maturation.

Finally estrogen functions as a sex steroid hormone, which is synthesized in the ovarian follicle, like that of progesterone. It is also imperative in the early development of the females reproductive tract, secondary sex characteristics, and sexual behavior.

D. Hormone Releasing Structures, LHRH/GnRH

The hypothalamus of the domestic hen produces two forms (I and II) of luteinizing hormone-releasing hormone (LHRH) (Sharp et al., 1989), also known as Gonadotropin releasing hormone (GnRH), which are structurally different from mammalian GnRH (m-GnRH) (Sharp et al., 1988). Chicken (c) LHRH-I was isolated in 1982 by King and Millar (1982) and by Miyamoto et al., (1982). Moreover, c-LHRH-II was recognized by Miyamoto et al. (1984). Both of these neuropeptides luteinizing hormone and follicle stimulating hormone are released in chickens (Hattori et al., 1986). In the course of numerous studies, it has been questioned whether c-LHRH-II (c-GNRH-II) is more biologically effective in chickens than c-LHRH-I (Chou et al., 1985; Millar et al., 1986; Sharp et al., 1986; Sharp et al., 1987). Yet, studies these studies have found that c-LHRH-I is more biologically efficient in promoting LH and FSH within that of the chicken. This suggests that c-GnRH-I is more important than that of c-GnRH-II for stimulation release of both LH and FSH.

According to Millar et al. (1986, 1989), the non-mammalian GnRHs have weak receptor-binding and gonadotropin-releasing action in sheep and rat pituitary cells, yet show signs of high activity in chicken bioassays.
It has also been proven that the non-mammalian GnRHs all vary from mammalian GnRH by the replacement of a neutral amino acid for the Arg in position 8 (Jacobs et al., 1994). Because of this observation, it has been concluded that Arg$^8$ is critical for activity in mammals (Jacobs et al., 1994). However, in non-mammalian vertebrates, the pituitary GnRH signals are less restricted and accept basic and neutral amino acids in the arrangement (Millar and King, 1987; Millar et al., 1989).

1. Mammalian GnRH

It has been well established that GnRH is essential to the fundamental patterns of hormone release. It is a neuropeptide that regulates the synthesis and secretions of pituitary gonadotrophins (Rissman et al., 1995). GnRH is synthesized by neurons located in the preoptic area of the hypothalamus and is transported to terminals which are located in the median eminence (Juorio et al., 1991). The hormone is released into the blood of the hypothalamic-pituitary portal system that carries it to the anterior pituitary where the hypophyseal gonadotropic cells respond with the release of luteinizing (LH) and follicle-stimulating (FSH) hormones (Schally, et al., 1971). Once GnRH is released, it regulates the function of the testes and ovaries (Sun, et al., 2000).

Because GnRH controls the reproductive process, its analogs can then be used either to enhance, or subsequently to decrease, reproductive efficiency in animals. Its investigation has provided an understanding of the mechanisms of hormone release, as well as applications of responsiveness to the target glands regulated. It has been proven that the biological half-life is very short, which is measured in a time span of minutes (Peczely, 1989).
a. Receptors and Mechanism of Action

The main site of GnRH action is the gonadotrope of the anterior pituitary gland. Furthermore, additional binding sites have been located in the adrenal and central nervous system (Reubi et al., 1987).

The mechanism of action requires the first step which is the release of GnRH. Next is the binding of GnRH to receptors on gonadotropes. These receptors are found within the plasma membrane portion of the cell (Marian and Conn, 1983).

Sialic acid residues on GnRH receptors are needed both for activation of the receptor and for appearance of receptors on the cell surface (Schvartz and Hazum, 1985). Schvartz and Hazum (1985) indicated that for binding to occur, both exterior hydrophilic head group, and fatty acids linked to the β-carbon of phospholipids must be present.

It has been proven that the GnRH receptor is linked to a G-Protein, because two carboxylic groups and two aromatic amino acids appear to be the ligand binding portion of the receptor (Hazum, 1982). Mammalian gonadotropin-releasing hormone receptors differ from other G protein-coupled receptors in that they lack the intracellular C-terminus, and show an exchange of two residues, Asp (D) and Asn (N) in transmembrane domains (Blomenrohr et al., 1997).

b. Importance of GnRH in Reproduction

Usually there are seasonal cycles in the hormone concentration, which stimulate specific breeding seasons in animals. GnRH is secreted from the hypothalamus, which then causes the secretion of two gonadotrophic hormones, FSH and LH, from the adenohypophysis in both male and female mammals (Arimura & Schally, 1974;
Awoniyi et al., 1988). These hormones have effects on the testes in males and the ovaries in females. In males LH stimulates androgen production by the interstitial cells and FSH acts on the seminiferous tubules to increase sperm production. In females FSH and LH act on the ovaries for follicular development; LH in addition causes progesterone production. Ishii (1993) determined that LH and FSH are glycoprotein hormones, with molecular weights of 37-38 kDa and 23.5-25 kDa, respectively (Hattori and Wakabayashi, 1979; Sakai and Ishii, 1980; Krishnan et al., 1992). Finally, it has been shown that GnRH can function as a neuromodulator, and in various species administration of an exogenous GnRH can cause sexual behavior (Pffaff, 1973; Cheng, 1977; Dudley and Moss, 1987; Jones 1987; Moore et al., 1987; King and Millar, 1992; Sherwood et al., 1993).

Thus far, at least eight forms of GnRH have been isolated from vertebrate brains (Miyamoto et al., 1984; Lovejoy et al., 1991; King and Millar, 1992; Sherwood et al., 1993; Sower et al., 1993), two of which include chicken GnRH-I (c-GnRH-I) and chicken GnRH-II (c-GnRH-II) (Fig. 3).

**Mammal**

\[
\text{pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH}_2
\]

**Chicken I**

\[
\text{pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH}_2
\]

**Chicken II**

\[
\text{pGlu-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly-NH}_2
\]

Fig. 3. Structure of vertebrate GnRH's. Illustrating different amino acid positions. (Millar et al. 1988).

c. Neural Endocrine Importance in Male Quail

The neural endocrine system in the male quail provides assistance in controlling reproduction by special feedback mechanisms. Gonadotrophin releasing hormone
(GnRH) provides the primary key for action within this system. In review of this system for the male Japanese quail, the hypothalamic secretion of GnRH is coordinated by the feedback outcome of sex steroid hormones, and these same hormones put forth their effects at the adenohypophysis level, modulating their effects of GnRH for the production of gonadotrophic hormones (Peczely, 1989). According to Hattori et al., (1985), gonadotrophin secretion in birds from the hypophysis is under primary control by a hypothalamic neurosecretory hormone, gonadotropin releasing hormone (GnRH), as is found in mammals. GnRH is secreted from axons within the median eminence of the hypothalamus. The GnRH secreted by these axons goes to certain target cells located in the adenohypophysis, by way of the hypothalamo-hypophyseal portal vessels (Gilbert, 1979; Kirby 2000). There it binds to specific receptors on gonadotropes located throughout the adenohypophysis (Mikami and Yamada, 1984).

Found within the gonadotropes are secretory granules that contain FSH and LH, which are released in reaction to GnRH (Hattori et al., 1985). Moreover, Follett (1970) recognized that chicken and quail hypothalamic secretions stimulated LH release from adenohypophysial halves. The gonadotropes are necessary for reproduction, in that results of Hill and Parkes, (1935), Brown and Follett (1977), Tanaka and Yasuda (1980), revealed a decrease of gonadotropins as a consequence of an adenohypophysectomy. Resulting from such procedure, additionally, is a collapse of the seminiferous epithelium, testicular decline, and minimization of the excurrent ducts.

d. Neural Endocrine Implications of the Female Quail
17β-estradiol (E2) and testosterone are produced and secreted by the gonads and adrenal glands throughout avian embryonic growth. Furthermore, they influence maturation and differentiation of the sex accessory gland makeup (Teng and Teng, 1979; Huston et al., 1985; Abdelnabi, 2000). According to Adkins (1979, 1985) and Schumacher et al. (1989) these same steroids are thought to coordinate those regions of the hypothalamus that control the neuroendocrine and behavioral components of reproduction (Abdelnabi, 2000).

GnRH secretion has been shown to regulate its effects on sexual steroids (Peczely, 1989). A study conducted by Kawashima et al. (1981) demonstrated that progesterone increases GnRH release by positive feedback mechanisms in birds. He was able to make this determination because of increased GnRH activity within the hypothalamus after an injection of progesterone.

Preovulatory peaks of luteinizing hormone (LH) correspond with changes in the GnRH source in the hypothalamus as studied in domestic hens (Peczely, 1989). According to Johnson and Advis (1985), the GnRH found within the hypothalamus is greater in hens laying eggs than in molting hens. They were able to determine that the GnRH content of the medial preoptic area increases during the preovulation period. However, when progesterone was given and GnRH measured, this caused a decrease in GnRH within the medial preoptic area (Johnson and Advis, 1985). Therefore, the reporting indicated that progesterone has an influence on LH by increasing GnRH secretion and controlling ovulation.

2. Chicken GnRH-I
Sharp et al. (1990) determined that measurements of GnRH (both c-I and c-II) in the chicken brain offer substantiation for their role. He was able to determine that c-GnRH-I is critical in the control of gonadotropin release in birds. The chemistry of c-GnRH-I is: pGlu-His-Trp-Ser-Tyr-Gly-Leu-Gln-Pro-Gly-NH₂ (King and Millar, 1982). Neurosecretory terminals within the median eminence release c-GnRH as a result of several stimuli (Scanes, 2000). One stimulus occurs during the photoperiodic induction of reproduction (Scanes, 2000). Another stimulus for release of GnRH is progesterone during the preovulatory surge (Scanes, 2000). Knight et al. (1984) and Millian et al. (1984) established that neurotransmitters are directly or indirectly involved in the influence of c-GnRH-I release. Norepinephrine appears to be the primary neurotransmitter, and it appears to act via α₁-adrenergic receptors (Knight et al., 1982 and Milliam et al., 1984).

The biological half-life of c-GnRH-I was determined (in vivo) by Sharp et al. (1990) for the domestic hen and cock. Sharp et al. (1990) determined that the biological half-life of c-GnRH-I in both female and male chickens is parallel, approximately 3 minutes. Moreover, through immunocytochemical studies done by Mikami et al. (1988), it was determined that the fractions of c-GnRH-I and II in the chicken hypothalamus are not identical. They determined the most significant difference was that the exterior layer of the median eminence contained c-GnRH-I, but not c-GnRH-II. Because of this finding, it has been suggested that c-GnRH-II may not be a structurally significant gonadotropin-releasing factor.

3. Chicken GnRH-II
Chicken GnRH-II is found within the cell bodies of lateral hypothalamus (Scanes, 2000). It is produced by a discrete population of neurons in the midbrain (Munz et al., 1981; Amano et al., 1991; Wright and Dernski, 1991; Dellovade et al., 1993; van Gils et al., 1993). Chou et al. (1985) and Millar et al. (1986) demonstrated that c-GnRH-II is more effective than c-GnRH-I in secreting LH release from chicken pituitary cells in vitro. However Chou et al. (1985) and Sharp et al. (1987) found little variation for in vivo action. It has been determined that more than one form of GnRH is present in individual species in most vertebræ studies (Millar et al., 2001). Of these, it is the structure isolated from chicken brain (c-GnRH-II) which is found to be universally present and uniquely conserved between taxa from boney fish to human (Sherwood et al., 1993; King & Millar, 1997; Sealfon et al., 1997; White et al., 1998).

Because of studies conducted by immunization with c-GnRH-II, it has been shown by Sharp et al. (1988) that GnRH-II is not a physiological important gonadotropin releasing hormone. This is further suggested by studies in which immunized laying hens against GnRH-I, ceased laying eggs; however, when hens were immunized against GnRH-II, they continued egg production (Sharp et al., 1988). In addition, studies by Millar and King, (1983), Miyamoto et al. (1984) and Millar et al. (1986), have shown that this peptide is simply one-third less successful than mammalian GnRH in stimulating gonadotropin release from chicken cells in vitro. Finally, evidence from radioimmunoassay (RIA) in chickens, along with high-performance liquid chromatography (HPLC), indicated that low amounts of c-GnRH-II in the hypothalamus and median eminence were present, but that elevated levels of c-GnRH-I were within the same area (Katz et al., 1990; Sharp et al., 1990). In light of the studies carried out and
data obtained, it appears that GnRH-II can cause gonadotropin release, but that its key function in some species may not be to cause gonadotropin hormone release. C-GnRH-II seems to be a phylogenetically ancient molecule that deviated from its ancestral genetic material early in vertebrae evolution to form a separate evolutionary branch, in that it is the most widely distributed form of the GnRHs (King and Millar 1991; Lovejoy et al., 1992).

F. The Growing Need for Wildlife Fertility Control

Because wildlife contraception can be a potential management tool for overabundant populations, contraception research is being done outside of State and Federal agencies (Guynn, 1997). Since hunting and natural mortality alone cannot manage wildlife populations, there is developing demand for advancement of non-lethal systems for population control of both free-roaming and captive wildlife (Becker and Katz, 1997). Wild horse herds in the western United States show evidence of growth rates that often exceed 15% per year (Garrott et al., 1991). This growing rate can result in population sizes that cause concerns about long-term habitat damage and territorial occupation (Gross, 2000). For example, the numbers of non-migrating (resident) Canada geese are escalating (Ankney, 1996). As the number resident geese increase, this causes problems of fouling water supplies, lawns, beaches, and golf courses with excreta, in addition to flocks feeding and damaging crops such as corn, soybeans, rice and wheat (Conover, 1988).

In the early 1960's, efforts were directed to controlling wildlife populations by use of oral contraceptives in the U.S. (Linhart et al., 1997). The animals targeted were the
coyote (*Canis latrans*) in the West because of their depredation of livestock, and the red fox (*Vulpes vulpes*) during the 1960’s, as they were the principal cause of rabies in the Northwest. Traditionally, the means of population control consisted of direct methods. This was done by lethal means of shooting, poisoning, trapping, gassing of dens, and habitat modification (Lewis, 1968; U.S. Department of Agriculture, 1992). However, these means of population control are viewed currently as undesirable as well as objectionable. Fertility control for excessive numbers of wildlife and domestic animals have now turned to contraception, in that it is considered a more humane and economical resolution to overpopulation than that of lethal methods (Maggitti, 1993).

**G. Non-Invasive Fertility Control Techniques**

1. **Nicarbazin**

   Increasing attention has turned to Canada geese and other avian species, because of their overabundance. The United States Department of Agriculture, Animal & Plant Health Inspection Service Wildlife Service (APHIS) has recently proposed a study using Canada geese in researching the use of Nicarbazin as a means of controlling avian populations for airport safety (Primus et al., 2001). Nicarbazin is an FDA-approved drug used to treat and prevent coccidiosis in broiler chickens. When once accidentally fed to breeder chickens, it was noticed that it reduce egg hatchability. Therefore, because of this occurrence, APHIS evaluated nicarbazin diet concentrations and how it correlated with blood levels in geese, to determine if this can be used as a potential antifertility substance. They found that the methods of determining nicarbazin (DNC) in avian plasma present were reliable.
2. DiazaCon

The National Wildlife Research Center also conducted a study in 2001 to test the effectiveness of DiazaCon in Japanese quail as a fertility reducing agent (Johnston, 2001). The purpose of the study was due to the lack of published analytical methodology for quantitating DiazaCon in feed and blood sera. Because cholesterol is a precursor for reproductive hormones synthesis, DiazaCon was used to potentially reduce the level of cholesterol available for hormone production. They were able to conclude that DiazaCon (azacosterol, ornitrol, 20,25 diazacholesterol, diazasterol) can be used as a contraceptive for nonlethal control of overabundant bird populations.

3. Immunocontraception

A recent study was carried out by Vizcarra et al., (2001) to determine whether immunization of male broiler breeders against mammalian gonadotropin releasing hormone (GnRH), proved to be effective in reducing fertility. The study was carried out for eighteen weeks in which 50 male broiler breeder birds were selected at 8 weeks of age and randomly assigned to treatments for immunization against either m-GnRH, c-GnRH-I, c-GnRH-II, or BSA (bovine serum albumin) alone. The GnRH peptides were conjugated to BSA (bovine serum albumin) and emulsified in Freund’s incomplete adjuvant and diethylaminoethyl-dextran (DEAE). Primary immunization was given at 10 weeks of age, and booster immunizations were given at 13, 16, and 24 weeks of age. It was concluded that immunization against mammalian GnRH significantly reduced testis
weight, testosterone, and β-FSH expressed in the pituitary compared to c-GnRH-I. And that c-GnRH-II was ineffective in affecting these variables.

In addition, GnRH vaccines have been developed, in which a modified molecule is prepared and is supposed to stimulate antibodies against the native GnRH (Killian et al., 2001). These antibodies inactivate GnRH secreted from the hypothalamus and discontinue the normal sequence of hormone release that is necessary for gonad control and gamete formation (Killian et al., 2001). An actual production of antibodies against GnRH has been accomplished in a number of species, including rabbit (Arimura et al., 1973), rat (Fraser et al., 1974), marmoset monkey (Hodges and Hearn, 1977), sheep (Clarke et al., 1978), and chickens (Vizcarra, 2001). This active immunization against GnRH, results in disruption of reproductive function characterized by low serum levels of gonadotropins and gonadal steroids, followed by gonadal suppression (Fraser, 1980). In addition, immunization against GnRH and its analogues (Ferro and Stimson, 1999) has been shown to have physiological effects on fertility in both males and females (Ferro et al., 1995; Ferro and Stimson, 1997, 1999).

Immunization against GnRH prevents circulating GnRH from binding to pituitary receptors and therefore, obstructs release of the two gonadotropins, FSH and LH (Talwar, 1985). According to Fraser (1982), it is thought that the most apparent site of action of antibodies to releasing hormone is within the hypophyseal portal blood constituent. Moreover, these antibodies can proceed at the level of the gonadotroph cell membrane, or possibly gain access into the median eminence where LHRH is accumulated in nerve terminals prior to secretion (Hokfelt et al., 1976). Belchetz et al. (1978) determined that a constant dose of anti-GnRH at high levels, reduces gonadotropin secretion, and results in
loss of gonadal function. It is thought that pituitary desensitization is caused by a loss of pituitary cell-surface receptors for GnRH by internalization of occupied receptors (Conn and Crowley, 1991). Later, as these receptors recover (Hazum and Conn, 1988), desensitization is sustained because the receptors become dissociated from their second messenger system (Conn and Crowley, 1991).

The decline or lack of serum FSH and LH leads to a regression of gonads and parallel infertility in both sexes, causing non-surgical castration in animals (Miller et al., 2000). Immunosuppression of testicular function has been established in lab settings and domestic animals by active immunization against gonadotropin releasing hormone (Keeling and Crighton, 1984). Immunization using GnRH seems to be specific, and does not appear to affect other hypothalamic releasing hormones (Miller et al., 2000). It has been shown by Awoniyi et al. (1993), that rats immunized with GnRH did not release FSH and LH, but that other pituitary hormones and their associated non-sexual actions were not impaired. In most studies conducted, active immunization against GnRH caused only a temporary suppression of reproduction, in that after a period of time, animals returned to their normal fertility (D'Occhio 1993, 1994). Moreover, a decline of circulating anti-GnRH antibodies, which is below the threshold requirement to neutralize GnRH in the hypophysial portal blood, caused animals to return to normal reproductive function (D'Occhio, 2001).

H. The Immune Response

An immune response is the result of a complex system of events involving antigen and at least three types of lymphoid or reticuloendothelial cells (Dresser, 1986). Antigen
is referred to the substance proficient in prompting an immune response (Sela, 1986).

The capability to cause an immune response depends upon the interaction between the chemistry of the antigen and the physiological state of the host (Sela, 1986). Most often, antigens involve T cell helper (T_H) to promote B cells into antibody formation (Dresser, 1986). A typical feature of the immune response, further known as a secondary response, occurs when a high number of memory cells rapidly respond to a second injection of antigen (Dresser, 1986). Because the immune response to a specific antigen is coordinated by intercellular interactions between T and B cells, the reaction to some antigens includes lymphocyte production that has been studied in vitro (Pearce et al., 1981; Dean et al., 1984; Aboudkhil et al., 1991; Olsen et al., 1991). Distinctive from mammals, the chicken has a unique organ, the bursa of Fabricius, in which the maturation of B lymphocytes occurs (Glick et al., 1956). Maturation of an additional major lymphocyte, T cells, takes place in the thymus, which is an attribute shared with the mammalian species (Glick et al., 1956).

Immune responses generally involve antigens, "in that an antigen indicates any molecule that can be distinctively recognized by the adaptive element of the immune system, which is by T cells or B cells" (Roitt et al., 1969). T cells that develop in the thymus do not identify foreign antigens independently (Arstila et al., 1994). According to Arstila et al. (1994), the antigen has to be introduced by host cells as an antigenic peptide bound to a major histocompatible complex (MHC) molecule. Because antigen-presenting cells initiate the T-cell response, this allows for activation of the CD4+ cells. These cells in turn induce the immune response (Lassila et al., 1988; Vianio et al., 1988).

A. Introduction

The once new technologies, such as implementing methods for increased fertility and fecal hormone analysis that were created for conservation of animals have now turned to developments of fertility control research (Kirkpatrick, 1993). For example, wildlife refuges and national parks that protected animals by laws and regulations, and the absence of large numbers of predators, resulted in populations of wildlife that occasionally exceed the habitats’ biological carrying capacities (Kirkpatrick, 1993).

Two recent technologies include wildlife contraception and non-invasive control. Of the animals not captured (non-invasive), the research is based on analysis of urinary or fecal hormone levels (Kirkpatrick, 1993). This has proven useful in gathering hormone levels before a target group of animals is considered for fertility control. One of the most successful contraceptives formulated has been the porcine zona pellucida (PZP) (Miller, 2000). This particular immunocontraceptive has been used with the feral horse and the white-tailed deer. In addition, immunocastration mechanisms have been used with sheep, swine, cattle, and pets (Miller, 2000). For example, Schanbacher (1982) conducted a study to determine the response of ram lambs to active immunization against testosterone and luteinizing hormone release. In addition, Zeng et al. (2001) investigated the efficacy of a newly developed anti-GnRH vaccine in Chinese pigs, under practical Chinese pig farming conditions. They were able to determine that this developed anti-GnRH vaccine worked well within Chinese pig farming conditions,
and can be used as an alternative to surgical castration of young male pigs. Based on these findings, and those of Vizcarra et al., (2001) the objective of the present study was to compare m-GnRH with c-GnRH-I and c-GnRH-II, with regard to reducing fertility in male and female Japanese quail. Their study was carried out for eighteen weeks in which 50 male broiler breeder birds were selected at 8 weeks of age and randomly assigned to treatments for immunization against either m-GnRH, c-GnRH-I, c-GnRH-II, or BSA (bovine serum albumin) alone. The GnRH peptides were conjugated to BSA (bovine serum albumin) and emulsified in Freund's incomplete adjuvant and diethylaminoethyl-dextran (DEAE). Primary immunization was given at 10 weeks of age, and booster immunizations were given at 13, 16, and 24 weeks of age. It was concluded that immunization against mammalian GnRH significantly reduced testis weight, testosterone, and β-FSH expressed in the pituitary compared to c-GnRH-I. And that c-GnRH-II was ineffective in affecting these variables.
B. Materials and Methods

1. Experimental animals

Fertile wild type Japanese quail eggs were obtained from Dr. B.C. Wentworth at the University of Wisconsin. The eggs were incubated and allowed to hatch at the University of Nebraska-Lincoln poultry complex. Once hatching occurred, chicks were transferred to a Petersime\(^1\) chick battery modified for quail and raised for two weeks. After reaching suitable age (6 weeks) and size (juvenile), they were housed four to a pen in a 6 X 10 (dimensions 65" X 24" X 73") quail cage/layer unit. Once sexual maturity was reached (2 months of age), birds were sexed and housed in male/female pairs. All birds were provided food and water \textit{ad libitum}, and housed under 16 h light: 8 h dark. A total of 80 quail were used for the study. The birds used in the study remained housed in the same pen to prevent injury due to territorial defense behavior.

2. Experimental protocol for immunization treatment

At the beginning of the study, a total of 80 birds were selected. A three month timeline was given for this study, beginning in the month of May, 2002. The birds were randomly assigned metal leg bands, numbered 1-80, (National Band and Tag Co)\(^2\). Treatment of birds was determined by the band number, and the treatments were applied to 20 birds/treatment. There was a total of four treatments (mammalian-GnRH, chicken GnRH-I, chicken GnRH-II, and controls). Before the initial injection of the

\(^1\) Petersime Inc. Genysberg, OH.
\(^2\) National Band and Tag Co., Newport, KY.
contraceptive/conjugated peptide, body weights were obtained and a plasma sample was collected from the jugular vein of all birds.

Birds were then given an initial injection of 30 µg of the peptide GnRH conjugated to KLH (Keyhole limpet hemocyanin) emulsified in 200 µL titermax gold. The injection was given intramuscularly in the breast. Care was taken not to puncture the heart with each injection. Two booster injections followed, every four weeks, with each bird receiving half of the initial injection of peptide; 15 µg conjugated peptide, emulsified in 200 µL titermax gold. The treatments were 1) mammalian (m)-GnRH, 2) chicken (c)-GnRH-I, 3) chicken (c)-GnRH-II, and 4) control (200 µL) titermax gold alone. Plasma samples were collected one week after initial injection and biweekly thereafter, and stored at -20 °C until assays could be conducted.

3. Antibody Determination

An ELISA (Miller et al., 2000) was used to assess the immune response to the GnRH vaccine. A 96-well plate was prepared by adding 25 µg/ml of BSA-GnRH antigen to each well and allowed to incubate overnight at 37 °C (Appendix A). Since KLH-GnRH was used in the vaccine, BSA-GnRH was coated onto the plates, thus allowing detection of antibodies to GnRH only. The plates were washed three times with buffer (PBS, containing 0.05% Tween 20) and blocked with 150 µL 1% blocking solution (PBS, containing Egg Ovalbumin) for 1 hr at room temperature in a humidified container (closed Tupperware® container), and washed as above. Quail plasma samples of 50 µL (dilution 1:100 in blocking solution) per well in duplicate were incubated for 1 hr at room temperature, washed three times with buffer, and incubated for 1 hr at room temperature with 1/10,000 dilution of peroxidase-labeled anti-chicken IgY (CytRx Co., Norcross, GA) and then incubated with 1% diaminobenzidine tetrahydrochloride (Sigma) for 1 min. Reactions were stopped with 1% trichloroacetic acid, and plates were washed three times with buffer. The absorbance was measured at 450 nm with a microplate spectrophotometer (Titertek Multiscan Excel, Flow Laboratories Inc., McLean, VA).

3 Commonwealth Biotechnologies, Inc. Richmond, VA.
4 CytRx Co. Norcross, GA.
temperature in the humidified container. The plates were washed three times with PBS-Tween. Rabbit-anti-chicken IgG\textsuperscript{6} was diluted 1:150 in blocking solution and 50 µL/well were incubated for 1 hr at room temperature in the humidified container. The plates were washed three times with wash buffer. Goat-anti-rabbit IgG\textsuperscript{7} was diluted 1:5000 in blocking solution and 50 µL/well were incubated for 1 hr at room temperature in the humidified container. The plates were washed three times with wash buffer and developed with equal parts TMB substrate\textsuperscript{8} (3,3',5,5'-tetramethylbenzidine in dimethylsulphoxide and Peroxidase Solution B (H₂O₂, 0.02% Citric Acid Buffer) 100 µL/well. The reaction was stopped with 100 µL 85% Phosphoric Acid (H₃PO₄) after color development that turned the TMB substrate yellow. The plates were read on an MR 700 microplate reader\textsuperscript{9} for optical density of \(A_{450}\). The duplicate samples were calculated as percent fold increase over background, in that background was considered initial reading in plasma obtained at the onset of the study.

4. Isolation of Testis and Granulosa Layer

At 4 weeks, approximately half of the birds from each treatment were weighed and then euthanized using CO₂. The testes were collected from the males, weighed, embedded in OCT\textsuperscript{10} (tissue tek embedding medium), and put on dry ice for quick freezing of the tissue until histological slides could be made. For the females the granulosa layer was isolated from the largest follicle present (Gilbert et al., 1977; Huang and Nalbandov, 1979; Zakar and Hertelendy, 1980; Tilly and Johnson, 1989; Novero et al., 1991) as follows: the

\textsuperscript{5} Commonwealth Biotechnologies, Inc. Richmond, VA.  
\textsuperscript{6} Sigma Co. St. Louis, MO.  
\textsuperscript{7} Sigma Co. St. Louis, MO.  
\textsuperscript{8} KPL, Gaithersburg, MD.
The follicle was placed in a labeled beaker of ice-cold 1% physiological saline solution, pH 7.4. The follicle was then held by forefingers over a Petri dish of 1% of physiological saline solution, pH 7.4. Insuring that the stigma was facing up, a slit along this area was made with a sharp surgical blade. The follicle was then flipped over to allow the granulosa layer and yolk to fall into the Petri dish. The granulosa layer was identified within the basal lamina and perivitelline layer as particularly white against the yellow yolk. With precision forceps, care was taken to peel this layer from the yolk and wash any residue remaining by gently shaking the layer in clean physiological saline.

5. Granulosa Layer staining

Once the granulosa layer, which is sandwiched between the basal lamina and perivitelline layer, was isolated, it was then stained for 3β Hydroxysteroid Dehydrogenase (3-β HSD) activity. The layer was incubated according to Huang and Nalbandov (1979) but with slight modification. The granulosa layer for approximately half of the birds at the midpoint of the study was collected in each treatment (m-GnRH, c-GnRH-I, and c-GnRH-II, control). It was then placed in 1.5 mL 3-β HSD stain (PBS, Pregnolone (P5), β-Nicotinamide Adenine Dinucleotide, and Nitroblue Tetrazolium) for 90 minutes. The layer was then mounted on glass microscope slides, and examined for staining of 3-β HSD active cells. No statistical analysis was conducted on these slides during the midpoint of the study; they were examined for 3β HSD activity, but the staining quality was not sufficient to allow for quantification.

9 Dynatech Laboratories, Europe
10 Sakura Co. Torrance, CA.
6. Granulosa cell dispersion

The female Japanese Quail remaining at the end of the study were euthanized using CO₂, and the granulosa cell layer was isolated as described above; however, the cells were dispersed instead. After separation of the layer, it was then dispersed enzymatically (Huang and Nalbandov, 1979) with some modifications. The granulosa layer was moved to a Petri dish containing incubation media (0.5 mL culture Medium RPMI with L-glutamine, 0.2% D-(+)-Glucose, 0.2% bovine serum albumin, 0.2% sodium bicarbonate, 0.01% trypsin inhibitor, 1 ml penicillin-streptomycin, and 99 ml dH₂O, at a pH of 7.4). The layer was then minced into smaller portions by a multipipette, by suctioning the layer multiple times and releasing it back into the Petri dish. After the granulosa layer was minced, it was transferred along with the incubation media to 15 mL tubes, consisting of 5 mL incubation media and 0.3% collagenase-type II (dispersion media), using a 1 mL micropipette. The cells and media within the 15 mL tubes were aspirated 10 times with the 1 mL micropipette to insure dispersion. Tubes were then incubated vertically in a shaking water bath (70 cycle/min) at 39°C for 30 minutes. During the incubation, the contents in the tubes were aspirated after 15 minutes had elapsed, and again at the end of the incubation. The tubes were centrifuged at 250 x g for 10 minutes at room temperature. Granulosa cells were collected after centrifugation, and washed twice with 8 mL incubation media. A viable cell determination was done by the trypan blue-exclusion technique using a hemocytometer.

7. Staining of Dispersed Granulosa Cells
After cells were dispersed, approximately 10,000 viable granulosa cells from each sample were incubated and stained for 3β-HSD activity using a 24-well culture plate. After incubation and staining of cells for 90 minutes, they were examined with an inverted microscope. Determination of stained cells is described by Gelety and Magoffin (1997), in that a dark blue formazan precipitate is formed when 3β-HSD activity is present within the cell. A count of at least 100 cells per treatment was examined to determine the percentage of cells that stained positive for this activity.

The percentage of positive 3β-HSD granulosa cells was determined by use of the following formula:

\[
\text{Percentage of stained cells} = \left( \frac{\text{Number of cells stained for 3β-HSD}}{\text{Total # of cells examined}} \right) \times 100.
\]

8. Staining Testes to Determine Gross Morphology

Testes stored at -70 °C embedded in OCT (tissue tek), were sliced in cross section (thickness 6 µm) in a Cryostat\(^\text{11}\) at -15 °C. Slides were allowed to dry on a microslide plate dryer\(^\text{12}\), and stained with Meyers Hemotoxylin\(^\text{13}\), counterstained with Eosin, and cover slipped with Permount. Morphological examination of the testicular sections was made under a bright-field microscope using a Spot RT Slider camera and the Scion Image\(^\text{14}\) program.

\(^{11}\) Triangle Biomedical Sciences, Inc. Durham, NC.
\(^{12}\) Fisher Co. Pittsburgh, PA.
\(^{13}\) Sigma Co. St. Louis, MO.
\(^{14}\) Diagnostic Instruments, Inc. Sterling Heights, MI.
9. Hormone Determinations

The concentrations of testosterone in plasma were determined by radioimmunoassay (RIA). Testosterone was validated for quail and assayed using a double antibody Testosterone Kit\(^1\). The assay was validated by pipeting sample volumes of 75, 50, 25, 12.5, and 6.25 µL and bringing the volume to 50 µL, by adding the standard A (0 ng/mL). Each sample volume was used with samples containing low or high concentrations of testosterone. Parallelism was determined by using the Allfit program (DeLean, 1978). Slopes of dilutions of plasma and the standard curve were not different as determined by the Allfit program (P=.114). Recovery of added mass averaged 115.3 for three different amounts of testosterone (.0625, .125, and .25 pg), added to 25 µL, from quail plasma of two different pools containing either high or low concentrations of testosterone. Assay determinations of 75, 50, 25, 12.5 µL of sample from low and high pools were highly correlated (r = .95 and .96, respectively).

The concentrations of Estradiol in plasma were determined by radioimmunoassay (RIA) which utilized an antiserum (Lilly Lot # 022367) provided by Dr. N. R. Mason (Lilly Research Laboratories)\(^1\). \(^{125}\)I-Estradiol\(^1\) (Catalog Number IM 135; E₂-6[O-carboxymethyl] Oximino\(^2\) \([^{125}\)I] iodosistamine) was used as a radiolabeled tracer and Estradiol\(^1\) was used as a standard. The assay was validated by pipetting sample volumes of 100, 75, 50 and 12.5 µL and bringing the volume to 100 µL, by adding the assay buffer. Each sample volume was used with samples containing low or high concentrations of estradiol. Parallelism was determined by using the Allfit program (DeLean, 1978). Slopes

\(^1\) Diagnostic Systems Laboratories, Inc., Webster, TX.
\(^1\) Lilly Research Laboratories, Indianapolis, IN.
\(^1\) Amersham Life Sciences, Piscataway, NJ
\(^1\) Sigma, St. Louis, MO.
of dilutions plasma and the standard curve were not different as determined by the Allfit program (P=.109). Recovery of added mass averaged 110.5 for four different amounts of testosterone (.25, 1.6, 3.2, and 6.4 pg), added to 50 µL, from quail plasma of two different pools containing either high or low concentrations of estradiol. Assay determinations of 100, 75, 50, 25, 12.5, and 6.25 µL of sample from low and high pools were highly correlated (r = .99 and .98, respectively).

10. Statistical Analysis

The study was conducted in a completely randomized design. Testis weights were adjusted for body weight (g) at the time of euthanasia to remove variation caused by body weight differences. Antibody concentrations were analyzed using repeated measures ANOVA. For this analysis, means of treatments (m-GnRH, c-GnRH-I, and c-GnRH-II), sex (male vs. female), and sampling week (1-13) were analyzed for differences. Hormones (estradiol for females; testosterone for males) were analyzed by repeated measures for ANCOVA. All analyses were conducted using Proc Mixed (SAS Institute Inc., 2001). Interactions were tested to determine whether there were any differences (P ≤ 0.100) in a single category of sampling week or treatment. The LSmeans of each sampling period was used to test for differences between treatment groups. The differences among LSmeans were determined using the least significant difference (LSD) test and LSmeans test. The p-value for determining significance was set at (P≤ 0.100). The Pearson Correlation Coefficients test was run to determine the correlation between antibody percentage and testosterone concentrations for males, moreover, antibody percentage and estradiol concentrations for females.
C. RESULTS

1. Antibody Determination

Antibody (Aby) concentrations are shown in Figures 4-11. Overall, the statistical analysis for antibody concentrations (expressed as percent fold increase over background) demonstrated that there was no significance between the sex of the quail (P=0.4716) or between weeks of treatment (P=0.6362). However there were significant differences in Aby concentrations between treatment groups (P=0.001) and a significant effect of the treatment by week (P=0.0094) (Fig. 4). There was no difference in the interactions of sex by treatment, sex by week, or sex by treatment by week (P=0.3086, P=0.6982, and P=0.8563, respectively). Fig. 4 represents the antibody concentrations throughout the course of the study. Control animals were not injected with any GnRH and thus did not develop antibodies specific to the conjugated KLH-GnRH.

At one WK (Fig. 5) post-injection of GnRH, Aby concentration was significantly higher in plasma from birds receiving m-GnRH than in all other groups (P=0.0278). Aby response in birds receiving c-GnRH-I and -II were not different from each other (P=0.8077) or from controls (P=0.2761).

By WK 3 (Fig. 6) post-injection, the Aby response in m-GnRH birds had increased further and continued to be significantly higher than either c-GnRH groups and controls (P=0.0007). Aby responses in birds receiving c-GnRH-I or -II were not different from each other (P=0.2148), and c-GnRH-I was not different from controls (P=0.428). There was a significant increase in Aby to c-GnRH-II compared to background (P=0.0441) however.
At 5 WK (Fig. 7) post-initial injection and one WK post-booster injection, Aby to m-GnRH remained significantly elevated over all other groups (P≤0.013). There was no obvious response to the booster injection; however c-GnRH-I and -II Aby concentrations were both significantly higher than controls (P≤0.0908).

By WK 7 (Fig. 8) post-initial injection, there was a change in Aby response, with c-GnRH-I-Aby beginning to rise sharply. Concurrently, c-GnRH-II-Aby also increased, but m-GnRH-Aby dropped. At this point there were no differences among these three treatment groups in Aby concentrations (P≥0.1495).

The Aby response to c-GnRH-I increased a maximum amount WK 9 post-initial injection (Fig 9) and one week post-second booster injection. At this point, there was a clear separation between the GnRH treatment responses, with c-GnRH-I showing the greatest response (P=0.0065); m-GnRH was intermediate (P≤0.0394); and c-GnRH-II was least and not significant from background (P=0.6839). M-GnRH-Aby concentrations were lower than c-GnRH-I (P=0.0690) and higher than c-GnRH-II (P=0.0783).

At WK 11 post-initial injection (Fig 10), there was no difference in Aby response to c-GnRH-I and m-GnRH (P=0.7808). Both remained significantly higher than c-GnRH-II (P≤0.0021 and 0.0937, respectively), which in turn, was not different from the controls (P=0.3697).

Finally, at 13 post-initial injection and the termination of the study (Fig. 11), there were no differences (P≥0.3574) between the GnRH treatment groups, all of which were significantly elevated over controls (P≤0.0822) between treatments of C-I and controls (0.0003), C-II and controls (p=0.0222), and between controls and m-GnRH (p=0.0065).
Fig. 4. Antibody concentrations in plasma of male and female quail sampled one week and then biweekly following initial immunization against various forms of GnRH. Control birds received adjuvant without GnRH. Treatment means are expressed as percent fold increase over background. Letters (a, b, c) indicate significant differences between treatments within week.
Fig. 5. Antibody concentrations (X± SEM) in plasma of quail immunized against GnRH at 1 WK post injection. Letters (a,b,c) indicate significant differences between treatments within week.

Fig. 6. Antibody concentrations (X± SEM) in plasma of quail immunized against GnRH at 3 WK post injection. Letters (a,b,c) indicate significant differences between treatments within week.
Fig. 7. Antibody concentrations (X± SEM) in plasma of quail immunized against GnRH at 5 WK post injection. Letters (a,b,c) indicate significant differences between treatments within week.

Fig. 8. Antibody concentrations (X± SEM) in plasma of quail immunized against GnRH at 7 WK post injection. Letters (a, b) indicate significant differences between treatments within week.
Fig. 9. Antibody concentrations (X± SEM) in plasma of quail immunized against GnRH at 9 WK post injection. Letters (a,b,c) indicate significant differences between treatments within week.

Fig. 10. Antibody concentrations (X± SEM) in plasma of quail immunized against GnRH at 11 WK post injection. Letters (a,b,c,d) indicate significant differences between treatments within week.
Fig. 11. Antibody concentrations (X± SEM) in plasma of quail immunized against GnRH at 13 WK post injection. Letters (a,b) indicate significant differences between treatments within week.

2. Male Response to GnRH

a. Testis Weights

Testis weights for treatment groups are given in Fig. 12. Because there was no significant difference in testis weight of males sacrificed at week 5 and those sacrificed at the end of the study (P=0.2259), (P=0.9995) the results were pooled. The overall means of quail testis weights in the c- GnRH treatments were quite similar, in that after immunization with c-GnRH-I, testis weight was 1.7795g and with c-GnRH-II, it was 1.7794g. However, there was tendency for an effect of treatment (P=0.0983). There were no differences between c-GnRH-I or c-GnRH-II and controls (P=0.1275 and P=0.1252, respectively). However, there were differences between c-GnRH-I and c-GnRH-II and m-GnRH treatments (P=0.0401 and P=0.0370) were the differences respectively. Finally,
there was no difference between control and m-GnRH treated male quail testis weights (P=0.4417).

**Fig. 12** Testis weights from quail immunized against GnRH. Controls animals received vehicle alone. Values are means (X ± SEM); like superscripts indicate no statistical differences between means.
b. Gross Morphology of Teste

There were no apparent differences in gross morphology of testicular tissue (sliced at 6 µm) stained with Hematoxylin and Eosin (Figs. 13a-20b).

Fig. 13a-b. Photomicrograph images of control treatment (sacrificed at WK 5) testicles sliced at 6µm, stained with Hematoxylin and Eosin. Fig. 13a Mag. 4X and Fig. 13b Mag. 10X

Fig. 14a-b. Photomicrograph images of m-GnRH treatment (sacrificed at WK 5) testicles sliced at 6µm, stained with Hematoxylin and Eosin. Fig. 14a Mag. 4X and Fig. 14b Mag. 10X

Fig. 15a-b. Photomicrograph images of c-GnRH-I treatment (sacrificed at WK 5) testicles sliced at 6µm, stained with Hematoxylin and Eosin. Fig. 15a Mag. 4X and Fig. 15b Mag. 10X

Fig. 16a-b. Photomicrograph images of c-GnRH-I treatment (sacrificed at WK 5) testicles sliced at 6µm, stained with Hematoxylin and Eosin. Fig. 16a Mag. 4X and Fig. 16b Mag. 10X
Fig. 17a-b. Photomicrograph images of control treatment (sacrificed at end of study) testicles sliced at 6µm, stained with Hemotoxylin and Eosin. Fig. 17a Mag. 4X and Fig. 17b Mag. 10X

Fig. 18a-b. Photomicrograph images of m-GnRH treatment (sacrificed at end of study) testicles sliced at 6µm, stained with Hemotoxylin and Eosin. Fig. 18a Mag. 4X and Fig. 18b Mag. 10X

Fig. 19a-b. Photomicrograph images of c-GnRH-I (sacrificed at end of study) testicles sliced at 6µm, stained with Hemotoxylin and Eosin. Fig. 19a Mag. 4X and Fig. 19b Mag. 10X

Fig. 20a-b. Photomicrograph images of c-GnRH-II (sacrificed at end of study) testicles sliced at 6µm, stained with Hemotoxylin and Eosin. Fig. 20a Mag. 4X and Fig. 20b Mag. 10X
From observation of the histological structure of the testes (Fig. 13-20), there was a clear indication of active spermatogenesis. From 10X magnification, (Fig. 14b, 15b, 16b, 17b, 18b, 19b, 20b) there were evident stages of spermatogenesis occurring in that there was detection of spermatids, secondary spermatocytes (II), primary spermatocytes (I), and spermatogonia. Comparing images from GnRH (Fig. 14a-b, 18a-b) treated male quail with those of control male (Fig. 13a-b, 17a-b) quail indicated that no regression had occurred within seminiferous tubules of the testes. This is apparent in that the seminiferous tubules have broad tubular diameters and very thick germinal epithelium (Baraldi-Artoni et al., 1993). Interstitial tissue is not very apparent in that low visibility of this tissue in sexually mature male birds seems to be caused by the great expansion of seminiferous tubules (Lake, 1971). Characterized regression as indicated by Eroschenko and Wilson (1974) would be a “decrease in the size of seminiferous tubules and an increased amount of cellular debris within their lumina.” There would also be deteriorating spermatozoa, spermatids, and spermatocytes, with large structures resembling that of degenerated cells.

c. Testosterone

Overall, the statistical analysis for testosterone concentrations is shown in Fig. 21. There was no significant effect of week (P=0.3551) or the treatment by week interaction (P=0.3876). However, there were significant differences in testosterone concentration between treatment groups (P=0.1071).

At one WK (Fig. 22), post injection of GnRH, testosterone concentration for c-GnRH-I, controls, and m-GnRH were not different from each other (P≤0.7932). However, testosterone concentrations for male quail treated with c-GnRH-II (P≤0.034), were
significantly lower than controls, c-GnRH-I, and m-GnRH. A small positive correlation was determined ($r \leq 0.28252$) from the Pearson correlations test for all GnRH treatments against antibodies. Therefore, as antibodies increased, testosterone levels did not decrease.

By three WK (Fig. 23) post-initial injection, the testosterone concentration decreased for c-GnRH-II, controls, and m-GnRH groups and demonstrated that they were not different from each other ($P \leq 0.7781$). Yet, testosterone levels for c-GnRH-I treated quail were significantly higher than other GnRH and controls ($P \leq 0.0913$). A negative correlation was determined ($r = -0.35830$) for m-GnRH treated males between testosterone levels and antibodies, in that as antibodies increased testosterone concentrations decreased. However, there was a positive correlation ($r \leq 0.3684$) between antibodies and c-GnRH testosterone concentrations, in that antibody levels did not decrease testosterone levels for these groups of treated males.

At five WK (Fig. 24) post-initial injection and one WK post-booster injection, testosterone concentrations for m-GnRH were not different from control testosterone concentrations ($P = 0.8409$) and c-GnRH-II testosterone concentrations ($P = 0.1405$). There was no obvious response to the booster injection in that testosterone levels for GnRH treatment groups did not drop below control testosterone concentrations. However, there was a difference in c-GnRH-I testosterone levels from all other treatment groups for WK five ($P \leq 0.0853$), in that it was significantly higher. A negative correlation was determined ($r \leq -0.00893$) between antibodies and testosterone concentrations for male quail immunized against all GnRH treatments.

By WK 7 (Fig. 25) post-initial injection, there were no differences in testosterone concentrations between any of the GnRH treatment groups and controls ($P = 0.9072$).
Average concentrations were 4±0.5 ng/mL. A negative correlation was detected for testosterone concentrations in both m-GnRH and c-GnRH-I immunized males and the respective GnRH antibodies ($r \leq -0.10908$). However, there was a positive correlation between antibodies and testosterone in the c-GnRH-II immunized males.

No treatment difference for testosterone concentrations was detected in samples collected in WK 9 post-initial injection ($P \leq 0.9915$) (Fig. 26). Testosterone concentrations were less in control groups compared to the m-GnRH ($P = 0.1010$). A negative correlation was observed between testosterone concentrations of m-GnRH and c-GnRH-I immunized males and the respective GnRH antibodies ($r \leq -0.28825$). There was a positive correlation between c-GnRH-II testosterone concentrations and antibodies ($r = 0.42305$).

At WK 11 post-initial injection (Fig. 27), there were no treatment effects in testosterone concentrations between c-GnRH-I, m-GnRH, and control treatment groups ($P \leq 0.8828$). Yet, testosterone concentration in c-GnRH-II treated males was significantly higher ($P = 0.0089$) than found in control birds and those immunized with c-GnRH-I ($P = 0.0106$). A negative correlation for testosterone concentrations in birds treated with m-GnRH and c-GnRH and GnRH antibodies ($r \leq -0.74566$) was determined. A positive correlation was determined for testosterone concentrations in males given c-GnRH-I and GnRH antibodies ($r = 0.16003$).

The study was terminated at WK 13. There were no differences ($P \geq 0.2325$) between testosterone concentrations of the GnRH treatment groups and controls (Fig. 28). A negative correlation for testosterone concentrations in birds treated with m-GnRH and c-GnRH-II and GnRH antibodies ($r \leq -0.55939$) was determined. A positive correlation was
determined between for testosterone concentrations in males given c-GnRH-I and GnRH antibodies ($r=0.71993$).
Fig. 21a. Testosterone (ng/mL) in plasma male quails immunized against m-GnRH, c-GnRH-I, c-GnRH-II, or vehicle (titermax alone) for controls. Week 1 samples were obtained one week after initial immunization, and samples were collected biweekly thereafter. Booster injections were given at WK 4 and WK 8 post-immunization. Letters (a,b,c) indicate significant differences between treatments within week.
Fig. 21b. Denotes relationship between antibody titer percentage and testosterone concentrations for males immunized against c-GnRH-I.

Fig. 21c. Denotes relationship between antibody titer percentage and testosterone concentrations for males immunized against c-GnRH-II.

Fig. 21d. Denotes relationship between antibody titer percentage and testosterone concentrations for males immunized against m-GnRH.
Fig. 22. Plasma testosterone (ng/mL) in quail immunized against various forms of GnRH or vehicle alone (controls). WK1 post-immunization. Like superscripts indicate no differences between means ± SEM.

Fig. 23. Plasma testosterone (ng/mL) in quail immunized against various forms of GnRH or vehicle alone (controls). WK 3 post-immunization. Like superscripts indicate no differences between means ± SEM.
Fig. 24. Plasma testosterone (ng/mL) in quail immunized against various forms of GnRH or vehicle alone (controls). WK 5 post-immunization. Like superscripts indicate no differences between means ± SEM.

Fig. 25. Plasma testosterone (ng/mL) in quail immunized against various forms of GnRH or vehicle alone (controls). WK 7 post-immunization. Like superscripts indicate no differences between means ± SEM.
Fig. 26. Plasma testosterone (ng/mL) in quail immunized against various forms of GnRH or vehicle alone (controls). WK 9 post-immunization. Like superscripts indicate no differences between means ± SEM.

Fig. 27. Plasma testosterone (ng/mL) in quail immunized against various forms of GnRH or vehicle alone (controls). WK 11 post-immunization. Like superscripts indicate no differences between means ± SEM.
Fig. 28. Plasma testosterone (ng/mL) in quail immunized against various forms of GnRH or vehicle alone (controls). WK 13 post-immunization and final week of study. Like superscripts indicate no differences between means ± SEM.

3. Female Response to GnRH

a. Granulosa cell staining

Granulosa cells that were dispersed and stained for 3β-Hydroxysteroid dehydrogenase (3β-HSD) at the end of the study were not different between treatment groups (p=0.6822) (Fig. 29-31). The individual mean tests for significance were as follows: between c-GnRH-I and -II (P=0.7673), c-GnRH-I and controls (P=0.3104), c-GnRH-I and m-GnRH (P=0.9812), c-GnRH-II and controls (P=0.4639), c-GnRH-II and m-GnRH (P=0.7495), and finally between controls and m-GnRH (P=0.3000).
Fig. 29. Percentages of 3β-Hydroxysteroid dehydrogenase (3β-HSD) positive stained granulosa cells by treatment. Values represent LSM ± SEM. No significant differences between treatments occurred (p=0.6822).

Fig. 30. Granulosa cells that stained positive for 3β-HSD (dark blue cells) activity. Arrows indicate positive staining.

Fig. 31. Cluster of granulosa cells that stained positive for 3β-HSD activity.
b. Estradiol

The overall profile for estradiol concentrations are shown in Fig. 32. There was no significant difference between week (P=0.3551), or interaction of treatment by week (P=0.3876). However, there were significant differences in mean estradiol concentration between treatment groups (P=0.1071).

Week one (Fig. 33), post injection of GnRH, estradiol concentration for female quail immunized against c-GnRH-II, m-GnRH, and controls were not different from each other (P≤0.9884). However, estradiol concentrations for female quail treated with c-GnRH-I were significantly lower than controls (P≤0.065). A negative correlation (r=-0.21292) for GnRH antibodies and estradiol concentrations from females of all GnRH groups. Therefore as antibodies developed against GnRH immunization, there was a decrease in estradiol concentrations in female quail.

By three WK (Fig. 34) post-initial injection, the estradiol concentration for all treatment groups demonstrated that they were not different from each other (P≤0.9985). A negative correlation (r=-0.54034) was determined for m-GnRH and c-GnRH-I immunized groups between estradiol concentrations and antibodies. Yet, a positive correlation (r=0.35960) was determined for c-GnRH-II immunized groups between antibodies and estradiol. This suggests that as antibodies developed, estradiol concentrations did not decrease as a result.

At WK 5 (Fig. 35) post-initial injection, there were no differences in estradiol levels between any of the GnRH treatment groups and controls (P≤0.8150). A negative correlation (r=-0.84415) was determined for both m-GnRH and c-GnRH-II immunized groups between estradiol concentrations and antibodies. However for this week, a positive
correlation ($r=0.12722$) was determined for c-GnRH-I treatment group between antibodies and estradiol.

The estradiol concentrations to GnRH treatments were not affected in WK 7 post-initial injection ($P \leq 0.9886$) (Fig. 36). A negative correlation ($r \leq -0.24492$) was determined for c-GnRH-I and c-GnRH-II treatment groups for antibodies and estradiol concentrations. However a positive correlation ($r=0.43019$) was determined for m-GnRH treatment group for antibodies and estradiol concentration. Therefore antibody titer did not decrease estradiol concentrations for this week.

At WK 9 post-initial injection of GnRH (Fig. 37), estradiol concentrations for c-GnRH-II, m-GnRH, and controls were not different from each other ($P \leq 0.5379$). However, estradiol concentrations for female quail treated with c-GnRH-I was significantly lower than m-GnRH, c-GnRH-II, and controls ($P=0.001, 0.074, \text{and } 0.0162$ respectively). A negative correlation ($r \leq -0.66973$) was found for m-GnRH and c-GnRH-I treatment groups between antibodies and estradiol. Yet, a positive correlation ($r=0.30195$) was determined for c-GnRH-II treatment group between antibodies and estradiol. Antibody titers developed against c-GnRH-II did not decreased estradiol concentrations during this week.

At WK 11 post-initial injection (Fig. 38), there were no differences between c-GnRH-II, m-GnRH, and control treatment groups for estradiol levels ($P \leq 0.1333$). Yet, m-GnRH treated female estradiol concentration was significantly higher ($P \leq 0.0019$) than c-GnRH-I and control estradiol levels. Moreover, a difference occurred between c-GnRH-I and c-GnRH-II treatment groups for estradiol concentrations ($P=0.0776$), in that c-GnRH-I immunized females estradiol concentrations were lower than C-GnRH-II. A negative correlation ($r \leq -0.49372$) was determined for all treatment groups between antibodies and
estradiol concentration. Therefore, as antibodies developed against all GnRH treatments, estradiol concentrations were decreased.

Finally at 13 WK post-initial injection (Fig. 39), and the termination of the study, there were no differences \((P \geq 0.2325)\) between estradiol concentrations of the treatment groups c-GnRH-II, m-GnRH, and controls \((P \leq 0.7752)\). However differences occurred between estradiol levels of treatments c-GnRH-I and -II \((P = 0.0559)\) and between treatments c-GnRH-I and controls \((P = 0.1037)\). A negative correlation \((r \leq -0.66943)\) was determined for all treatment groups between antibodies and estradiol concentration. Once again, as antibodies developed against all GnRH treatments, estradiol concentrations were decreased.
**Fig. 32a.** Estradiol (pg/mL) in plasma levels of female quail immunized against m-GnRH, c-GnRH-I, c-GnRH-II, or vehicle (titermax alone). Week 1 samples were obtained one week after initial immunization and samples were collected biweekly thereafter. Booster injections were given at WK 4 and WK 8 post-immunization. Letters (a,b,c) indicate significant differences between treatments within week.
Fig. 40b. Denotes correlation between antibody titer percentage and estradiol concentrations for females immunized against c-GnRH-I.

Fig. 40c. Denotes correlation between antibody titer percentage and estradiol concentrations for females immunized against c-GnRH-II.

Fig. 40d. Denotes correlation between antibody titer percentage and estradiol concentrations for females immunized against m-GnRH.
Fig. 33. Plasma Estradiol (pg/mL) in female quail immunized against various forms of GnRH or vehicle alone (controls). WK 1 post-immunization. Like superscripts indicate no difference between means ± SEM.

Fig. 34. Plasma Estradiol (pg/mL) in female quail immunized against various forms of GnRH or vehicle alone (controls). WK 3 post-immunization. Like superscripts indicate no difference between means ± SEM.
Fig. 35. Plasma Estradiol (pg/mL) in female quail immunized against various forms of GnRH or vehicle alone (controls). WK 5 post-immunization. Like superscripts indicate no difference between means ± SEM.

Fig. 36. Plasma Estradiol (pg/mL) in female quail immunized against various forms of GnRH or vehicle alone (controls). WK 7 post-immunization. Like superscripts indicate no difference between means ± SEM.
**Fig. 37.** Plasma Estradiol (pg/mL) in female quail immunized against various forms of GnRH or vehicle alone (controls). WK 9 post-immunization. Like superscripts indicate no difference between means ± SEM.

**Fig. 38.** Plasma Estradiol (pg/mL) in female quail immunized against various forms of GnRH or vehicle alone (controls). WK 11 post-immunization. Like superscripts indicate no difference between means ± SEM.
D. Discussion

With regard to the end-point responses (reproductive changes), the male birds responded somewhat more than the females. There were some effects of treatment on testis weights and testosterone levels in plasma. In contrast, no effects were noted on granulosa cell function or estradiol. With regard to the treatment responses, mGnRH responded early with higher circulating antibodies and lower circulating testosterone. The peptide structure sequence of m-GnRH is foreign to chickens. Testis weights were less in mGnRH-treated birds by the end of the study. This is interesting because neither the antibody elevation nor the testosterone suppression was sustained over the time of the study. C-GnRH-I, considered to be the most biologically relevant form of cGnRH in birds with regard to regulatory activity (Teruyama and Beck, 2000), did not have any effect on testes.
However, the antibody response was more similar to what was expected. C-GnRH-II, the function of which is not entirely clear in birds (Teruyama and Beck, 2000), neither elicited a distinct antibody response nor resulted in a clear pattern of reproductive response.

Antibodies developed immediately to m-GnRH, increasing from 30% to approximately 55% above background and were significantly higher than either cGnRH treatment group but declined steadily thereafter. This may have occurred because of the structural arrangement of m-GnRH conjugated to KLH (Keyhole Limpet Hemocyanin), and the amino acid difference at the Arg position. Because a specific immune response is elicited by a specific antigen, catabolic destruction of the antigen by specific macrophages may compromise the response (Dresser, 1986). Moreover, Dresser explains that although repeated injections of antigen can increase antibody levels in plasma, sometimes to high levels, a point is reached when no amount of antigenic stimulation will lead to further increases. A third possibility could be that antigen supplied in the second booster injection complexed with circulating antibody, masking any further antigenic response (Kittok, personal communication). However it is also possible that methodology was a factor and that if repeated booster injections of m-GnRH antigen had been given over several more weeks, it might have kept antibodies levels elevated and reduced hormone levels of estradiol in females and testosterone in males.

The results obtained with mGnRH in this study differed from those of Vizcarra et al. (2001), who reported a gradual increase of antibody titers against m-GnRH by week sixteen after initial immunization and week two after the second booster immunization. Titers developed to approximately 35% by week 21. Differences between this study and that of Vizcarra et al. (2001) in antibody development could be related to dose of
conjugated peptide, age of animals when the study was carried out, and/or type of bird used. When Miller et al. (2000) immunized white-tailed deer with a GnRH vaccine, they found variations in immune responses of individual deer. According to Miller et al. (2000) this occurred as a result of inherent differences in reproductive physiology, and was thought to be due to genetic differences among individual animals.

Antibodies developed to c-GnRH-I began to increase by the third week after initial injection. By week 9 after initial injection and after the second booster, titers to c-GnRH-I increased to approximately 42% above background. This antibody titer increase closely followed that of Vizcarra et al. (2001), in that there was a gradual increase of titer in the birds in his study to c-GnRH-I conjugated antigen and a larger titer response after the second booster injection.

Antibodies against c-GnRH-II were significantly lower than either m-GnRH or cGnRH-I antibodies. Sharp et al. (1990) conducted a study to determine the physiological role of chicken LHRH-I and -II in the control of gonadotropin release in the domestic chicken and found the same thing; that is, that immunization against c-GnRH-II failed to induce significant physiological effects. The overall response of antibodies to m-GnRH occurred as hypothesized, with the sharp increase of titers after two weeks of the initial injection. Because this form of GnRH is not naturally occurring in birds, the titer response was greater at first. However, c-GnRH-I antibodies did develop over time, and increased by week nine after initial injection of the conjugated peptide and after two consecutive boosters. This probably occurred due to the fact c-GnRH-I is a naturally occurring GnRH, and that the secretion of gonadotropins in birds is directly controlled by this form of the releasing hormone (Teruyama and Beck, 2000). Finally, absence of antibodies against
cGnRH-II was supported by Vizcarra et al. (2001) and Sharp et al. (1990) in that those studies did not observe a significant development of antibodies against c-GnRH-II either.

Testis weights of m-GnRH treated male quail were decreased significantly compared to those of c-GnRH treated birds. This finding agrees with Vizcarra et al. (2000), because testis weights were significantly decreased in broiler breeders immunized against mGnRH in that study. However, Vizcarra et al. (2000) also found that m-GnRH and c-GnRH-I treated male broilers had testis weights significantly lower than did control birds. This also occurred for Dowsett et al. (1996) when a GnRH vaccine was administered in colts to suppress testicular function using a two dose rate of water soluble GnRH. They were able to determine that suppression occurred in colts when antibody titers were effective. However, they did find variability in response of the immunosuppression of testicular function caused by two different dose rates. In this study with quail, immunization against mGnRH did have an effect on the testis, but neither cGnRH antigen did, and even the testicular weight response in mGnRH treated birds was not different from controls. The reason for this is not clear, but based on the antibody data, the large response to mGnRH had dropped off by week 7, even though the titer was higher than background throughout the rest of the study. Perhaps the response to mGnRH overall was not strong enough to reduce LH and FSH and involve the testis in a significant way.

On careful examination of testicular histology, no regression occurred in somniferous tubules between any of the treatments to male quail. There appeared to be active spermatogenesis within numerous tubules, and apparent Leydig cells within the interstitial space between tubules. Several studies have reported a relationship between testis weight and testicular function. Mezquita et al. (2001), in a study focused on constitutive and heat
shock induced expression of HSP70 mRNA during chicken testicular development and regression, found that administration of estrogen agonist DES to roosters for 2 weeks induced a 90% reduction in testis weight and a severe atrophy in seminiferous tubule diameter, a collapse in tubular lumen and a decrease in germ cell numbers. Moreover, findings of Tanaka and Fujioka (1981), in a study to determine histological changes in the testis of the domestic fowl after partial adenohypophysectomy, found that intact controls had full spermatogenic activity and the seminiferous tubules contained all stages of sperm production. In contrast, the birds that were fully adenohypophysectomized had a decrease in testicular weight and the spermatozoa produced were partially abnormal. They determined from this study that to maintain a fully active testicular function, more than 75% of the adenohypophysis is needed. Based on the relationships described in these studies and the effect of mGnRH on testis weight, the testes in the mGnRH-treated quail in this study should have shown evidence of decreased testicular function; the fact that this did not happen may be related to the fact that testes of mGnRH-treated birds were not different from controls or that treatment was not long enough.

Testosterone concentrations in quail did show significant differences of treatments during different weeks and again the response varied with type of GnRH used, with cGnRH-II eliciting no clear response. Testosterone in mGnRH treated birds responded logically the presence of antibodies against GnRH, with inverse relationships observed in both the early and late segments of the study. Unfortunately the negative correlations seen early in the study between antibody titer and testosterone were not sustained throughout the study. The explanation for this observation is similar to that for testicular morphology; perhaps the exposure was not long enough or mGnRH immunization simply did not
effectively reduce LH or FSH. CGnRH-I as an antigen did appear to produce a more expected effect on testosterone, with the negative correlations occurring later in the study. This is consistent with expected antibody development and testosterone response as determined by Vizcarra et al. (2001), in that the same response appeared to follow the expected direction.

The results for testosterone show variations between treatments and antibody correlations. This is parallel to the results of Vizcarra et al. (2001). They found a large variation in titers and testosterone concentrations in birds immunized against m-GnRH. They had, however, a stronger relationship between the two parameters that indicate that birds with higher titers had less testosterone concentrations in blood. In the study conducted by Dowsett et al. (1996) to suppress testicular function in colts using a two dose rate of water soluble GnRH vaccine, they determined that the testosterone concentration decreased during the first and second suppression periods while antibody titers were above 1000 (expressed as geometric mean) but that hormone concentrations increased and returned to normal levels by the time antibody titers were undetectable. This suggests that antibody levels must be sufficiently high to successfully reduce testosterone concentrations. In the present study, the early response to mGnRH and the delayed response to cGnRH-I appeared to be similar, in that as antibody levels increased to over 50% above background for m-GnRH treated birds and to over 35% above background for cGnRH-I treated birds, testosterone concentrations decreased.

Immunization of the female birds against GnRH, as indicated by granulosa cell staining for 3\(\beta\)-hydroxysteroid dehydrogenase (3\(\beta\)-HSD), did not successfully disrupt steroid activity within the cells. Granulosa cells from prehierarchal follicles express the
3β-HSD activity because steroid production is under the control of LH acting through the adenyl cyclase/cAMP second messenger signaling pathway (Bahr and Calvo, 1984). This was found from their study to determine the correlation between adenyl cyclase activity and responsiveness to gonadotropins during follicular maturation in the domestic hen. The granulosa layer produces primarily progesterone, which is the primary steroid needed to initiate the preovulatory surge of LH that precedes ovulation by 4-5 hrs. Moreover, in a study conducted by Marrone and Sebring (1989) to determine the quantitative chemistry of 3β-HSD activity in avian granulosa cells during follicular maturation, they found that in every case, 100% of granulosa cells from each preovulatory follicle showed positive staining for the presence of 3β-HSD activity when the pregnenolone substrate was added and that staining intensity increased with increasing follicular maturation. The immunization protocol in this study did not significantly decrease the percentage of cells that showed steroidogenic activity, compared to controls, but numerically both cGnRH-I and mGnRH were slightly lower than cGnRH-II and controls. Thus, some inhibition of LH action on these cells appears to have occurred, but not enough to successfully inhibit the entire follicle.

Estradiol concentrations in female quail immunized with m-GnRH were negatively correlated with antibody titer early in the study. However, when estradiol from mGnRH-treated birds was compared with that from other groups, it was not depressed, indicating that this negative correlation was not a real response.

As with its effect on testosterone, c-GnRH-I appeared to act more as expected than either of the other treatments did, with a delayed increase in antibody coinciding with a
decreased level of circulating estradiol. From week 9 through the end of the study, 
estradiol was significantly lower in cGnRH-I treated females compared to the other three 
groups. This was true even though the negative correlation between antibody titer and 
estradiol was significant only at week 9. Estradiol concentrations in m-GnRH immunized 
females decreased as antibodies developed against m-GnRH and remained low throughout 
the first five weeks after initial immunization, increasing at week 7 even though antibody 
titers did not remain elevated past week 3. This suggests that as antibodies declined for m-
GnRH treated female quail, estradiol was able to increase thereafter. It should be noted 
that that this increase was a sharp increase of estradiol for this treatment of birds compared
to all other treatments. It may be that female quail immunized with m-GnRH were actually 
stimulated in producing estrogen after antibodies declined against m-GnRH. There is, 
however, not enough evidence to support this, in that practically no research has been 
conducted to understand this mechanism. Therefore, at this point it can only be indicated 
by observation in this study.

C-GnRH-II immunized birds did not respond with changes in estradiol overall; the 
minor negative and positive correlations were not consistent and probably did not indicate 
biological significance. The lack of response to cGnRH-II is consistent with the other 
results of this study with regard to cGnRH-II. This may be due to the fact that c-GnRH-II 
is naturally occurring in birds and is three amino acids different from m-GnRH. Moreover, 
in the study conducted by Sharp et al. (1990), immunization against c-GnRH-II failed to 
induce significant physiological effects when compared to c-GnRH-I. In addition, they 
found that immunization of laying hens against c-GnRH-I caused successful suppression of 
plasma LH levels, a stop in egg laying and a complete regression of the reproductive
system. The findings of immunization against c-GnRH-II in this study correspond with that of Sharp et al. (1990), in that no reproductive physiological effects occurred for female birds within this treatment.

E. Conclusion

In this study, immunization of Japanese quail using m-GnRH, c-GnRH-I, and c-GnRH-II affected sex steroid hormones in both male and female birds, but failed to significantly reduce fertility. This was evident in that the testicular morphology of male quail was not altered or regressed. Moreover, granulosa cells in female quail were still able to synthesize LH and thus progesterone, as evidenced by positive staining for steroidogenesis in these cells. M-GnRH and c-GnRH-I treated quail appeared to give the greatest response of reduced hormone levels, but not enough to fully reduce reproduction. This corresponds with Sharp et al. (1990), in that they were also unable to cause involution of the reproductive system in male Japanese quail, even with a high dose of GnRH application (220 µg GnRH/kg body weight). In addition, Sharp et al. (1990) were not able to cause a desensitization of the neuroendocrine system of quail even after a 2-week application of GnRH, which is in accord with results in other poultry species (Peczely, 1989). Although Vizcarra et al. (2001) were able to successfully reduce testis weight and β-FSH expression in the pituitary of broiler breeder males, the same application did not apply to Japanese quail.

Therefore, with these findings more studies are needed to adequately determine whether immunization using GnRH has potential for use in fertility control of birds. Some specific experiments that should be conducted include the following:
1. Repeat the study, possibly using one sex only (even though no sex differences were noted in terms of antibody response) to compare ELISA with $^{125}$I-RIA for antibody titer. This is one of the differences between this study and that of Vizcarra et al. (2001) and it should be addressed.

2. Repeat the study, but with a dose-response design to determine both most effective amount of antigen to administer and also to determine optimum booster schedule. Timing of sampling should also be considered.

3. Once technical details have been determined, a study should be conducted to better quantify the reproductive response. This might include hormone-receptor interactions, determination of specific antibody classes relative to immunization (IgG, IgM, etc.), and more sensitive indicators such as mRNA.

4. Consider using an antagonist to GnRH to assess its efficacy in reducing fertility. Because success has been determined in mammals with immunization against m-GnRH and because of the encouraging results with cGnRH-I in this study, further studies should be conducted determine if the use of immunization against GnRH can be used as a fertility control in birds.
F. Literature Cited


Fraser, H. M. 1980. Inhibition of reproductive function by antibodies to luteinizing hormone releasing hormone. In: Immunological aspects of reproduction and fertility control. J.P. Hern (Ed) pp143-171.


Appendix 1

Elisa GnRH Antibody Titer

Day 1: coating of wells with GnRH-BSA
1. Add 50 µl of 25 µg/mL of GnRH-BSA to each well (96 well plate).
2. Vortex, incubate 12-16 hrs. at 37 °C.

Day 2
1. Pipet 150 µl of PBS-1% OVA (egg ovalbumin)
2. Incubate at room temperature for 1 hour in a humidified container.
3. Wash plate (150µl) three times with PBS-0.05% Tween.
4. Add 50 µl of diluted sample (4µl /396 ul PBS-1% OVA) to the appropriate well.
5. Incubate as in step 2.
6. Wash plate as in step 3.
7. Add 50 µl /well Rabbit-anti-chicken IgG diluted 1:100 in PBS-1% OVA.
8. Incubate as in step 2.
9. Wash plate as in step 3.
10. Add 50 µl /well Goat-anti-rabbit IgG diluted 1:5000 in PBS-1% OVA.
11. Incubate as in step 2.
12. Wash plate as in step 3.
13. Add 100 µl /well TMB substrate.
14. Add 100 µl /well Phosphoric Acid (H2PO4) to stop reaction.
15. Read at A 450.
Appendix 2.

Granulosa cell layer isolation

1. Euthanize hens by cervical dislocation.

2. Remove the largest follicle (F1) and immediately place it in ice-cold 1% physiological saline, pH 7.4 (Appendix 3).

3. Hold F1 by fingers over petri dish containing 1% physiological saline, pH 7.4, with stigma facing up.

4. Make a slit along the stigma with a surgical blade.

5. Flip F1 upside down to allow the yolk and enclosing granulosa layer to fall in the petri dish leaving the thecal tissue behind.

6. Granulosa cell layer with its basal lamina and perivitelline layers are the top white layers that surrounding the yolk mass (black color background would help to locate the edges of the layer).

7. Using fine forceps, carefully and slowly peel (step by step from all corners) granulosa cell tissue from the yolk, and agitate in 1% physiological saline to remove any yolk residual.
Appendix 3.

Dispersion of granulosa cells

1. Transfer granulosa cells layer to petri dish containing 0.5 ml of fresh incubation media (Appendix 4).
2. Mince granulosa cell layer by microdissection scissors (about 2 mm²).
3. Use a new pipet tip for every sample suspension, media, and solution.
4. After all granulosa layers from all hens are minced, transfer the content of the petri dishes (incubation media and minced granulosa tissues) to 15ml tubes containing 5 ml dispersion media (Appendix 4) using 1ml micropipette.
5. Aspirate the content of the 15ml tubes approximately 10 times with 1 ml micropipette.
6. Cap the tubes and incubate them vertically in shaking water bath (70 cycle/min) for 30 min at 39°C.
7. Aspirate again after 15 min and at the end of incubation period.
8. Collect granulosa cells by centrifugation at 250 x g for 10 min at room temperature.
9. Decant the supernatant and add 8 ml incubation media to the cell pellet and resuspend by vortex (first wash).
10. Centrifuge the cell suspension at 250 x g for 10 min.
11. Decant the supernatant and add 8 ml fresh incubation media to the cell pellet and resuspend (second wash).
12. Decant the supernatant and add 2 ml of incubation media to the cell pellet and resuspend.
13. Count the number of viable cells by trypan blue-exclusion technique (Appendix 5).
14. Dilute the granulosa cells with an appropriate volume of fresh incubation media to a final concentration of approximately 100,000 viable cell/1 ml incubation media.
Appendix 4.

Medias and Solutions

1% Physiological Saline (pH 7.4)

\[
\begin{align*}
\text{NaCl} & : 10 \text{ g} \\
\text{DH}_2\text{O} & : 1 \text{ L}
\end{align*}
\]

10% Formal Saline

\[
\begin{align*}
\text{1% Physiological Saline (see above)} & : 900 \text{ ml} \\
\text{Formaldehyde Solution (37% w/w)} & : 100 \text{ ml}
\end{align*}
\]

Phosphate Buffered Saline (PBS) (pH 7.4)

\[
\begin{align*}
\text{NaCl} & : 8.766 \text{ g} \\
\text{NaH}_2\text{PO}_4 & : 0.276 \text{ g} \\
\text{Na}_2\text{HPO}_4 & : 1.136 \text{ g} \\
\text{DH}_2\text{O} & : 1 \text{ L}
\end{align*}
\]

Luteinizing hormone (LH) preparation

1. Dissolve ovine LH (NIH-LH-S16) in PBS (see above) at a concentration of 1000 ng of LH/1 ml of PBS.
2. Store at -20°C in 12x75 polypropylene test tubes (1 ml of LH solution/tube).
3. Thaw when needed.

Interleukin-1 (IL-1) preparation

1. Dissolve human synthetic IL-1β (IL-1β, fragment 163-171) (SIGMA Chemical Co., St. Louis, MO) in PBS (see above) at a concentration of 1000 ng of IL-1/1 ml of PBS.
2. Store at -20°C in 12x75 polypropylene test tubes (1 ml of IL-1 solution/tube).
3. Thaw when needed.
Oil Red O Stain ................................................. 0.7 g

Oil red O (SIGMA Chemical Co., St. Louis, MO) . . . . . .
Propylene Glycol (SIGMA Chemical Co., St. Louis,
MO) ........................................................................ 100 ml

1. Dissolve Oil red O in Propylene Glycol at 100°C.
2. Filter through no. 2 filter paper.
3. Reduce temperature to 60°C and use immediately or
store at 60°C in oven.
4. Avoid contact and inhalation.

85% Propylene Glycol

Propylene Glycol ............................................... 85 ml
DH2O ........................................................................ 15 ml

1. Mix well.
2. Avoid contact and inhalation.

Harris’s Hematoxylin Stain

Hematoxylin Solution (SIGMA Chemical Co., St.
Louis, MO) (0.1% Hematoxylin) .................................. 10 ml
DH2O ........................................................................ 20 ml

Glycerol Gelatin

Gelatin ................................................................. 10 g
Glycerol ................................................................. 62.50 ml
01.25 ml
Phenol Crystals ......................................................
DH2O ........................................................................

3β-HSD Stain ......................................................... 40.0 ml

PBS (see above) ...................................................... 0.5 mg
Pregnenolone (P5) (SIGMA Chemical Co., St. Louis,
MO) ........................................................................
β-Nicotinamide Adenine Dinucleotide (β-NAD) (SIGMA Chemical Co., St. Louis, MO) 28.0 mg
Nitroblue Tetrazolium (SIGMA Chemical Co., St. Louis, MO) 10.0 mg

1. Add 40 ml PBS to 50 ml tube.
2. Dissolve 20 mg pregnenolone in 4 ml of 100% ethyl alcohol.
3. Add 100 ul of the dissolved P5, 28 mg β-NAD, and one tablet containing 10 mg Nitroblue Tetrazolium to the PBS.
4. Mix well.
5. Open the tube for a while to allow ethanol to evaporates.

Incubation Medium (pH 7.4)

Medium RPMI with L-glutamine (SIGMA Chemical Co., St. Louis, MO) 1.64 g
D-(+)-Glucose (SIGMA Chemical Co., St. Louis, MO) 0.20 g
Bovine serum albumin (SIGMA Chemical Co., St. Louis, MO) 0.20 g
Sodium bicarbonate 0.01 g
Trypsin inhibitor
Penicillin-streptomycin (SIGMA Chemical Co., St. Louis, MO) 1 ml
DH2O 99 ml

Dispersion Medium

Incubation medium (see above) 0.3 g
Collagenase II (SIGMA Chemical Co., St. Louis, MO) 0.3 g
Appendix 5.

Determination of viable cells by trypan blue-exclusion

1. Add 90 ul of 0.4% trypan blue dye solution (SIGMA Chemical Co., St. Louis, MO) to 12x75 polypropylene test tube.

2. Add 90 ul dH2O to dilute trypan blue dye to 0.2%.

3. Add 20 ul of granulosa cells suspension to the dye, mix, and allow dye to penetrate dead cells for 1-2 min.

4. Transfer 10 ul of cells and dye suspension to the haemocytometer chamber.

5. Don’t leave cells in the dye longer than 5 min.

6. Count number of live cells (translucent) and dead cells (blue).

7. Total number of live cells/1 ml of cell suspension =

   \[
   \text{Number of viable cells} \times 10 \times 10,000
   \]
Appendix 6.

Staining granulosa cells for 3β-hydroxysteroid dehydrogenase (3β-HSD)

1. Isolate and disperse granulosa cells (Appendix 2 and 3, respectively).

2. Using 24-well, flat bottom plate, add 1.5 ml of 3β-HSD stain (Appendix 4) to each well.

3. Add 100 ul of granulosa cells suspension containing approximately 10,000 viable cells from each treatment to the wells (each well should have 1.5 ml 3β-HSD stain and 100 ul of granulosa cells suspension).

4. Aspirate each well 2 times using 1 ml micropipette.

5. Incubate the plate(s) at 39°C for 90 min.

6. Using inverted microscope, examine a total of at least 100 cells per sample for dark blue formazan precipitate (dark blue cells) to determine the percentage of cells that stained positive for 3β-HSD (cells exhibit 3β-HSD activity).