

Superovulatory response in New Zealand White rabbits
under environmental heat stress

by

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For the Graduate College

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INTRODUCTION

The term hormone was introduced by Starling in 1905 and comes from the Greek meaning to “excite” or “arouse”. A characteristic of hormones is that they are synthesized and secreted into the bloodstream by endocrine glands to stimulate, inhibit, or interact with the functional activity of specific target organs, which produce a wide range of physiologic responses. The pituitary gland or hypophysis is one of the major endocrine glands recessed within the sella turcica of the sphenoid bone situated beneath the hypothalamus near the optic chiasm. It is divided into two lobes, the anterior (adenohypophysis) and the posterior (neurohypophysis). The anterior pituitary receives blood directly from the hypothalamus via blood vessels in the pituitary stalk. In response to the releasing hormones from the hypothalamus, secretes a variety of different hormones, in particular the so-called “tropic hormones”.

Follicle stimulating hormone (FSH) and luteinizing hormone (LH) are synthesized by the anterior pituitary. They are released into the blood stream and act upon the gonads “gonadotropic” of both the female and male. In the female, they stimulate follicular growth, steroid secretion, ovulation, and luteinization of the Graafian follicle.

Superovulation is the increased ovulatory response, above the number that would be expected to occur naturally, generated in an animal by the administration of exogenous gonadotropins. The main objective of a superovulatory treatment is to increase the number of oocytes released by an animal, and thereby the potential number of embryos. Superovulation is accomplished by subcutaneous or intramuscular injections of follicle stimulating hormone. In

some species, this treatment is followed by administration of luteinizing hormone to induce ovulation.

A major constraint to reproductive efficiency is seasonal periods of heat stress, where the reproductive functions of males and females are adversely affected. The specific reaction may depend upon the degree of temperature and humidity increases. In the female, reproductive functions consist of strongly interrelated and interdependent factors, which are involved with the estrous cycle and subsequent pregnancies. Heat stress may affect many of these factors, such as endocrine patterns, physiological parameters or direct effects on maternal tissue, oocytes and embryos, which can eventually lead to pregnancy failure.

The objective of the present study was to determine the response to superovulatory treatment in female New Zealand White (NZW) rabbits under conditions of environmental heat stress.

Thesis organization

This thesis includes an introduction, review of the literature, two experiments composed of materials and methods, results and discussion, and general conclusions. Also included are two appendixes, references cited, and acknowledgments.

LITERATURE REVIEW

Historical aspects of gonadotropins

In early studies, two groups of scientists (Smith, 1926; Zondek and Ashheim, 1926) demonstrated that daily implants of fresh adenohipophysis tissue promoted growth and maturation of the ovaries and uterus. Gonadal atrophy resulting from ablation of the pituitary gland in rats and its repair by daily homoplastic implants were briefly described by Smith, as was the ovarian enlargement and precocious sexual maturation induced in immature mice by Zondek and Aschheim using similar implants. These observations had a tremendous impact on the volume and direction of research on the adenohipophysis. They constituted the first demonstration of a positive influence of the adenohipophysis on the gonads. In 1927, these studies were extended to include immature rats and mice of both sexes and the demonstration that pituitaries from many other species would produce same gonadal response (Smith, 1927; Smith and Engle, 1927; Zondek, 1927; Zondek and Aschheim, 1927).

During 1927, Aschheim and Zondek discovered a similar gonad-stimulating activity in the urine of pregnant women and soon demonstrated that this could be used as a test for pregnancy. Widening their search for gonad-stimulating activity in blood or urine from many different sources, they were soon rewarded with the uncovering of such an activity in the urine of ovariectomized or postmenopausal women. Another gonad-stimulating activity was described by Cole and Hart (1930) in the serum of pregnant mares.

By 1927, it was clear that the pituitary gland produced a substance that stimulated the gonads via the blood stream and hypophysectomy removed the source of the stimulus. During

the following years supporting evidence came in abundance and was extended to other species (Allen, 1928; Brooks, 1938; Dempsey, 1939; Gaupp and Spatz, 1955; Greep and Barnett, 1951; Hartman, 1930; Reichert, 1928).

The classical experiments of Smith (1926), and Zondek and Ashheim (1926), revealed the presence of gonadotropic substances in the adenohypophysis. In 1931, the chemical evidence for the existence of separate gonadotropins was shown when two hormones were isolated from extracts of the adenohypophysis (Fevold *et al.*, 1931). The separation was obtained when an aqueous (50 %) pyridine extract of ovine pituitary powder was evaporated to dryness and the residue extracted with water. The active material was precipitated by adding several volumes of absolute alcohol. This precipitate or “crude” extract was dried and shown to contain both FSH and LH activities as demonstrated by its ability to produce enlarged and massively luteinized ovaries in immature rats. Fractionation of FSH and LH was based on their differential solubility in water. The FSH entered into solution while the LH remained in the residue. The test of fractionation was based solely on biological criteria. The ovaries of immature rats receiving the soluble fraction for 4-5 days were enlarged and filled with many clear follicles, while those of animals receiving the residue in suspension were unstimulated and the uteri small. When the two fractions were combined, the ovarian response was the same as that produced by the crude extract.

The discovery of gonadotropic hormones generated the idea that it would be possible to induce fertility in immature or anestrous animals and to further improve the fertility in those already capable of breeding. After superovulation was accomplished in mice (Smith and Engle, 1927), many reports followed extending this observation to the rat (Evans and

Simpson, 1940; Wilson and Zarrow, 1962), guinea pig (Deanesly, 1963), sheep (Casida *et al.*, 1944; Hammond *et al.*, 1942; Zawadowsky, 1941) and cattle (Dowling, 1949; Hammond, Jr., 1949). In the rabbit, it was shown that superovulation could also be accomplished by administration of exogenous gonadotropins (Foster and Fevold, 1938; Greenwald, 1961; Hertz and Hisaw, 1934; Parkes, 1942; Pincus, 1940).

Distribution and source of gonadotropins

FSH and LH are found in the blood after synthesis and secretion from basophilic cells located in the adenohypophysis (Chow, *et al.*, 1942; Ellis, 1958; Fevold *et al.*, 1931; Li *et al.*, 1949).

Biologically active material containing both FSH and LH activities are present in human urine, notably in the urine of postmenopausal women (Aschheim and Zondek, 1927). This material is termed human menopausal gonadotropin (HMG). The blood and the urine of pregnant women contains another gonadotropin which is synthesized in the chorionic villi of the placenta, human chorionic gonadotropin (hCG) and has a similar biological activity to pituitary LH (Aschheim and Zondek, 1928; Bahl, 1977; Diczfalusy and Troen, 1961). This hormone can be detected in women beginning on day 8 after ovulation (Jaffe, 1978). Recently Birken *et al.* (1996) reported the isolation and characterization of a human pituitary chorionic gonadotropin possessing 50 % of the activity of purified hCG from the urine of pregnant women based on cyclic adenosine monophosphate (cAMP) assays.

In the mare a gonadotropin called pregnant mare serum gonadotropin (PMSG) is produced during early pregnancy (days 40-140) by fetal trophoblastic cells termed endometrial

cups embedded within the uterus. This hormone has been renamed equine chorionic gonadotropin (eCG) due to its close relationship with hCG and has predominantly FSH-like activity (Cole and Hart, 1930; Sherwood and McShan, 1977; Zondek, 1930). The hormone is also present in the donkey (Calisti and Oliva, 1955) and in the zebra (King, 1965).

Structure of gonadotropins as related to function

Purified preparations of LH from different species and human FSH allowed the determination of some aspects of the chemical structure of the gonadotropins. These hormones are glycoproteins composed of α and β subunits (Pierce and Parsons, 1981; Sairam and Papkoff, 1974). They share a common α subunit which is similar between the same and different species (Combarous, 1988; Papkoff *et al.*, 1977; Sairam and Papkoff, 1974; Ward *et al.*, 1973). The β subunit is hormonal and species specific (Papkoff *et al.*, 1977; Pierce and Parsons, 1981).

The peptide component of these two gonadotropins is first synthesized under direct genetic control. Glycosylation, which generates a final molecular mass of 32 kDa, is believed to be a postribosomal event controlled by glycosyltransferase enzymes localized in the Golgi cisternae (Matzuk *et al.*, 1988). The carbohydrate moieties have been identified as D-mannose, D-galactose, L-fucose, N-acetyl neuraminic acid, D-glucosamine, N-acetylated D-galactosamine and sialic acid (Matzuk *et al.*, 1988; Pierce and Parsons, 1981; Sairam and Bhargavi, 1985). It has been shown that sialic acid apparently prolongs the half-life of the circulating gonadotropins (Niswender *et al.*, 1974). The α subunit contains two oligosaccharides that are N-linked to asparagines and these hormones may contain O-sulfated

(attached through serine to the peptide chain) hexosamines (Smith and Baenziger, 1988).

Removal of the carbohydrate from the α subunit generates a molecule that is still able to bind to their respective receptors, but is unable to stimulate adenylate cyclase (Sairam and Bhargavi, 1985). It has been proposed that the α subunit also provides the hormone-specific β subunit with the conformation necessary to bind the receptor and the α -subunit itself is necessary for the stimulation of adenylate cyclase (Sairam and Bhargavi, 1985).

Recombinations of α and β subunits demonstrated that the specificity of the hormone for its receptor is conferred by the β subunit (Combarrous, 1988).

The estimated molecular weight of LH for the ovine is 28000-32500, bovine 25200-30000, swine 27000-34000, and equine 27000-33500 (Sherwood and McShan, 1977). The estimated molecular weight for ovine FSH ranges from 32700-33800, equine 33800-33200 (Sherwood and McShan, 1977), and 37300 for bovine (Grimek *et al.*, 1979).

Actions of pituitary gonadotropins on the ovary

The release of the gonadotropic hormones into the blood is not done at constant rate but rather by a sequence of small dosages, varying amplitudes and frequencies (Foster *et al.*, 1975; Hackett and Hafs, 1969; Orstead *et al.*, 1988; Parlow *et al.*, 1964). Binding of FSH and LH to respective receptors stimulate the ovaries by promoting follicular growth, oocyte maturation, steroid secretion, ovulation and luteinization of the ovulated follicle, and progesterone secretion by the corpus luteum (Davis and Hellbaum, 1944; Dempsey, 1937; Greep *et al.*, 1942; Hisaw, 1947; Mandl and Zuckerman, 1952; Marden, 1952; Moon and Li, 1952). Follicular growth is a sequential event and once a follicle has been selected from the

pool of inactive follicles it begins to grow and will ovulate or undergo atresia (Hirshfield, 1991). The gonadotropins will determine which event will take place, although they have no influence on the selective process from the inactive pool of follicles (Hirshfield, 1991).

FSH is required for follicular growth and antrum formation (Glasier *et al.*, 1989). In addition, FSH acts with estrogens to stimulate the formation of FSH and LH receptors in granulosa cells (Glasier *et al.*, 1989; Richards and Midgley, 1976). Both gonadotropins act upon the follicle to stimulate estrogen secretion (Short, 1972). In most species, levels of estrogens increase a few days before ovulation and this increase stimulates the release of LH from the adenohypophysis and induces ovulation (Adams, 1972; Goding *et al.*, 1969; Hirshfield, 1991; Hodgen, 1989). Under the influence of LH, granulosa cells luteinize and begin to secrete progesterone (Goding *et al.*, 1969; Mandl and Zuckerman, 1952; McNatty, 1978). In the rabbit model, it has been shown that serum concentration of FSH does not vary greatly during estrous, while a sharp increase in LH concentration is observed approximately 90 to 120 minutes after mating (Dufy-Barbe *et al.*, 1973; Hilliard, *et al.*, 1964; Orstead *et al.*, 1988, Scaramuzzi *et al.*, 1972). Levels of estrogens are highly variable during estrous in the rabbit (Orstead *et al.*, 1988).

The requirements for development of an active corpus luteum are established during the follicular phase. There is a need for adequate numbers of granulosa cells (FSH and estrogen actions), response to LH by the development of LH receptors (FSH stimulation) and the ability to secrete progesterone after LH stimulus (Hirshfield, 1991; McNatty, 1978).

Evidence exists for the regulation of the ovarian blood flow by LH, since administration of

anti-LH to sheep reduced not only the blood flow to the ovaries but the progesterone concentration (Niswender *et al.*, 1976).

Mechanism of signal transduction

Both LH and FSH bind to their respective receptors located in the cellular membrane of target organs (Catt and Dufau, 1976; Catt and Pierce, 1978) and activate an enzyme “adenylyl cyclase” which catalyzes the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP). Cyclic adenosine monophosphate is considered a “second messenger” and plays an important function in the mechanism of signal transduction of many hormones (Sutherland and Rall, 1960).

Granulosa cells are the only cells in the female body known to possess FSH receptors. When FSH is bound to its receptor on the cell surface, adenylyl cyclase and cAMP-dependent protein kinases are activated, leading to the increased expression of the diverse messenger ribonucleic acids (mRNAs) that encode the proteins crucial to cytoproliferation and differentiation. The list of FSH-responsive genes is extensive and so far includes aromatase (P450arom), the steroidogenic cytochrome P450 crucial for estrogen synthesis (Hickey *et al.*, 1988; Steinkampf *et al.*, 1988), cholesterol side chain cleavage (P450scc), which is rate-limiting in progesterone synthesis (Richards *et al.*, 1987), the LH receptor (Segaloff *et al.*, 1990), several polypeptide growth factors and their binding proteins, such as the insulin-like growth factors (IGFs) and IGFBPs (Hernandez *et al.*, 1992; Oliver *et al.*, 1987; Voutilainen and Miller, 1987); proteolytic enzymes and inhibitors implicated in the mechanism of follicular rupture at ovulation, such as tissue plasminogen activator-TPA (O’Connell *et al.*, 1987) and

plasminogen activator inhibitor-PAI (Ny *et al.*, 1985), local regulatory peptides such as inhibin, activin, and follistatin (Ying, 1988), and the heat-shock protein hsp 90 (Ben-Ze'ev and Amsterdam, 1989).

Receptors for LH can be found on mature granulosa and thecal/interstitial cells where binding of the hormone activates adenylyl cyclase, which increases precursor-cholesterol uptake and sustains characteristically high steroidogenic enzyme activity in these cells (Gwynne and Strauss, 1982). In addition, it activates the transmission of intracellular signals in thecal (Hofeditz *et al.*, 1988) and luteal (Davis *et al.*, 1989; Wheeler and Veldhuis, 1989) cells through inositol lipid hydrolysis and the diacylglycerol/protein kinase C pathway (Leung and Steele, 1992).

One of the best known pathways of cAMP is through the activation of protein kinases which phosphorylates other enzymes necessary for the production of steroids (Dufau *et al.*, 1977). The mechanism of enzyme activation and their substrates are still being identified. There is evidence that once FSH or LH binds to its receptor, the complex is transported inside the cell and degraded (Ascoli and Puett, 1978; Chen *et al.*, 1978).

Superovulation in the rabbit model

Initial studies comparing the superovulatory response with PMSG and follicle stimulating extracts from sheep and horses, demonstrated that these gonadotropic preparations were able to induce superovulation in prepubertal and pubertal rabbits when properly administered (Pincus, 1940). Treatments using a single subcutaneous or

intramuscular injection of PMSG 50-200 IU, followed by 50 IU of hCG at the time of mating, 66 to 80 hours later, superovulated rabbits (Adams, 1982).

Administration of extracts from the horse pituitary to increase the number of mature follicles in ovaries of adult Albino, Lop, Dutch, Himalayan and cross-bred rabbits, indicated that the optimum duration of the priming process was 5 days and further injections evoked insensitivity to the extract which could not be overcome by an increase in the dosage (Parkes, 1942). Superovulation was obtained successfully in donor rabbits utilizing FSH extracted from porcine pituitary cells (400 µg per injection) during 4 consecutive days followed by one injection of hCG (100 to 120 IU) 6 hours after the last FSH injection (Voss *et al.*, 1990). Sexually mature rabbits of mixed breeds were superovulated by the administration of 0.3 mg of FSH subcutaneously twice daily during 3 consecutive days followed by an intravenous injection of 75 IU of hCG and mating. An average number of 22 embryos were obtained from this procedure (Fischer, 1987). Superovulation was induced in 222 does by 0.3 mg injections of FSH administered subcutaneously twice daily during 3 consecutive days with a minimum of an 8 hour interval between injections, followed by an intravenous injection of 75 IU of hCG and mating or artificial insemination. An average of 30 ovulations and 22.7 embryos were recovered per FSH treated donor rabbit (Fisher and Meuser-Odenkirchen, 1988).

Influence of heat stress on serum gonadotropins

The effects of environmental heat stress on serum gonadotropin levels, has been demonstrated in many species. Decreased secretion of basal as well as preovulatory surges of LH has been reported in Guernsey cows after exposure to 33.5° C and 55 % relative humidity

(RH) during two successive estrous cycles (Madan and Johnson, 1973). The preovulatory levels of LH were 6.4 ng/ml in thermoneutral conditions compared to 2.7 ng/ml in Angus heifers after exposure to a temperature variation of 21-34° C during the estrous cycle (Miller and Alliston, 1974). In the rabbit, exposure to heat stress for 2 days during the formation of the corpus luteum, significantly reduced the size of the luteal cells and plasma progesterone concentrations (Lublin, *et al.*, 1984). In the pig, exposure to high temperature of 35° C and 80 % RH for 3 hours, 5 times during a 41 hour period between the onset of estrus and ovulation not only lowered plasma levels of LH, but also estrogen levels (Ogasa *et al.*, 1989). Sows exposed to 33° C and 80 % RH in a climatic chamber during 3 hours daily during 1 week beginning on day 4 before ovulation and 35° C and 80 % RH for 3 hours, 5 times during 41 hours between the onset of estrus and ovulation demonstrated that exposure to elevated temperature and relative humidity may inhibit ovulation and lead to the production of cystic follicles. Peak plasma levels of LH were reduced and of a shorter duration than in the control animal (Ogasa *et al.*, 1989). Chronic exposure to elevated environmental temperatures has been shown to diminish the ability of the hypothalamo-hypophyseal axis to secrete FSH and LH, which influence follicular growth in gilts (Flowers and Day, 1990). Decreased serum concentrations of FSH and LH were observed in rats exposed to an elevated temperature of 35° C for 0.5, 2, 4, 6 and 24 hours. The concentration of FSH and LH decreased significantly within the first 30 minutes of exposure. During the next 3.5 hours of heat stress the concentration of gonadotropins increased gradually, but still remained below that of control animals (Bedrak and Chap, 1980). After 6 hours of heat stress exposure, the concentration of FSH and LH decreased once again. In animals exposed to heat stress during 24 consecutive

hours, serum concentrations of FSH and LH were below control animals (Bedrak and Chap, 1980). In the rabbit model, there are no reports on the effects of short or long term exposure to environmental heat stress on serum concentrations of FSH and LH.

Influence of heat stress on the response to gonadotropin treatment

There are few reports on the effects of heat stress during superovulatory treatments. Studies with lactating Holstein cows under environmental heat stress, demonstrated that elevated temperatures appear to alter the efficiency of follicular selection, dominance, and influence the quality of ovarian follicles (Badinga *et al.*, 1993). From a total of 34 embryos recovered from superovulated cows during low heat stress, 15 were transferable when compared to 1 of 4 recovered during high heat stress. In the study, heat stress apparently reduced the responsiveness of the ovaries to FSH (Kumar, 1987).

Oocyte maturation and preovulatory modifications in the follicle were considered critical and the most sensitive stages to heat stress (Doney *et al.*, 1973). Exposure of adult female rats to elevated temperatures for two hours daily during 5 consecutive days and superovulatory treatments with FSH and LH, demonstrated that in the rat, gonadotropic hormones tends to counteract the adverse effects of heat stress on folliculogenesis, but the superovulatory response was not fully expressed under heat stress conditions (Antoine and Pattabiraman, 1994).

MATERIALS AND METHODS

Experiment 1. Superovulatory response in New Zealand White rabbits with two commercial gonadotropins: FSH-P™ vs. SUPER-OV®

In this experiment, a comparison study was made between two commercial gonadotropins in order to determine the follicle stimulating hormone to be used in Experiment 2. The two commercial gonadotropins were:

1. FSH-P™ (Schering-Plough Animal Health Corp., Kenilworth, NJ)
2. SUPER-OV® (AUSA International Inc., Tyler, TX)

Hypothesis

The efficacy of SUPER-OV to superovulate adult female NZW rabbits is comparable to results obtained with FSH-P.

Experimental design

The experimental design is presented in Figure 1. Adult New Zealand White does were superovulated with either FSH-P or SUPER-OV during three consecutive days under thermoneutral conditions with a minimum interval of 8 hours between injections. Six hours after the last FSH injection, 25 IU/kg of hCG was given intramuscular to ensure ovulation followed by pairing overnight with a male of proven fertility. Nineteen hours after hCG administration, the reproductive tracts were recovered, the ovarian structures classified, and

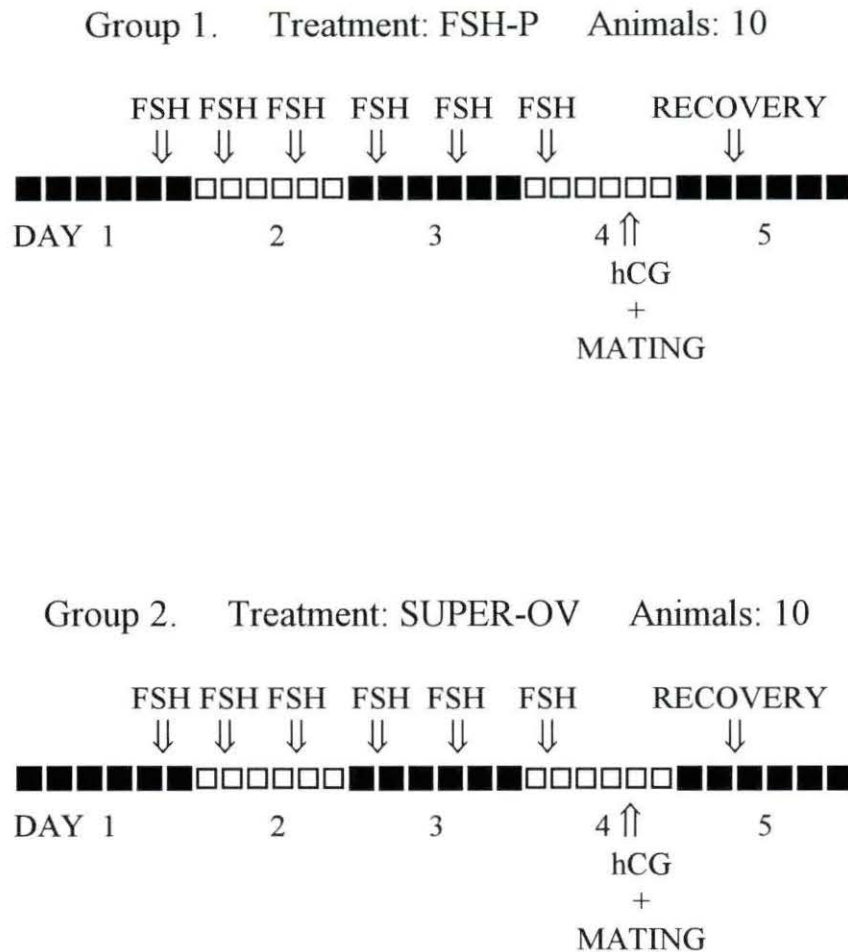


Figure 1. Description of experimental design, indicating treatments, number of animals, hormone injections, day of mating and day of recovery of the reproductive tracts. Does were treated with FSH-P or Super-OV beginning on the afternoon of Day 1 throughout the morning of Day 4. In the afternoon of Day 4, all does were given an intramuscular injection of hCG (25 IU/kg of body weight) to ensure ovulation and paired overnight with a male of proven fertility. The reproductive tracts were recovered during the morning of the following day.

the oviducts flushed to recover embryos.

Experimental animals

A total of 20 female New Zealand White rabbits (>3.0 kg of body weight) were allocated to one of two groups of ten animals. All does were kept undisturbed during a minimum of 17 days after arrival to avoid risks of pseudopregnancy due to transport. Each female was caged individually and housed under thermoneutral conditions of $21 \pm 1^\circ \text{C}$, 15-70 % RH, 12 h light/12 h dark cycle, provided commercial feed and water *ad libitum*. Sexually mature New Zealand White bucks were used for mating. The males were housed in a separate room under same environmental conditions as the females. Animal care was provided by the Laboratory Animal Resources staff.

Gonadotropin preparation and storage

Vials containing 50 mg Armour Standard units of FSH-P or SUPER-OV powder were diluted in 50 ml of sterile water (SyntroVet Incorporated, Lenexa, KS), giving a final concentration of 1 mg/ml. The preparation was divided into 25 aliquots of 2 ml each and stored in cryotubes at -20°C . Prior to use, the hormone was removed from the freezer, thawed at 37°C and then kept refrigerated at 4°C between injections.

Gonadotropin treatment

Superovulation was induced by subcutaneous injections of 0.3 ml of either FSH-P or SUPER-OV twice daily over three consecutive days (Fischer and Meuser-Odenkirchen,

1988; Kennelly and Foote, 1965). As described previously, a minimum interval of 8 hours were maintained between successive FSH injections.

Induction of ovulation

To ensure ovulation, all does received a single intramuscular injection of 25 IU/kg of hCG (Solvay Animal Health, Inc., Princeton, NJ) six hours after the last FSH injection. All does were then paired overnight with a male of proven fertility.

Recovery of the reproductive tracts

Does were transferred to the surgical room 19 hours after hCG administration and given an intramuscular injection of 5 mg/kg of body weight of acepromazine maleate (CEVA Laboratories, Inc., Overland Park, KS) for preanesthesia. After a 15 minute period, 40 mg/kg of body weight of ketamine hydrochloride (Fort Dodge Laboratories, Inc., Fort Dodge, IA) was given by intramuscular injection to induce anesthesia.

After reaching the surgical plane of anesthesia, does were euthanized by CO₂ inhalation, the abdominal cavity was opened and the reproductive tracts recovered (Kamolpatana, 1995). The ovary and the oviduct with a short segment of the proximal portion of the uterine horn from each side were placed in collecting bowls containing HEPES-buffered saline (pH 7.4) at 37° C. The oviducts were flushed from the uterotubal junction using a 24 gauge blunt needle and a 5 ml syringe (Air Tite, Virginia Beach, VA) containing 3 ml of HEPES-buffered saline solution (pH 7.4). A 2 ml air chase was used to confirm the passage of the flushing solution through the oviducts. A bowl containing a

bicarbonate-buffered saline solution (pH 7.4) at 37° C supplemented with 0.3 % (w/v) of gelatin type B (Sigma Chemical Inc., St. Louis, MO) was used to collect the embryos and flushing solution. The oocytes and embryos were separated from the debris with the aid of a stereomicroscope and transferred to a 24 well, flat bottom tissue culture plate with a low evaporation lid (#3047 Becton Dickinson Labware, Lincoln Park, NJ) and examined for stage of development and morphology using an inverted microscope at 100X magnification.

Recovered embryos were expected to be at the 1 or 2-cell stage of development. Oocytes that had spermatozoa in the perivitelline space, that had extruded the second polar body, or had two visible pronuclei were considered to be fertilized. Embryos that had two blastomeres of comparable size were classified as 2-cell embryos.

Classification of ovarian structures

Ovaries were separated from the reproductive tracts and examined under a stereomicroscope at 20X magnification. The number of ovulated follicles, and the number of non-ovulated and hemorrhagic follicles were recorded for each ovary. The total ovarian stimulation (number of ovulations + non-ovulated follicles + hemorrhagic follicles) from FSH-P and SUPER-OV treated does was calculated after completion of the experiment. In addition, the number of oocytes and/or embryos recovered from the flushing of the oviducts were recorded.

Endpoints

1. Classification of ovarian structures from FSH-P and SUPER-OV treated animals.
2. Determination of the number of embryos and/or oocytes recovered from FSH-P and SUPER-OV treated animals.

Statistical analysis

In Experiment 1, the individual rabbits were the experimental units, and they were allocated to treatment using stratified random sampling. Data were analyzed by using a complete randomized design for analysis of variance based on general linear model (SAS Institute Inc., Cary, NC) and Students *t*-test (Steel and Torrie, 1980) for comparisons between treatment means. The P values were calculated based on total observations from right and left ovaries or oviducts. Statistical significance was established at $P \leq 0.05$ and results from the classification of ovarian structures were expressed using treatment mean \pm SD.

Experiment 2. Superovulatory response in New Zealand White rabbits under environmental heat stress

Hypotheses

1. The superovulatory response with FSH/hCG treatment is affected by exposure to environmental heat stress.

2. Exposure of donor rabbits to environmental heat stress before mating affects the fertilization rate and development of embryos during a 96-hour period of *in vitro* culture.

Experimental design

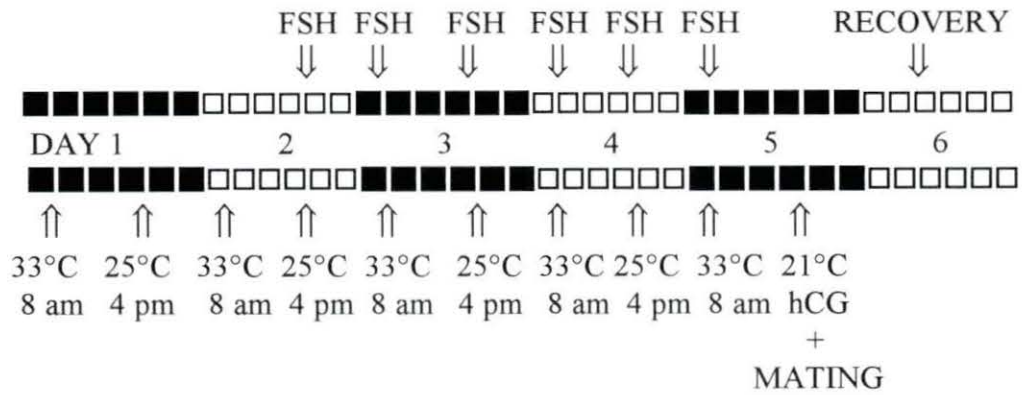
The design for Experiment 2 is presented in Figure 2. Adult New Zealand White does were allocated using stratified random sampling to 4 groups of 10 animals and assigned to one of the following treatments: Group (1) FSH/hCG + heat stress, Group (2) FSH/hCG, Group (3) sterile saline + heat stress and Group (4) sterile saline. Heat stress consisted of daily exposure to $33 \pm 1^\circ \text{C}$ and 10-30 % RH during 8 consecutive hours followed by 16 hours at $25 \pm 1^\circ \text{C}$ and 10-30 % RH. Does were heat-stressed inside the chamber during 5 consecutive days. Animals that were not assigned to heat stress were housed inside the same chamber but at different periods under thermoneutral conditions of $21 \pm 1^\circ \text{C}$ and 15-70 % RH. A 12 h light/12 h dark cycle was maintained during the experiment.

Superovulatory treatment, mating, recovery of the reproductive tracts, flushing of the oviducts and classification of the ovarian structures followed the same procedures cited in Experiment 1. Embryos and/or oocytes recovered from all does were cultured during a 96-hour period. Embryonic viability was determined prior to and after culture.

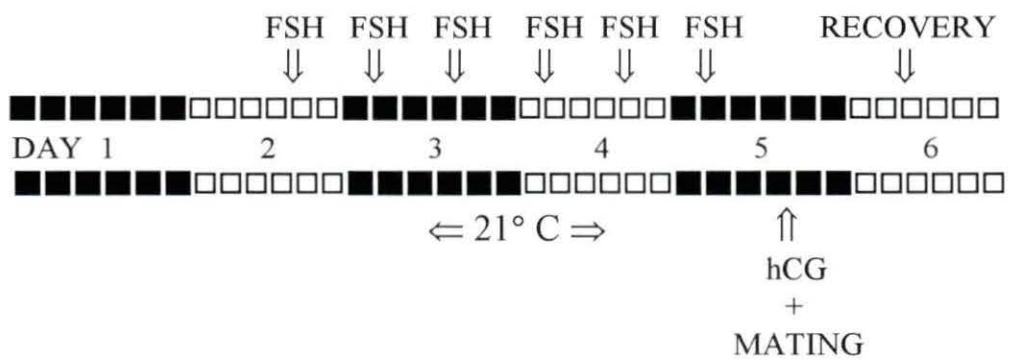
Experimental animals

A total of 40 female New Zealand White rabbits (> 3.0 kg of body weight) were used in this experiment. All does were kept undisturbed during a minimum of 17 days after arrival to avoid risks of pseudopregnancy due to transport. Each animal was caged individually and

Group 1. Treatment: FSH + HS Animals: 10



Group 2. Treatment: FSH + TN Animals: 10



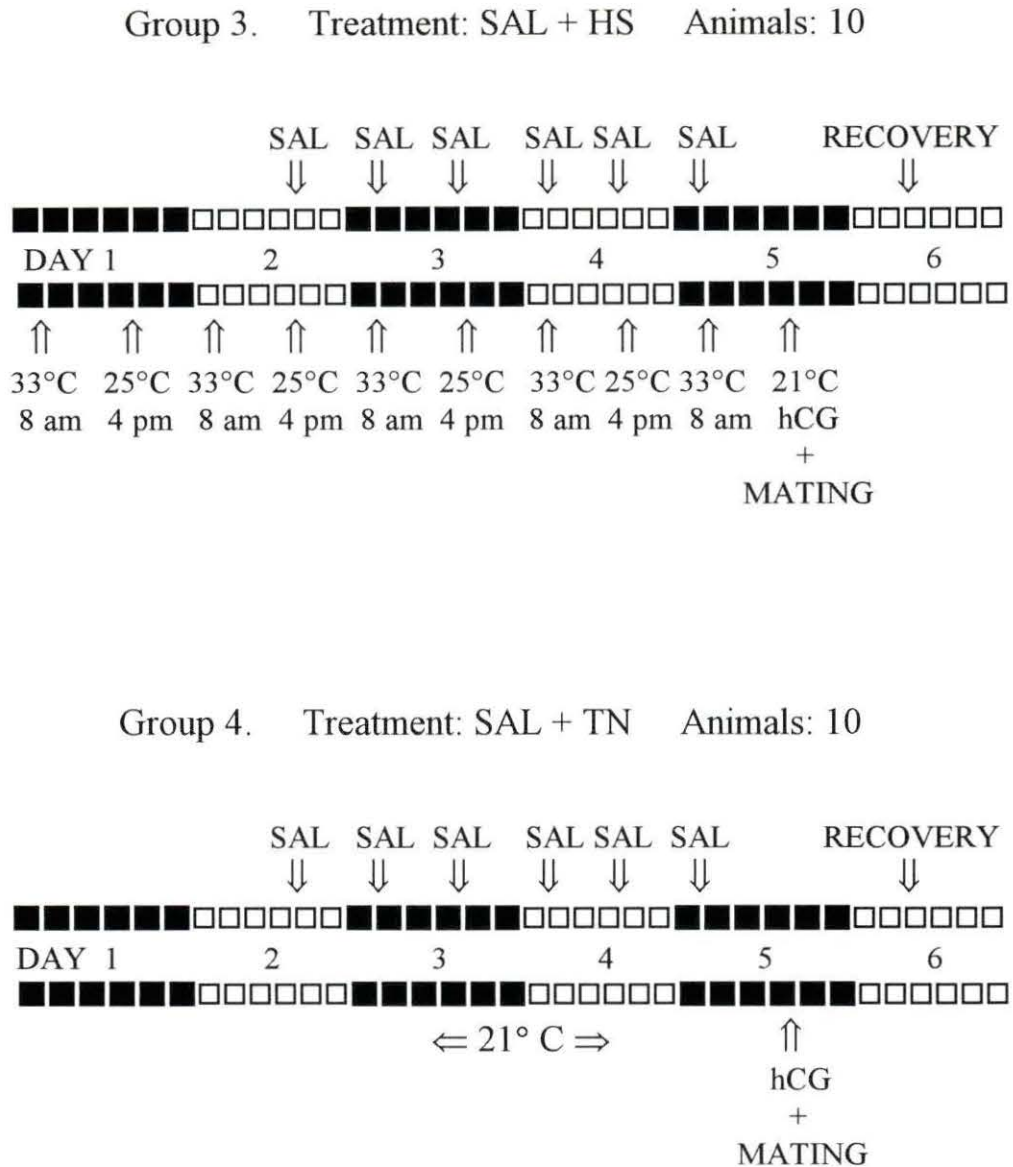


Figure 2. Description of experimental design, indicating the 4 groups of animals, treatments, number of animals, hormone injections, day of mating and recovery of the reproductive tracts. All does were fed commercial feed and water *ad libitum* during the experiment. Does were superovulated (FSH) or saline injected (SAL) under heat stress (HS) or thermoneutral conditions (TN).

housed under thermoneutral conditions of $21 \pm 1^\circ \text{C}$, 15-70 % RH, 12 h light/12 h dark cycle, fed commercial feed and water *ad libitum* before assignment to treatment. Sexually mature New Zealand White bucks were used for mating. The males were housed in a separate room under the same environmental conditions as the females. Animal care was provided by the Laboratory Animal Resources staff.

Gonadotropin treatment

Superovulation was induced by 0.3 ml subcutaneous injections containing 0.3 mg Standard Armour units of FSH-P twice daily during three consecutive days with a minimum 8 hour interval between FSH injections (Fischer and Meuser-Odenkirchen, 1988; Kennelly and Foote, 1965). A total dosage of 1.8 mg of FSH was given to each FSH-P treated doe. Animals that were not assigned to superovulatory treatment received 0.3 ml subcutaneous injections of sterile saline (Baxter, Deerfield, IL). Six hours after the last FSH-P or saline injection, all does were given hCG and paired overnight with a fertile male.

Exposure to heat stress

A temperature controlled chamber 12'9" x 8'10" was used for heat-stress exposure. Does were housed during 5 consecutive days inside the chamber under $33 \pm 1^\circ \text{C}$ and 10-30 % RH from 8:00 am to 4:00 pm and $25 \pm 1^\circ \text{C}$ and 10-30 % RH from 4:00 pm to 8:00 am. Animals that were not assigned to heat stress were housed inside the experimental chamber under constant thermoneutral conditions of $21 \pm 1^\circ \text{C}$ and 15-70 % RH. The air flow was

adjusted to ensure a minimum of 20 air changes per hour inside the chamber and a 12 h light/12 h dark cycle was maintained.

Culture media

The media used for embryo culture was prepared by adding 2.5 mM of L (+) Glutamine (Fisher Scientific Company, Fair Lawn, NJ) to 100 ml of HL-1 culture media (Bio Whittaker, Walkersville, MD). The preparation was mixed and 0.5 ml of gentamicin solution (Sigma-Aldrich Co., Irvine, UK) at a concentration of 10 mg/ml was added. A glass fiber prefilter and a 0.2 μm cellulose nitrate filter apparatus (Nalge, Rochester, NY) were used to filtrate the culture media. After filtration, the media was gassed during 20 minutes with 5 % CO_2 and stored at 4° C until used.

Embryo culture

After flushing the oviducts, the embryos and/or oocytes recovered were washed 3 times with bicarbonate-buffered saline solution (pH 7.4) supplemented with 0.3 % (w/v) of gelatin type B maintained at 37° C using a 24 well, flat bottom tissue culture plate with a low evaporation lid (#3047 Becton Dickinson Labware, Lincoln Park, NJ). The embryos and/or oocytes were then transferred to a 2-well depression slide containing 35 μl of a 240 μM solution of eosin B in bicarbonate-buffered saline in each well. Embryonic viability was determined by exposure of the embryos and/or oocytes to the eosin B solution over a 10 minute period (Dooley, 1988; Dooley *et al.*, 1984) and examined under an inverted microscope at 100X of magnification for staining and classification of the viability while inside

the depression slide. Embryos and/or oocytes that were unstained or partially stained were classified as viable, where as those that were completely stained by the eosin B solution were considered non-viable. After exposure to the eosin B solution, all embryos and/or oocytes were washed 2 times with bicarbonate-buffered saline solution and randomly assigned to *in vitro* culture during a 96-hour period in a 24 well, flat bottom tissue culture plate with a low evaporation lid (#3047 Becton Dickinson Labware, Lincoln Park, NJ). Each embryo was cultured individually inside a well containing 500 µl of culture media during a 96-hour period. Once the embryos were allocated inside the tissue culture plate, the stage of development and morphology of each embryo was recorded using an inverted microscope at 100X magnification. The incubator used for culture was adjusted to 37° C and supplemented with 5 % CO₂ and humidified air atmosphere. Embryos were evaluated for stage of development at 0, 24, 48, 72 and 96 hours of culture. After the 96-hour evaluation, viability was determined using the eosin B assay.

Endpoints

1. Classification of ovarian structures from animals of groups 1, 2, 3 and 4.
2. Evaluation of embryonic development at 0, 24, 48, 72 and 96 hour of culture.
3. Embryonic viability prior to and after a 96-hour period of *in vitro* culture.

Statistical analysis

In Experiment 2, a split-plot experimental design with subsampling and repeated measures was used. The experimental units in the whole plot were 4 blocks of rabbits which

were exposed to heat stress or thermoneutral conditions inside the chamber. In the subplot, each individual rabbit was the experimental unit and were treated with FSH or sterile saline. The embryos recovered from donor rabbits were considered a subsample and the repeated measures were the stages of development during the 96-hour period of *in vitro* culture.

Data were analyzed by using a split-plot experimental design for analysis of variance based on general linear model (SAS Institute Inc., Cary, NC) and Students *t*-test (Steel and Torrie, 1980) for comparisons between treatment means. Chi-square analyses of ratios (Steel and Torrie, 1980) was used to compare developmental stages and viability of embryos. The P values were calculated based on total observations from right and left ovaries. Statistical significance was established at $P \leq 0.05$ and results from the classification of ovarian structures were expressed using treatment mean \pm SD.

RESULTS AND DISCUSSION

Experiment 1. Superovulatory response in New Zealand White rabbits with two commercial gonadotropins: FSH-P™ vs. SUPER-OV®

Results

Number of ovulated follicles

The number of ovulated follicles was greater ($P < 0.005$) in FSH-P (34 ± 12) than in SUPER-OV (19 ± 6) treated does (Figure 3). However, no effects ($P > 0.1$) were found for the influence of ovary or the ovary x treatment interaction.

Number of non-ovulated follicles

The number of non-ovulated follicles (≥ 1.5 mm in diameter) present on the ovaries of FSH-P (5 ± 3) and SUPER-OV (5 ± 4) treated does did not differ greatly (Figure 4). No effects were found ($P > 0.1$) for treatment, ovary, or the ovary x treatment interaction.

Number of hemorrhagic follicles

In both treatment groups, hemorrhagic (bloody) follicles were present on the ovaries. Overall, a higher incidence ($P < 0.05$) was recorded with FSH-P (11 ± 16) than with SUPER-OV treatment (2 ± 0.3), Figure 5. Statistical analysis showed no effect ($P > 0.1$) for the influence of ovary or the ovary x treatment interaction.

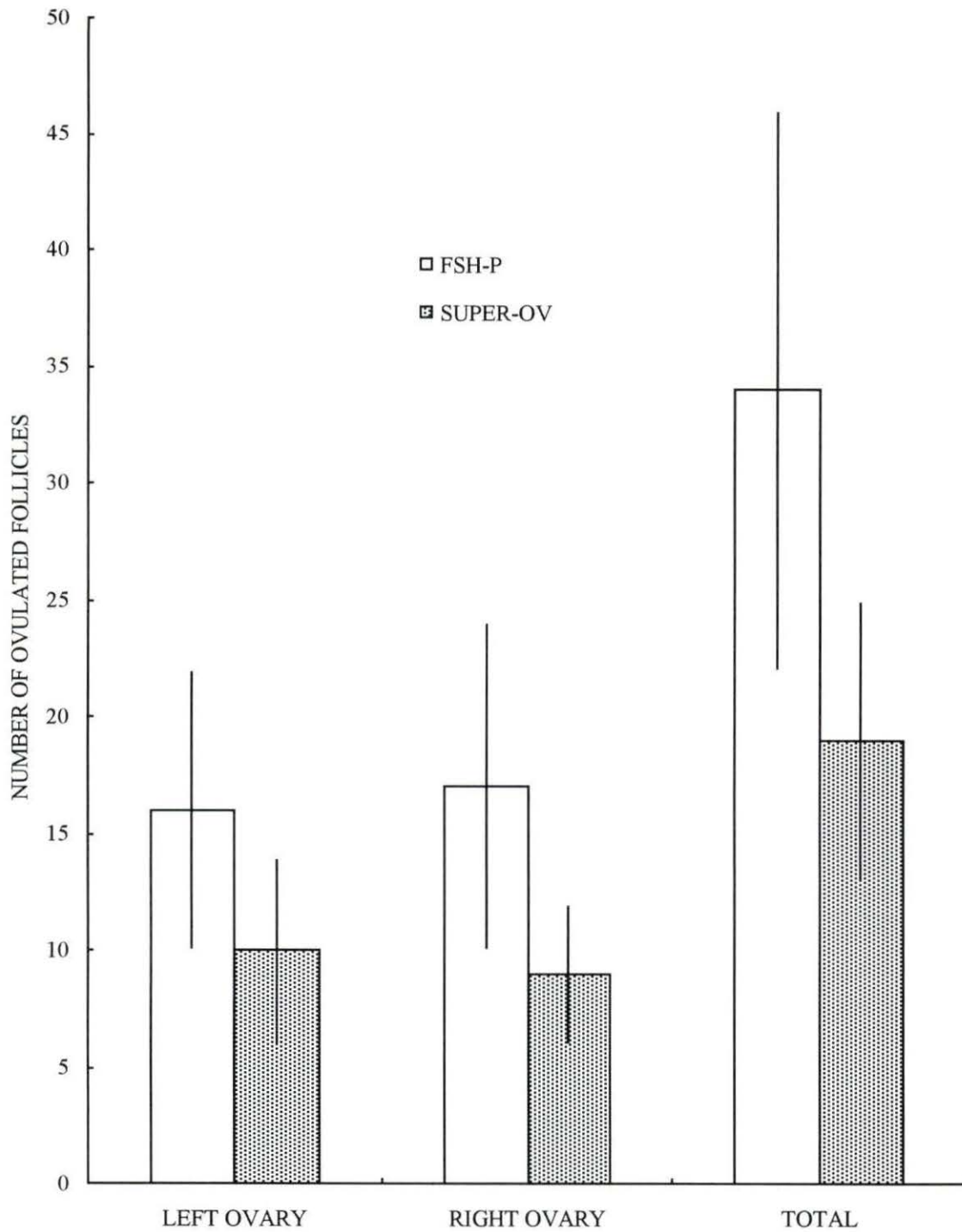


Figure 3. Ovulatory rates for FSH-P and SUPER-OV treated animals (mean \pm SD; n=10/Group). Treatment with FSH-P resulted in more ($P < 0.005$) ovulations than for SUPER-OV treated does.

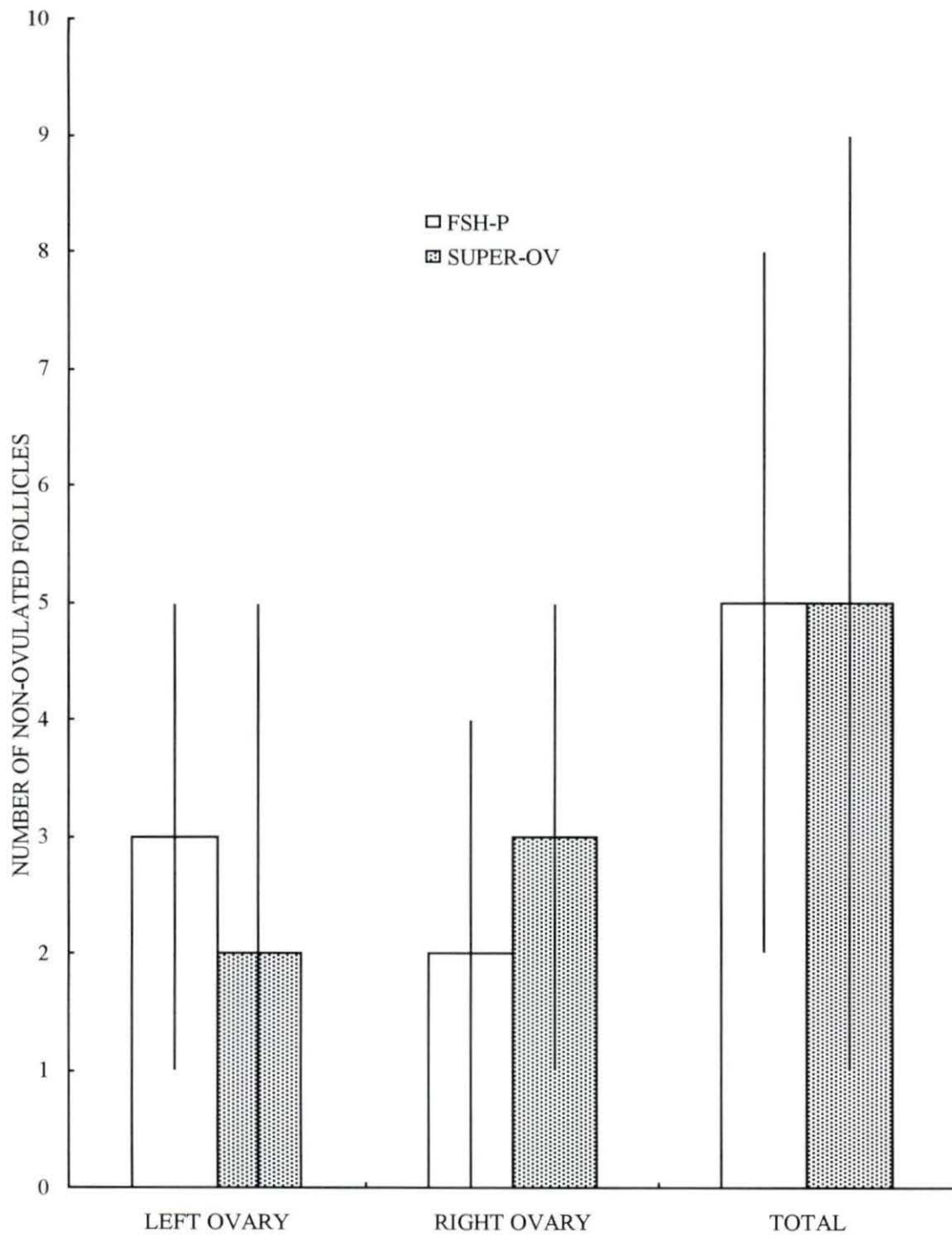


Figure 4. Non-ovulated follicles in FSH-P and SUPER-OV treated animals (mean \pm SD; n=10/Group). Responses did not differ ($P > 0.1$) between treatments.

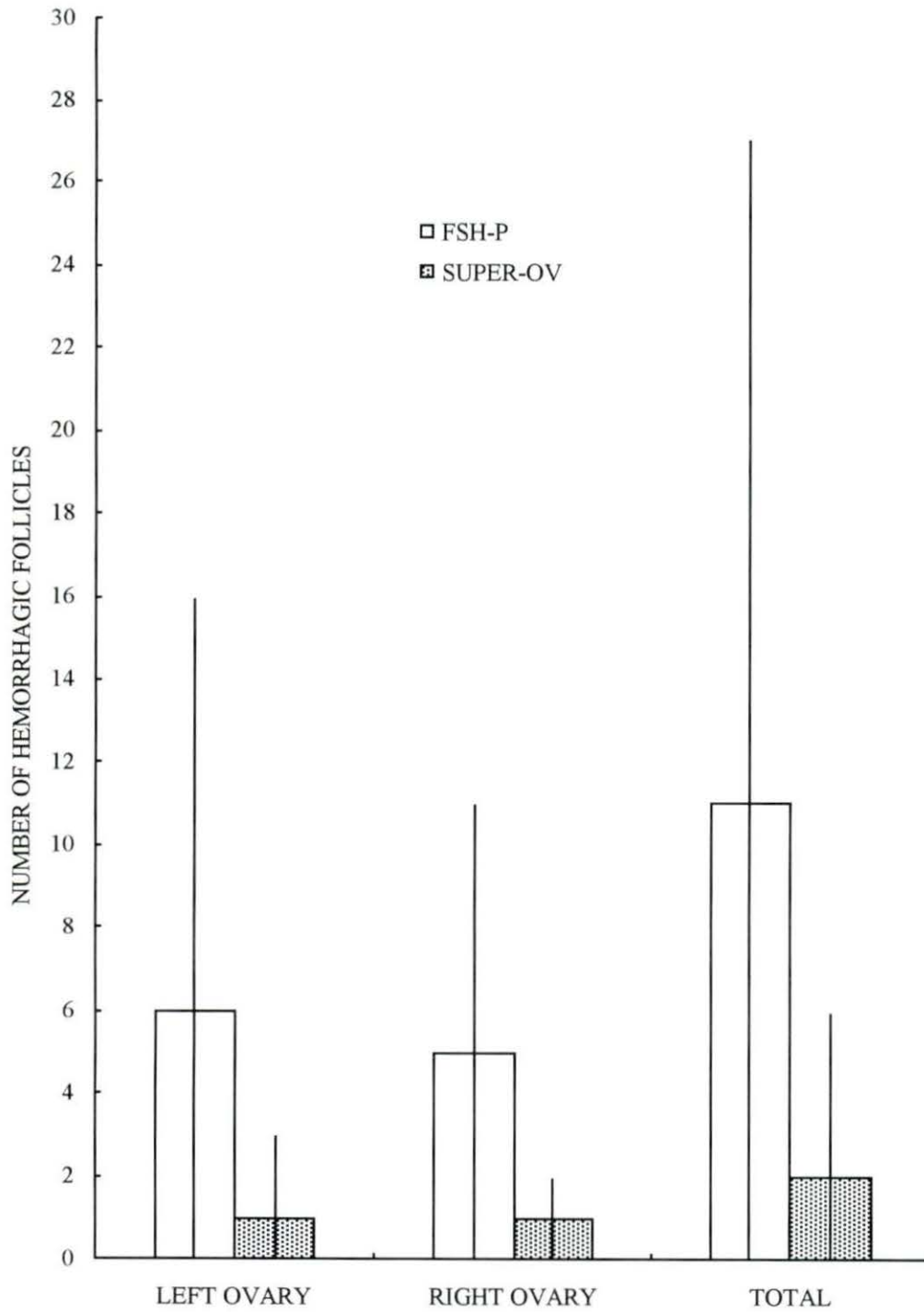


Figure 5. Hemorrhagic follicles in FSH-P and SUPER-OV treated animals (mean \pm SD; n=10/Group). Treatment with FSH-P resulted in more ($P < 0.05$) hemorrhagic follicles than for SUPER-OV treated does.

Total follicular stimulation

Evaluation of total follicular stimulation from FSH-P and SUPER-OV treated does, indicated that FSH-P (49 ± 16) resulted in significantly greater ($P < 0.005$) follicular stimulation than for SUPER-OV treated does (24 ± 9), Figure 6. The influence of ovary and the ovary x treatment interaction were not significant ($P > 0.1$).

Number of embryos and/or oocytes recovered

Flushing fluids from FSH-P treated does contained significantly more ($P < 0.005$) embryos and/or oocytes recovered (291) than fluids from SUPER-OV (154) treated does (Table 1). No effects were found ($P > 0.1$) for the influence of oviduct or the oviduct x treatment interaction. In addition, a higher fertilization rate was present in FSH-P (92 %) than in SUPER-OV (62 %) treated does ($P < 0.005$), Table 1.

Discussion

Because the rabbit is an induced ovulator, all females were given hCG to ensure ovulation of mature follicles even if the doe did not mate. In general, superovulation was obtained in animals administered either FSH-P or SUPER-OV. Ovulatory rates in the rabbit normally varies between 4-10 (Carney and Foote, 1990; Fisher and Meuser-Odenkirchen, 1988; Kennelly and Foote, 1965). Evaluation of ovarian structures and flushings of the oviducts indicated that FSH-P treatment resulted in twice as many ovulations, embryos and/or oocytes recovered, and follicles stimulated when compared to SUPER-OV treated does. In addition, a greater number of hemorrhagic follicles were present with FSH-P

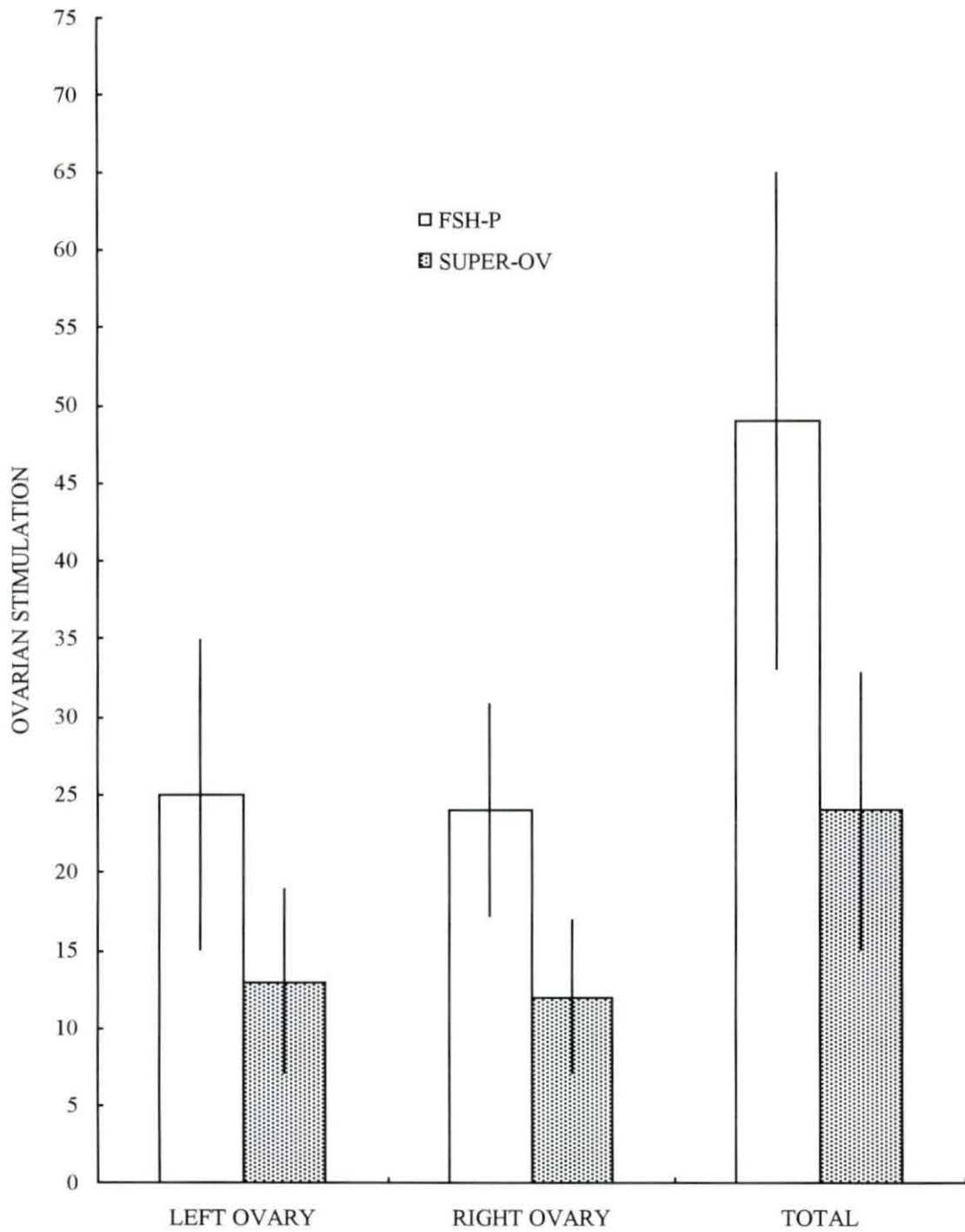


Figure 6. Ovarian stimulation (total number of ovulated, non-ovulated, hemorrhagic follicles) in FSH-P and SUPER-OV treated animals (mean \pm SD; n=10/Group). Treatment with FSH-P resulted in greater ($P < 0.005$) ovarian stimulation than for SUPER-OV.

Table 1. Embryos and/or oocytes recovered from FSH-P and SUPER-OV treated animals (n=10/Group). Treatment with FSH-P resulted in more embryos and/or oocytes recovered than for SUPER-OV. Fertilization rates were also higher with FSH-P treatment.

Treatment	No. of Oocytes + Embryos	No. of Embryos	Fertilization Rate (%)
FSH-P	291	267	92
SUPER-OV	154	96	62
P	< 0.005	< 0.005	< 0.005

treatment.

The higher level of follicular stimulation, increased rates of ovulation, and embryos and/or oocytes recovered from FSH-P treated animals may be due to the fact that the concentration of FSH and LH and/or ratio between them were higher than in SUPER-OV.

In the rabbit, when ovaries are over-stimulated by gonadotropin preparations, hemorrhagic follicles are present (Parkes, 1942; Pincus, 1940). Additional studies are needed to determine if the occurrence of these bloody follicles are primarily related to the overall concentration and/or ratio of FSH and LH in the preparation.

The observed difference between the number of ovulations and embryos or oocytes recovered in superovulated rabbits seems to be a general feature after gonadotropin treatment. Fischer and Meuser-Odenkirchen (1988), reported that does given 0.3 mg of FSH-P subcutaneously, twice daily, during 3 consecutive days, followed by an intravenous injection of 75 IU of hCG, had an average of 30 ovulations and 22.7 embryos were recovered per female (75.6 %). The administration of 0.25 mg Armour of FSH subcutaneously twice daily during 3 consecutive days followed by 2.5 mg Armour of equine pituitary luteinizing

hormone to female rabbits (Kennelly and Foote, 1965), produced an average of 53.7 ovulations and 39.8 embryos recovered (74.1 %). In this study, an average of 34 ovulations and 29 embryos were recovered (85.2%) from FSH-P treated does, while 19 ovulations and 15 embryos were recovered (78.9%) from SUPER-OV treated does.

Embryonic losses due to retention in the uterine tubes was unlikely, because flushing of the oviducts was repeated until no additional embryos were found. The discrepancy between the number of ovulations and embryos recovered may be due to losses during recovery and manipulation of the reproductive tracts or failure of the ovulated oocytes to enter the oviducts. No attempt was made to determine if oocytes might have been trapped within follicles despite the induction of ovulation and evidence for follicular rupture.

Experiment 2. Superovulatory response in New Zealand White rabbits under environmental heat stress

Results

Number of ovulated follicles

The ovulatory rates were greater ($P < 0.001$) for does treated with FSH + HS (27 ± 11) or FSH + TN (28 ± 10) than in SAL + HS (8 ± 2) or SAL + TN (9 ± 3) treated animals, Figure 7. Although the number of ovulated follicles was significantly influenced by hormone treatment, differences between total ovulations from FSH + HS and FSH + TN and between SAL + HS and SAL + TN treated groups were not significant ($P > 0.1$). No effects were found for the influence of heat stress ($P > 0.1$) or the heat stress x FSH interaction ($P > 0.1$).

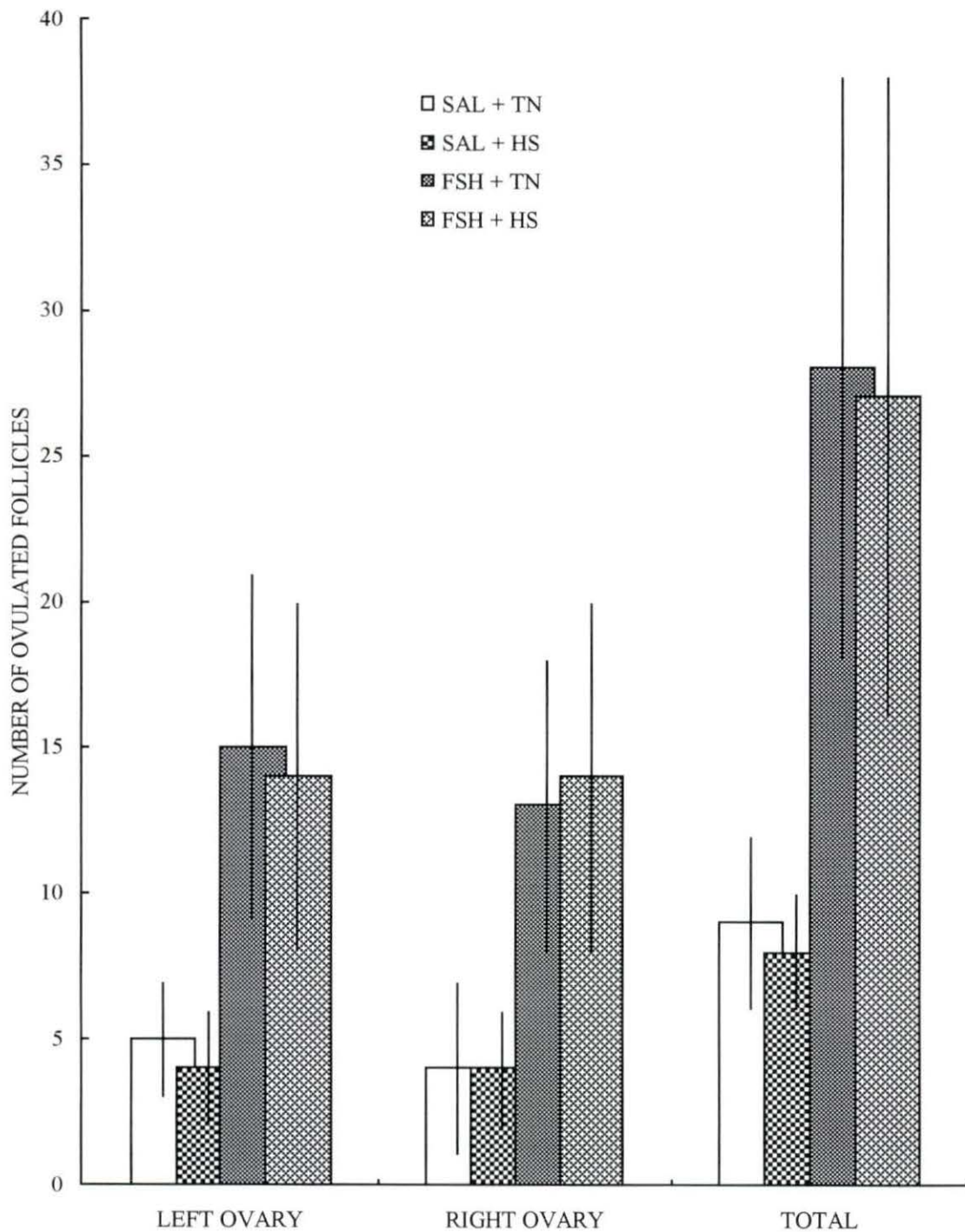


Figure 7. Ovulatory rates from does treated with FSH or saline under heat stress or thermoneutral conditions (mean \pm SD; n=10/Group). A greater number of ovulated follicles were present with hormone treatment ($P < 0.001$). Heat stress did not influence the ovulatory response ($P > 0.1$).

Number of non-ovulated follicles

There was a moderate increase in the number of non-ovulated follicles (≥ 1.5 mm in diameter) on the ovaries of does treated with FSH + HS (6 ± 8) or FSH + TN (5 ± 6) when compared to the SAL + HS (3 ± 3) or SAL + TN (2 ± 2) treatments, Figure 8. The number non-ovulated follicles was influenced by hormone treatment ($P < 0.05$), where as the differences observed between means for FSH + HS and FSH + TN or between SAL + HS and SAL + TN treated groups were not significant ($P > 0.1$). No effects were found for the influence of heat stress ($P > 0.1$) or the heat stress x FSH interaction ($P > 0.1$).

Number of hemorrhagic follicles

A moderate increase in the number of hemorrhagic follicles was observed on the ovaries of does treated with FSH + HS (3 ± 3) or FSH + TN (3 ± 5) when compared to SAL + HS (0.3 ± 0.6) or SAL + TN (2 ± 6) treatments, Figure 9. The number of hemorrhagic follicles was influenced ($P < 0.01$) by hormone treatment. The total means between FSH treated groups did not differ, while the difference observed between the saline injected groups were not significant ($P > 0.1$). No effects were found for an influence of heat stress ($P > 0.1$) or a heat stress x FSH interaction ($P > 0.1$).

Total follicular stimulation

In Experiment 2, does that received FSH + HS (36 ± 12) or FSH + TN (36 ± 10) had a greater ($P < 0.001$) follicular stimulation than SAL + HS (11 ± 2) or SAL + TN (13 ± 9) treated animals, Figure 10. The differences between total stimulation from the saline-treated

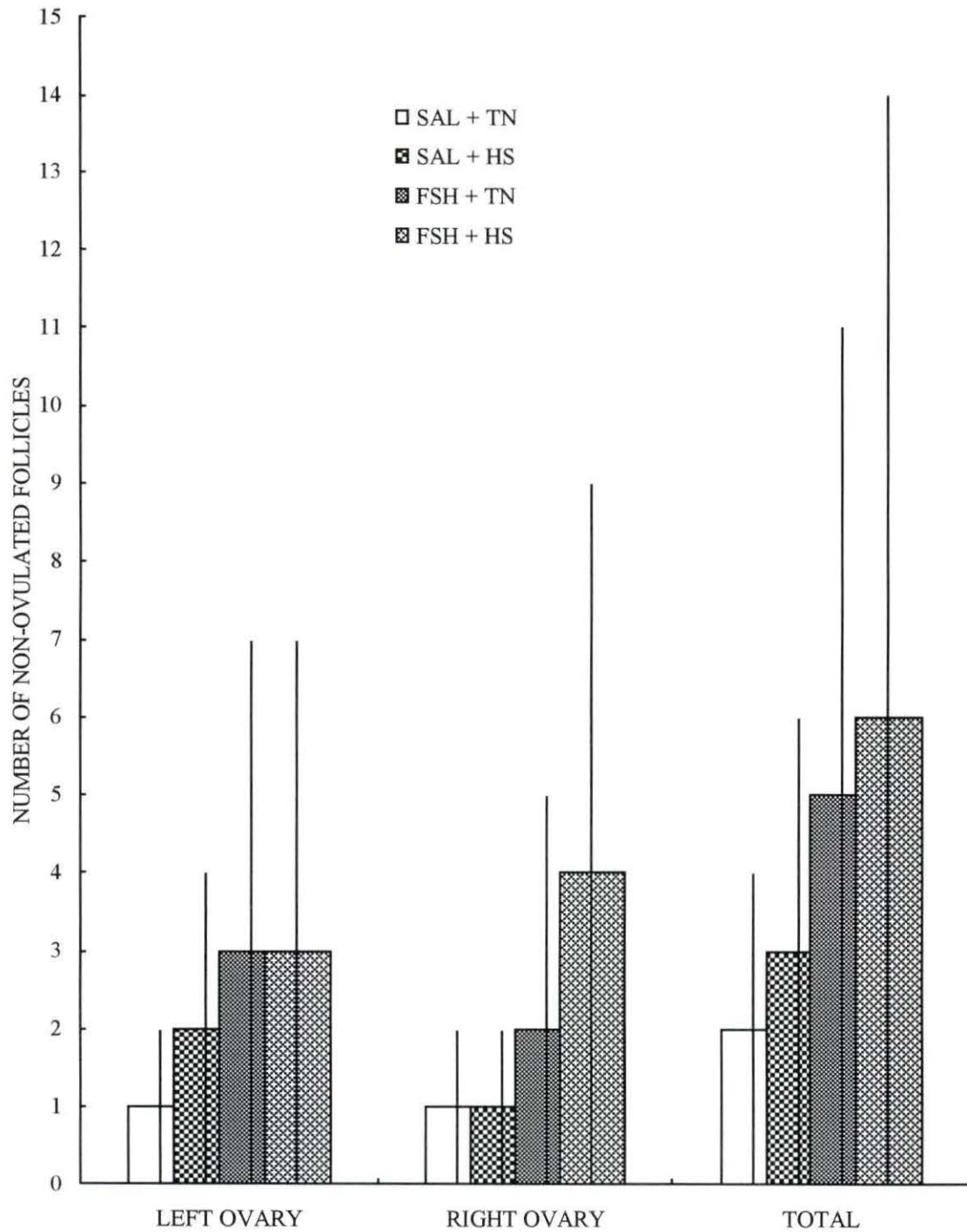


Figure 8. Non-ovulated follicles (≥ 1.5 mm in diameter) present on the ovaries of does treated with FSH or saline under heat stress or thermoneutral conditions (mean \pm SD; $n=10$ /Group). A hormone effect was present ($P < 0.05$). Heat stress did not influence the occurrence of non-ovulated follicles ($P > 0.1$).

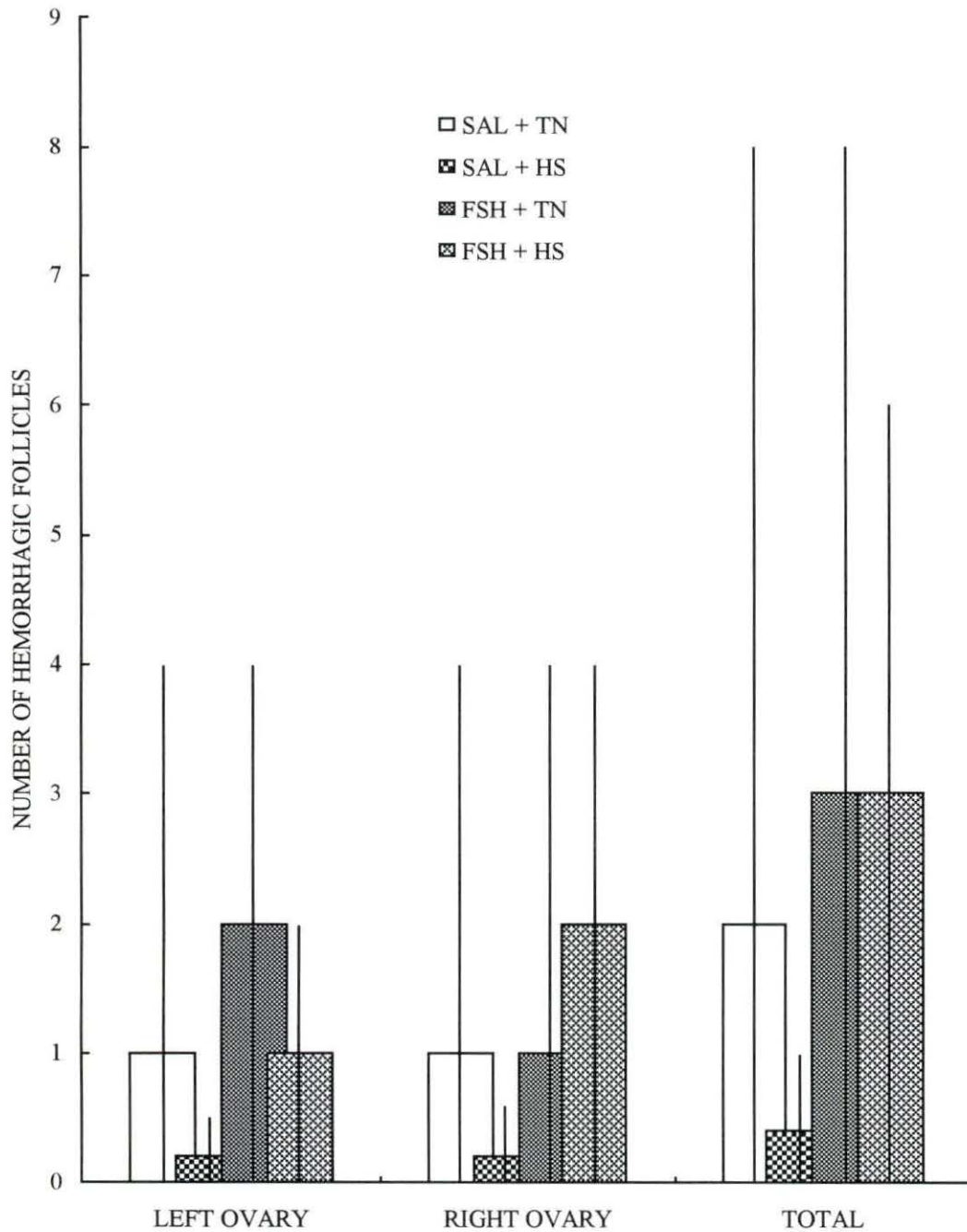


Figure 9. Hemorrhagic follicles present on the ovaries of does treated with FSH or saline under heat stress or thermoneutral conditions (mean \pm SD; n=10/Group). The effects of FSH treatment were significant ($P < 0.01$), however exposure to heat stress did not influence the number of hemorrhagic follicles ($P > 0.1$).

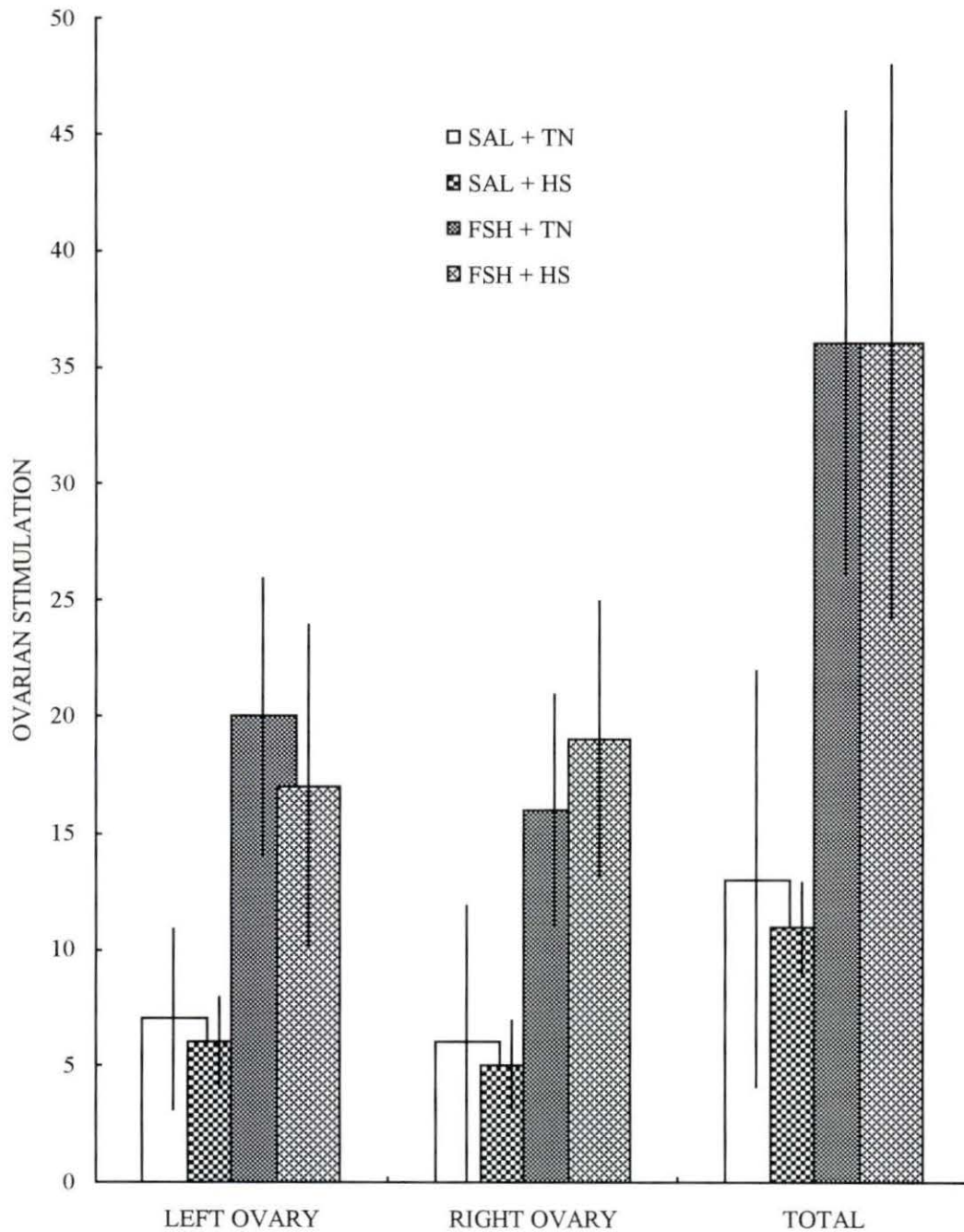


Figure 10. Ovarian stimulation (total number of ovulated + non-ovulated + hemorrhagic follicles) from FSH or saline-treated does under heat stress or thermoneutral conditions (mean \pm SD; n=10/Group). A greater ovarian stimulation was present with FSH treatment ($P < 0.001$). No effects of heat stress were found ($P > 0.1$).

groups were not significant ($P > 0.1$). The higher levels of follicular stimulation were clearly due to hormone treatment ($P < 0.001$), rather than heat stress exposure ($P > 0.1$) or to a heat stress x FSH interaction ($P > 0.1$).

Number of embryos and/or oocytes recovered

A greater number of embryos and/or oocytes were recovered from flushing fluids of the oviducts of FSH + HS (250) and FSH + TN (210) treated does when compared to SAL + HS (80) and SAL + TN (80) treated animals, Table 2. The number of embryos and/or oocytes recovered was significantly influenced by hormone treatment ($P < 0.001$). However, the difference found between total embryos from FSH + TN and FSH + HS was not significant ($P < 0.1$). No effects were found for the influence of heat stress ($P > 0.1$) or the heat stress x FSH interaction ($P > 0.1$). In addition, higher fertilization rates were present in FSH + HS (88 %) and FSH + TN (88 %) when compared to SAL + HS (56 %) and SAL + TN (54 %) treated does ($P < 0.005$), Table 2. There were no significant effects of heat stress exposure on the fertilization rates ($P > 0.1$).

Embryo culture

A total of 492, 1-cell and 2-cell embryos were cultured *in vitro* during a 96-hour period in Experiment 2. Each embryo was evaluated individually at 0, 24, 48, 72 and 96 hour for stage of development (Tables 3-6). The number of morulae, blastocysts and degenerate embryos found in each group after a 96-hour period of culture is shown in Table 7.

Table 2. Embryos and/or oocytes recovered from oviducts of FSH + HS, FSH + TN, SAL + HS and SAL + TN treated does (n=10/Group). Fertilization rates were higher in groups of animals treated with FSH under heat stress or thermoneutral conditions.

Treatment	No. of Oocytes + Embryos	No. of Embryos	Fertilization rate (%)
FSH + HS	250	220	88
FSH + TN	210	184	88
P	< 0.1	> 0.1	> 0.1
SAL + HS	80	45	56
SAL + TN	80	43	54
P	> 0.1	> 0.1	> 0.1

FSH + HS = Superovulatory treatment with follicle stimulating hormone under heat stress conditions.

FSH + TN = Superovulatory treatment with follicle stimulating hormone under thermoneutral conditions.

SAL + HS = Saline injected under heat stress conditions.

SAL + TN = Saline injected under thermoneutral conditions.

Table 3. Embryonic development of 220 embryos recovered from superovulated (FSH) donor rabbits under heat stress conditions (HS) and cultured during a 96-hour period.

Stage of Development	Hour of Culture				
	0	24	48	72	96
1-2 cell	220 (100 %)	9 (4 %)	4 (2 %)	0	0
3-8 cell	0	208 (95 %)	51 (23 %)	7 (3 %)	0
16-32 cell	0	0	150 (68 %)	42 (19 %)	3 (1 %)
Morula	0	0	1 (1 %)	102 (46 %)	71 (32 %)
Blastocyst	0	0	0	4 (2 %)	61 (28 %)
Degenerate	0	3 (1 %)	14 (6 %)	65 (30 %)	85 (39 %)

Table 4. Embryonic development of 184 embryos recovered from superovulated (FSH) donor rabbits under thermoneutral conditions (TN) and cultured during a 96-hour period.

Stage of Development	Hour of Culture				
	0	24	48	72	96
1-2 cell	184 (100 %)	7 (4 %)	3 (2 %)	3 (2 %)	3 (2 %)
3-8 cell	0	165 (90 %)	39 (21 %)	2 (1 %)	1 (1 %)
16-32 cell	0	0	97 (53 %)	14 (8 %)	3 (2 %)
Morula	0	0	17 (9 %)	114 (62 %)	48 (26 %)
Blastocyst	0	0	0	11 (6 %)	89 (48 %)
Degenerate	0	12 (6 %)	28 (15 %)	40 (21 %)	40 (21 %)

Table 5. Embryonic development of 45 embryos recovered from saline treated (SAL) donor rabbits under heat stress conditions (HS) and cultured during a 96-hour period.

Stage of Development	Hour of Culture				
	0	24	48	72	96
1-2 cell	45 (100 %)	1 (2 %)	0	0	0
3-8 cell	0	39 (87 %)	9 (20 %)	0	0
16-32 cell	0	0	25 (56 %)	6 (13 %)	0
Morula	0	0	2 (4 %)	25 (56 %)	9 (20 %)
Blastocyst	0	0	0	0	16 (36 %)
Degenerate	0	5 (11 %)	9 (20 %)	14 (31 %)	20 (44 %)

Table 6. Embryonic development of 43 embryos recovered from saline treated (SAL) donor rabbits under thermoneutral conditions (TN) and cultured during a 96-hour period.

Stage of Development	Hour of Culture				
	0	24	48	72	96
1-2 cell	43 (100 %)	0	0	0	0
3-8 cell	0	42 (98 %)	18 (42 %)	1 (2 %)	0
16-32 cell	0	0	20 (47 %)	10 (24 %)	0
Morula	0	0	1 (2 %)	28 (65 %)	14 (33 %)
Blastocyst	0	0	0	0	25 (58 %)
Degenerate	0	1 (2 %)	4 (9 %)	4 (9 %)	4 (9 %)

Table 7. Number of morulae, blastocysts and degenerate embryos obtained after a 96-hour period of culture. Embryos were recovered from does that were superovulated (FSH) or saline-injected (SAL) under heat stress (HS) or thermoneutral (TN) conditions.

Donor Treatment	Number of Embryos	Morula		Blastocyst		Degenerate Embryos	
		No.	(%)	No.	(%)	No.	(%)
FSH + HS	220	71	(32)	61	(28)	85	(39)
FSH + TN	184	48	(26)	89	(48)	40	(21)
SAL + HS	45	9	(20)	16	(36)	20	(44)
SAL + TN	43	14	(33)	25	(58)	4	(9)
P		> 0.1		< 0.005		< 0.005	

Morula = Multicellular embryo containing > 16 blastomeres, without a blastocoel. At this stage the extent of cellular associations formed between blastomeres results in greater membrane continuity between cells such that identification and counting of individual blastomeres is no longer possible.

Blastocyst = Number of embryos which developed to the blastocyst stage (formed a clearly defined blastocoel) within the 96-hour period of *in vitro* culture.

Degenerate = Embryos in which fragmentation of the blastomeres were observed during the 96-hour period of *in vitro* culture.

At the end of the 96-hour period of culture, the percentage of embryos to reach the blastocyst stage were 28 % (FSH + HS), 48 % (FSH + TN), 36 % (SAL + HS) and 58 % (SAL + TN) respectively ($P < 0.005$), where the percentage of embryos to reach the morula stage did not differ greatly ($P > 0.1$), Table 7. In addition, there were significant increases ($P < 0.005$) on the number of degenerate embryos recovered from does treated with FSH + HS (39 %) and SAL + HS (44 %) when compared to FSH + TN (21 %) and SAL + TN (9 %) treated does, Table 7. The differences observed between groups were mainly due to heat stress effects ($P < 0.005$) increasing embryonic degeneration during culture. An increase on the number of degenerate embryos was also observed with hormone treatment alone, although no statistical significance was present ($P < 0.1$). The percentage of degenerate embryos in the FSH + HS (39 %) group was lower ($P > 0.1$) than for those in the SAL + HS (44 %) group.

All 1-cell and 2-cell embryos were viable before culture. The viability of embryos at the end of the 96-hour period of culture is presented in Table 8. There was a higher percentage of partially stained than unstained embryos in each group but overall, a relatively small number of completely stained embryos were found ($P > 0.1$).

Discussion

In Experiment 2, exposure of New Zealand White does to heat stress conditions of $33 \pm 1^\circ \text{C}$ and 10-30 % RH for 8 hours followed by 16 hours of $25 \pm 1^\circ \text{C}$ and 10-30 % RH during 5 consecutive days apparently did not influence the superovulatory response. The fertilization rates seemed equally unaffected by heat stress. After a 96-hour period of *in vitro* culture, it was shown that embryonic development was significantly influenced by exposure of

Table 8. Viability of embryos at the end of 96-hour period of culture. Embryos were recovered from does that were superovulated or saline-injected under heat stress or thermoneutral conditions.

Donor Treatment	Number of Embryos	Viable		Non-Viable	
		Unstained No. (%)	Partially Stained No. (%)	Completely Stained No. (%)	
FSH + HS	220	41 (19)	176 (80)	3 (1)	
FSH + TN	184	36 (20)	144 (78)	3 (2)	
SAL + HS	45	11 (25)	33 (73)	1 (2)	
SAL + TN	43	18 (42)	25 (58)	0 (0)	
P		< 0.05	< 0.05	> 0.1	

Unstained = Number of embryos for which all of the blastomeres were viable at the end of the 96-hour period of *in vitro* culture.

Partially stained = Number of viable embryos in which one or more blastomeres had died during the 96-hour period of *in vitro* culture.

Completely stained = Number of embryos which had died during the 96-hour period of *in vitro* culture.

FSH + HS = Superovulatory treatment with follicle stimulating hormone under heat stress conditions.

FSH + TN = Superovulatory treatment with follicle stimulating hormone under thermoneutral conditions.

SAL + HS = Saline injected under heat stress conditions.

SAL + TN = Saline injected under thermoneutral conditions.

donor rabbits to heat stress, which could be confirmed by the increased number of degenerate embryos found in FSH + HS and SAL + HS groups.

Classification of ovarian structures indicated that an average of 27 total ovulations were obtained from groups that received FSH treatment under heat stress or thermoneutral conditions. These results are consistent with the results of Experiment 1 and those of Fisher and Meuser-Odenkirchen (1988). A moderate increase in the total number for non-ovulated and hemorrhagic follicles was present with superovulatory treatment. Overall, a greater ovarian stimulation and more embryos were recovered from FSH-treated does regardless of the environmental temperature.

Exposure to elevated temperatures for short or long periods has been shown to decrease plasma concentrations of gonadotropins in the rat (Bedrak and Chap, 1980), cow (Madan and Johnson, 1973; Miller and Alliston, 1974) and sow (Flowers and Day, 1990; Ogasa *et al.*, 1989). Antoine and Pattabiraman in 1994 reported that exposure of female rats to 40° C for 2 hours daily during a 5-day period had a negative effect on folliculogenesis. When FSH/LH treatment was given, it tended to counteract the adverse effects of heat stress on folliculogenesis, but the superovulatory response was not fully expressed. The results obtained in Experiment 2 indicates that the superovulatory response in New Zealand White does was not influenced under conditions of heat stress. There were no significant differences between the number of ovarian structures found in FSH + HS and FSH + TN or between SAL + HS and SAL + TN treated groups.

Results from the embryo culture demonstrated that exposure of New Zealand White does to heat-stress before mating significantly increased the number of degenerate embryos.

Oocyte degeneration, as well as embryonic mortality at several stages of development were observed in the mouse by Baumgartner and Chrisman (1987) after exposure of donor mice to $35 \pm 1^\circ \text{C}$ and $65 \pm 3 \% \text{RH}$ for 12.5 hours beginning immediately after synchronization of ovulation with PMSG and hCG. Bellve (1972), reported that exposure of mouse oocytes to heat stress indirectly through the female resulted in extensive developmental retardation and/or arrest among embryos recovered from heat-stressed females 54 hours after the vaginal plug was observed. In our study, we confirm the detrimental effects caused by exposure of the oocyte indirectly to heat stress in the rabbit through the increased number of degenerate embryos found. Although we did not observe significant rates for embryonic mortality as described by Baumgartner and Chrisman (1987).

Evaluating the effects of superovulatory treatment on embryonic development, Carney and Foote (1990), reported significantly fewer cell numbers and smaller intrazonal volumes in embryos recovered from FSH/LH treated does than in control animals. Fugimoto *et al.* (1974) noted an increase in chromosomal abnormalities of rabbit blastocysts collected from does superovulated with PMSG/hCG. The abnormal embryos also had lower mitotic indices, but differences in the gross appearance between superovulated and control embryos were not found. In Experiment 2, an increase in the number of degenerate embryos recovered from FSH + TN treated does was observed, although this increase was not significant when compared to embryos recovered from SAL + TN treated animals. Interestingly, the percentage of degenerate embryos found in the FSH + HS (39 %) group was lower than in the SAL + HS (44 %) group, indicating no additive effects of hormone treatment on embryonic degeneration.

GENERAL CONCLUSIONS

Experiment 1. Superovulatory response in New Zealand rabbits with two commercial gonadotropins: FSH-P™ vs. SUPER-OV®

Results from this study indicate that for the dosages given, FSH-P produced more ovulations and recoverable embryos than SUPER-OV in New Zealand White does. The differences in response of animals given these two products may be due to the amount of gonadotropins administered or loss of gonadotropic activity during processing and storage. Additional studies utilizing different concentrations of SUPER-OV will be needed to confirm whether comparable results can be obtained and if the differences shown were related to an inadequate concentration of FSH or due to the low concentration of LH in SUPER-OV preparations.

Experiment 2. Superovulatory response in New Zealand rabbits under environmental heat stress

The superovulatory response was not influenced by exposure of New Zealand White does to $33 \pm 1^\circ \text{C}$ and 10-30 % RH for 8 hours followed by exposure for 16 hours of $25 \pm 1^\circ \text{C}$ and 10-30 % RH during 5 consecutive days. Classification of ovarian structures (number of ovulations, non-ovulated and hemorrhagic follicles) and the number of embryos and/or oocytes recovered did not differ greatly between superovulated or between saline injected

does under heat stress or thermoneutral conditions. Fertilization rates seemed equally unaffected by heat stress treatment.

Exposure of New Zealand White does to heat stress before mating significantly increased the number of degenerate embryos during a 96-hour period of *in vitro* culture. An increased number of degenerate embryos was also observed with superovulatory treatment alone, although this increase was not significant. When FSH was given under heat stress conditions, there were no increases in the number of degenerate embryos when compared to heat stress treatment alone. No significant levels of embryonic mortality were observed during culture.

APPENDIX A

EFFECTS OF HEAT STRESS EXPOSURE ON BODY TEMPERATURE AND RELATIVE HUMIDITY

In order to induce hyperthermia, New Zealand White does were subject to temperatures of $33 \pm 1^\circ \text{C}$ and 10-30 % RH from 8:00 am until 4:00 pm, followed by 16 hours of $25 \pm 1^\circ \text{C}$ and 10-30 % RH from 4:00 pm until 8:00 am during 5 consecutive days. Rabbits that were not assigned to heat stress were housed under constant thermoneutral conditions of $21 \pm 1^\circ \text{C}$ and 15-70 % RH. A temperature controlled chamber 12'9" x 8'10" was used for heat stress exposure. A minimum of 20 air changes per hour were allowed and a 12 hour light/12 h dark cycle was maintained inside the experimental chamber. Rectal temperatures were recorded twice daily from each doe. The first measurement was taken around 8:00 am prior to the increase of environmental temperature from 25°C to 33°C and the second measurement was taken around 4:00 pm at the end of the 8-hour exposure to 33°C .

The effects of heat stress were confirmed by an increase of about 1°C above normal body temperature (Figure 11). Similar increases in body temperature were reported in rabbits during heat stress studies (Burfening *et al.*, 1969; Ulberg and Sheean, 1973; Wolfenson and Blum, 1988). The means for body temperature did not differ under 21°C ($39.3^\circ \text{C} \pm 0.4$) and 25°C ($39.3^\circ \text{C} \pm 0.3$), where exposure to 33°C increased body temperature to 40.2°C (± 0.3). The relative humidity gradually decreased with the increase of environment temperature (Figure 12).

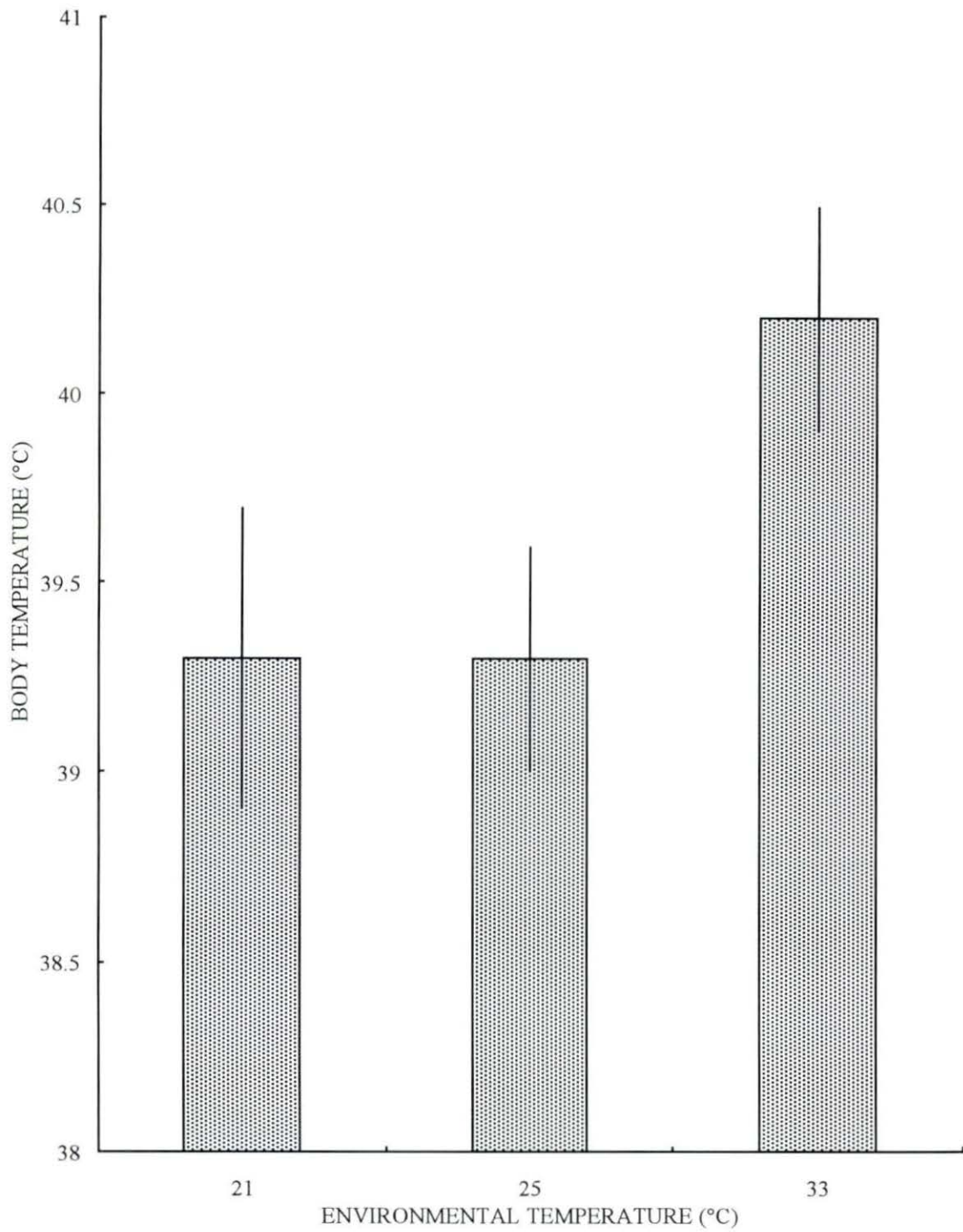


Figure 11. Body temperature of New Zealand White does during exposure to 21, 25 or 33° C (mean \pm SD).

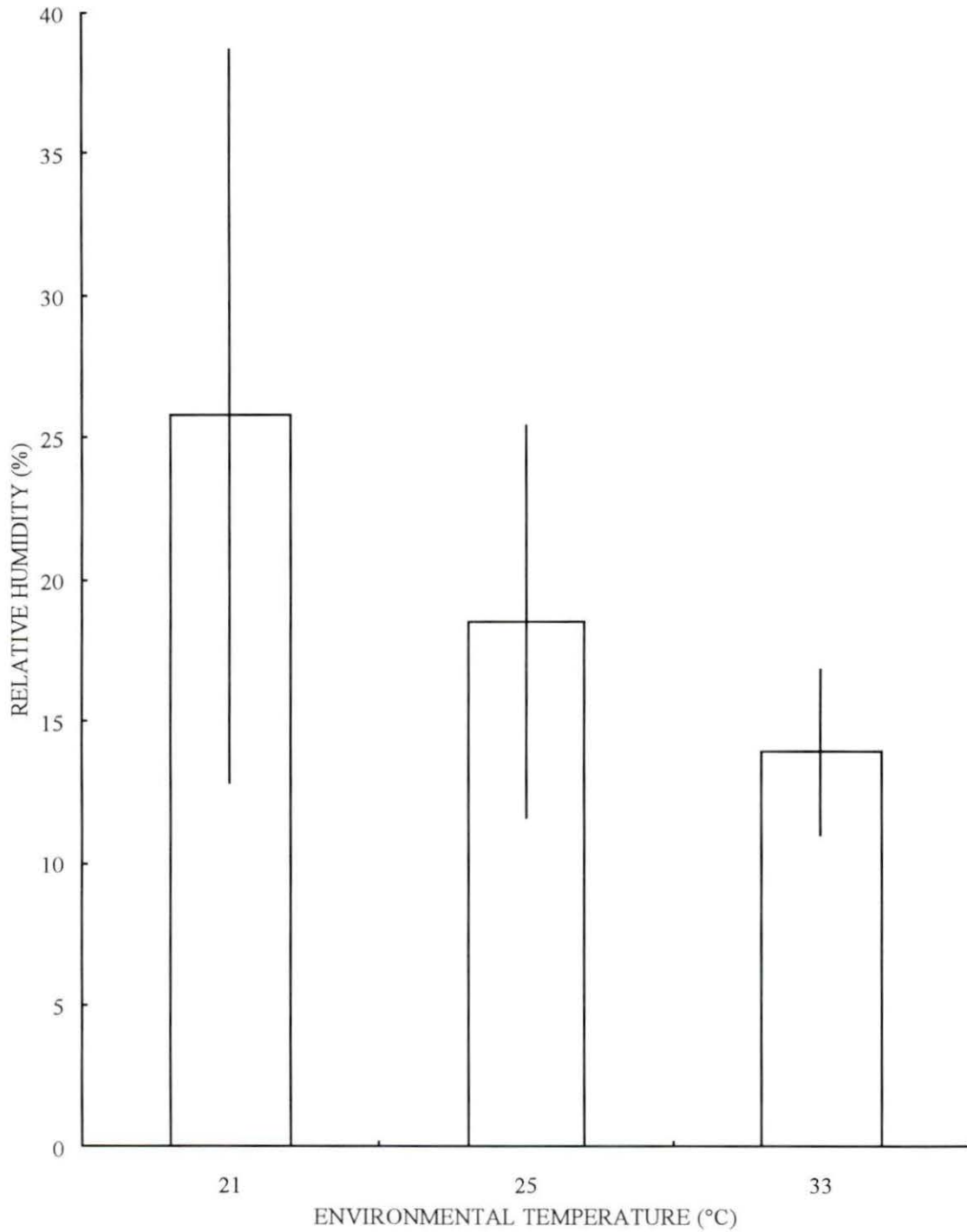


Figure 12. Relative humidity inside the experimental chamber during ambient temperatures of 21, 25 and 33° C (mean \pm SD).

APPENDIX B

EFFECTS OF HEAT STRESS ON PLASMA LEVELS OF GONADOTROPINS

Hypotheses

1. Exposure of female New Zealand White rabbits to heat stress affects the concentration of LH and FSH during estrous.
2. Exposure of female New Zealand White rabbits to heat stress affects the concentration of LH and FSH during the post-mating period.
3. Exposure of female New Zealand White rabbits to heat stress affects implantation rates.

Experimental design

New Zealand White does were subjected to $33 \pm 1^\circ \text{C}$ and 10-25 % RH or $21 \pm 1^\circ \text{C}$ and 15-40 % RH during an 8-day period. Venous blood samples were collected via an indwelling cannula for radioimmunoassay (RIA) of LH and FSH, twice daily for the first five days. On day 6, females were removed from the experimental chamber and mated with a fertile male rabbit under thermoneutral conditions of $21 \pm 1^\circ \text{C}$ and 15-40 % RH. After copulation, blood samples were taken at 15-minute intervals during 6 consecutive hours. Does were then returned to the chamber and blood samples were collected twice daily on days 7 and 8. Two weeks after mating all does were euthanized and the reproductive tracts recovered. The number of corpora lutea present on the ovaries, the presence of non-ovulated follicles, and the number of embryos implanted in the uterus were recorded.

Due to construction at the laboratory where the RIA was to be performed, it was not possible to present the hormone analysis data from this study at the time this thesis was written. The material and methods, and the results obtained to date from the evaluation of the reproductive tracts are cited.

Experimental animals

A total of 12 female New Zealand rabbits (> 3.0 kg of body weight) were allocated to one of two groups, 6 animals per group. All does were kept undisturbed during a minimum of 17 days after arrival to avoid risks of pseudopregnancy due to transport. Each female was caged individually and housed under thermoneutral conditions of $21 \pm 1^\circ \text{C}$ and 15-40 % RH, with a 12 h light/12 h dark cycle, and provided commercial feed and water *ad libitum*. Sexually mature New Zealand White bucks were used for mating. The males were housed in a separate room under similar environmental conditions as the females. Animal care was provided by Laboratory Animal Resources staff. An indwelling cannula was implanted in each doe in the posterior facial vein according to a modification of procedures from Hall *et al.*, 1974.

Catheter preparation

A total of 12 30 cm Silastic® Medical-Grade Tubing (Dow Corning Corp., Midland, MI), 0.04" i.d. x 0.85" o.d. catheters were used for cannulation. All catheters were washed with neutral soap and deionized water and rinsed several times with ultrapure water. The cannulas were then autoclaved during 20 minutes at 20 lbs of pressure and stored until used.

Immediately prior to implantation, the catheters were filled with saline containing 1500 IU/ml of heparin (Elkins-Sinn, Inc., Cherry Hill, NJ).

Cannulation procedures

All animals received a subcutaneous injection of 50 mg of chloramphenicol sodium succinate (Fujisawa USA, Inc., Deerfield, IL) 24 hours before surgery. Each doe was fasted for 3 hours before surgery. An intramuscular injection of 5 mg/kg of body weight of acepromazine maleate (CEVA Laboratories Inc., Overland Park, KS) was given for pre-anesthesia. Fifteen minutes later, an intramuscular injection of 40 mg/kg of body weight of ketamine hydrochloride (Fort Dodge Laboratories Inc., Fort Dodge, IA) was given to induce anesthesia. The incision site was infiltrated with a subcutaneous injection of 1.0 ml of 2 % lidocaine solution (Abbott Laboratories, North Chicago, IL) to further reduce the incisional pain. After the rabbit had reached the surgical plane of anesthesia, the neck and interscapular area was initially shaved with hair clippers and the area cleaned with 70 % alcohol and 2 % chlorhexidine (Fort Dodge Laboratories Inc., Fort Dodge, IA). The incision site was then coated with shaving foam and shaved with disposable razor. The doe was then positioned on the surgical table and prepared for surgery. A 4 cm median ventral skin incision starting from the level of the hyoid bone and extending toward the manubrium of the sternum was made. The depressor conchae posterior muscle was divided by cleavage on the right side, exposing the jugular vein. The bifurcation of the anterior and posterior facial veins was then located and cleared of connective tissue about 2 cm from the posterior facial vein, starting at the bifurcation. Two 4-0 suture lines were passed under the posterior facial vein and a small

incision was made in the vein with a Potts-Smith scissors, the catheter was inserted into the incision and advanced so that the tip of the cannula was estimated to be within the right anterior vena cava. After the catheter was inserted, the lower and upper ligatures were tied. The catheter was then secured at the site of insertion by tying it with the long ends of the upper ligature. A 15.5 cm glass rod, 0.06" i.d. x 0.95" o.d. was used to guide the catheter through the subcutaneous space of the neck and exteriorized dorsally at the middle of the neck and over the spine. A single 4 cm loop of catheter was formed in a subcutaneous pocket on the side of the neck and the incision site was irrigated with 0.9 % sodium chloride solution (Baxter Health Care Corporation, Deerfield, IL) and closed with skin clips.

The catheter loop within the subcutaneous pocket offered a spring action so that movements of the animals neck and shoulders did not place any undue strain on the catheter where it is sutured into the vein.

In order to stabilize the exteriorized portion of the catheter, two ligatures were tied 1 cm apart and 1 cm behind the terminal loop. The catheter was positioned along the midline with the end loop pointing posteriorly and a small pocket was made with 4-0 suture line, 2 cm away from the second ligature. The end loop was inserted inside the pocket and two knots were tied using 1-0 suture line to fix the cannula and restrict the flow, the first knot was made between the second ligature and the pocket ligature and the second knot at the terminal loop.

After the catheter was implanted, the incision and catheter exteriorization sites were coated with nitrofurazone-furacin soluble powder (SmithKline Beecham Animal Health, West Chester, PA) and all does were treated with 50 mg of chloramphenicol sodium succinate, intravenous twice daily during 7 consecutive days following surgery. Cannula patency was

maintained by replacing the heparin inside the cannula twice daily. A 5-day recovery period was allowed for each doe before assignment to treatment inside the experimental chamber.

Heat stress exposure

A temperature controlled chamber 12'9" x 8'10" was used to study the effects of environmental heat stress on plasma levels of gonadotropins. Chamber temperature was adjusted daily to $33 \pm 1^\circ \text{C}$ and 10-40 % at 8:00 am and then to $25 \pm 1^\circ \text{C}$ and 10-40 % relative humidity at 4:00 pm for heat stress exposure. Control animals were exposed to constant thermoneutral conditions of $21 \pm 1^\circ \text{C}$ and 20-40 % relative humidity.

Blood sampling

During the first 5 days inside the chamber, blood samples were collected twice daily. The first sample of the day was collected between 7:00 am and 8:00 am and the second sample between 3:00 pm and 4:00 pm. On day 6, between 8:00 am and 12:00 pm does were removed from the chamber and mated with a fertile male under thermoneutral conditions of $21 \pm 1^\circ \text{C}$ and 15-40 % RH. After copulation was observed, does were then separated from the bucks and blood samples were collected at 15-minute intervals during 6 consecutive hours within the mating room. All does were returned to the chamber after the 6-hour blood sampling. On days 7 and 8, blood samples were collected twice daily as described for the first 5 days. All samples were collected in 2 ml ice cold culture tubes containing 50 IU of heparin and centrifuged at $1500 \times g$ (TJ-6R Beckman Instruments, Inc., Palo Alto, CA) for 10 minutes at 4°C . The plasma was harvested with a Pasteur pipette, and transferred into 1.8 ml

cryotubes (Nunc A/S, Roskilde, Denmark) and stored at -20°C until assayed for FSH and LH.

Endpoints

1. Concentration of LH and FSH during heat stress 5 days prior to mating.
2. Concentration of LH and FSH during a 6-hour period after mating.
3. Concentration of LH and FSH during heat stress 2 days after mating.

Results

Two weeks after mating, all does were euthanized and the reproductive tracts recovered. The number of corpora lutea present on the ovaries and the number of embryos implanted in the uterine horns of does are shown in Table 9. The presence of corpora lutea was observed on the ovaries of all 6 does exposed to heat stress where for 2 of 6 treated females no embryos had implanted, despite the presence of corpora lutea on the ovaries. In the group of animals that were exposed to thermoneutral conditions, only 2 of 6 does had corpora lutea on the ovaries and both does had embryos implanted. Of the remaining females, 3 does had only non-ovulated follicles present on their ovaries and in 1 doe, no ovarian structures were found.

Discussion

Evaluation of the ovaries from heat-stressed does indicated that ovulation occurred after mating in all animals as confirmed by the presence of corpora lutea. For the does that

Table 9. Number of corpora lutea and number of embryos recorded from the uterus two weeks after mating. Does were exposed to thermoneutral conditions (TN) or heat stress conditions (HS) during a 8 day period.

Doe	Treatment	No. of Corpora Lutea		No. of Embryos		Implantation Rate (%)
		Left Ovary	Right Ovary	Left Horn	Right Horn	
2895	TN	0	0	0	0	0
2892	TN	0	0	0	0	0
2896	TN	4	4	4	4	100
2888	TN	0	0	0	0	0
2898	TN	0	0	0	0	0
2932	TN	3	4	3	0	43
2901	HS	4	2	3	0	50
2891	HS	6	5	4	3	64
2902	HS	4	3	0	0	0
2884	HS	5	2	1	2	43
2934	HS	8	2	0	0	0
2938	HS	4	5	1	2	33

ovulated, the number of embryos found in the uterine horns ranged from 0 to 60 % in heat-stressed does and from 43 to 100 % in control animals. The number of corpora lutea present on the ovaries of heat-stressed does exceeded the number of embryos found in the uterine horns, which suggests that exposure to heat stress probably induced embryonic mortality or resorption during early pregnancy.

Although copulation was observed in all females in the thermoneutral group, 4 does failed to ovulate. Of these, 3 does had only non-ovulated follicles on their ovaries. A fourth doe had no corpora lutea or non-ovulated follicles ≥ 1.5 mm in diameter on the ovaries. No attempt was made to confirm intromission and ejaculation during mating. All does were paired with a fertile male rabbit and immediately after the first copulation, they were separated from the male and blood samples taken during 6 consecutive hours. In the rabbit, an ovulatory stimulus such as mating induces an LH surge between 90 and 120 minutes later (Dufy-Barbe *et al.*, 1973; Hilliard, *et al.*, 1964; Orstead *et al.*, 1988, Scaramuzzi *et al.*, 1972). This LH surge is required for ovulation (Dufy-Barbe *et al.*, 1973; Hilliard, *et al.*, 1964). Since blood samples were taken within a 15-minute interval during a 6 hour period, data from the RIA will confirm if no ovulatory stimulus was obtained in animals that failed to ovulate. In Experiment 2 of this study, the fertilization rates were not affected by heat stress. Thus, evidence for fertilization failure and/or embryonic mortality was found in all heat-stressed does.

REFERENCES

- Adams, C. E. (1972) *In: Reproduction in Mammals, Book 4.* Austin and Short (eds.), Cambridge, Cambridge University Press pp. 128-156.
- Adams, C. E. (1982) *Mammalian Egg Transfer.* Boca Raton, FL, CRC Press pp. 29-48.
- Allen, E. (1928) Precocious sexual development from anterior hypophysis implants in a monkey. *Anat. Record* 39: 315-323.
- Antoine, D., and Pattabiraman, S. R. (1994) Effect of heat stress and gonadotropic hormone treatment on folliculogenesis. *Ind. J. Anim. Reprod.* 15: 141-143.
- Aschheim, S., and Zondek, B. (1927) Hypophysenvorderlappenhormon und ovarialhormone im harn von schwangeren. *Klin. Wochschr.* 6: 1322.
- Aschheim, S., and Zondek, B. (1928) Die schwangerschaftsdiagnose aus dem harn durch nachweis des hypophysenvorderlappenhormons. *Klin. Wochschr.* 7: 1404-1410.
- Ascoli, M., and Puett, D. (1978) Degradation of receptor-bound human choriogonadotropin by murine Leydig tumor cells. *J. Biol. Chem.* 253: 4892-4899.
- Badinga, L., Thatcher, W. W., Diaz, T., Drost, M., and Wolfenson, D. (1993) Effect of environmental heat stress on follicular development and steroidogenesis in lactating Holstein cows. *Theriogenology* 39: 797-810.
- Bahl, O. P. (1977) The chemistry and biology of human chorionic gonadotropin and its subunits. *In: Frontiers in Reproduction and Fertility Control.* Greep, R. O. and Koblinsky, M. A. (eds), Cambridge, Mass., MIT Press pp. 11-24.
- Baumgartner, A. P., and Chrisman, C. L. (1987) Embryonic mortality caused by maternal heat stress during mouse oocyte maturation. *Anim. Reprod. Sci.* 14: 309-316.
- Bedrak, E., and Chap, Z. (1980) Serum concentration of gonadotrophins and the hypothalamic content of gonadotrophin releasing hormone in male rats exposed to 35° C. *J. Endocr.* 84: 9-16.
- Ben-Ze'ev, A., and Amsterdam, A. (1989) Regulation of heat shock protein synthesis by gonadotropins in cultured granulosa cells. *Endocrinology* 124: 2584-2594.

- Birken, S., Maydelman, Y., Gawinowicz, M. A., Pound, A., Liu, Y., and Hartree, A. S. (1996) Isolation and characterization of human pituitary chorionic gonadotropin. *Endocrinology* 137: 1402-1411.
- Brooks, C. M. (1938) A study of the mechanism whereby coitus excites the ovulation-producing activity of the rabbit's pituitary. *Am. J. Physiol.* 121: 157-177.
- Burfening, P. J., Alliston, C. W., and Ulberg, L. C. (1969) Gross morphology and predictability for survival of 4-day rabbit embryos following heat-stress during the first cleavage division. *J. Exp. Zool.* 170: 55-60.
- Calisti, V., and Oliva, O. (1955) Ricerca istochimica sulla localizzazione delle gonadotropine nella placenta degli equini. *Clin. Vet.* 78: 65-76.
- Carney, E. W., and Foote, R. H. (1990) Effects of superovulation, embryo recovery, culture system and embryo transfer on development of rabbit embryos *in vivo* and *in vitro*. *J. Reprod. Fertil.* 89: 543-551.
- Casida, L. E., Warwick, E. J., and Meyer, R. K. (1944) Survival of multiple pregnancies induced in the ewe following treatment with pituitary gonadotropins. *J. Anim. Sci.* 3: 22-28.
- Catt, K. J., and Dufau, M. L. (1976) Basic concepts of the mechanism of action of peptide hormones. *Biol. Reprod.* 14: 1-15.
- Catt, K. J., and Pierce, J. G. (1978) Gonadotropic hormones of the adenohypophysis (FSH, LH and prolactin). *In: Reproductive Endocrinology.* Yen, S. S. C. and Jaffe, R. B. (eds), Philadelphia, W. B. Saunders pp. 34-62.
- Chen, T. T., Endres, D. B., Abel, J. H., Jr., and Niswender, G. D. (1978) Binding and degradation of human chorionic gonadotropin by ovine luteal cells. Abstr. 337, 60th Annual Meeting, Endocrine Society, Miami, FL.
- Chow, B. F., van Dyke, H. B., Greep, R. O., Rothen, A., and Shedlovsky, T. (1942) Gonadotropins of swine pituitary. II. Preparation, and biological and physicochemical characterization of a protein apparently identical with metakentrin (ICSH). *Endocrinology* 30: 650-656.
- Cole, H. H., and Hart, G. H. (1930) Potency of blood serum of mares in progressive stages of pregnancy in affecting sexual maturity of immature rats. *Am. J. Physiol.* 93: 57-68.
- Combarrous, Y. (1988) Structure and structure-function relationships in gonadotropins. *Reprod. Nutr. Develop.* 23: 211-228.

- Davis, J. S., Tedesco, T. A., West, L. A., Maroulis, G. B., and Weakland, L. L. (1989) Effects of human chorionic gonadotropin, prostaglandin F-2-alpha and protein kinase C activators on the cyclic AMP and inositol phosphate 2nd messenger systems in cultured human granulosa-luteal cells. *Mol. Cell. Endocrinol.* 65: 187-194.
- Davis, M. E., and Hellbaum, A. A. (1944) Observations on experimental use of gonadotropic extracts in human female. *J. Clin. Endocrinol.* 4: 400-409.
- Deanesly, R. (1963) Further observations of the effect of oestradiol on tubal eggs and implantation in the guinea-pig. *J. Reprod. Fertil.* 5: 49-57.
- Dempsey, E. W. (1937) Follicular growth rate and ovulation after various experimental procedures in guinea pig. *Am. J. Physiol.* 120: 126-132.
- Dempsey, E. W. (1939) Relationship between central nervous system and reproductive cycle in female guinea pig. *Am. J. Physiol.* 126: 758-765.
- Diczfalusy, E., and Troen, P. (1961) Endocrine functions of the human placenta. *Vitamins and Hormones* 19: 229-311.
- Doney, J. M., Gunn, R. G., and Griffiths, J. G. (1973) The effect of pre-mating stress on the onset of oestrous and on ovulation rate in Scottish Black Face ewes. *J. Reprod. Fertil.* 35: 381-384.
- Dooley, M. P. (1988) The use of eosin B to assess the viability and developmental potential of rat embryos. Ph.D. Dissertation, Iowa State University, Ames, Iowa.
- Dooley, M. P., Pineda, M. H., and Martin, P. A. (1984) A dye-exclusion assay using eosin B to estimate the viability of rat embryos. 10th International Congress of Animal Reproduction and Artificial Insemination. University of Illinois, Urbana/Champaign. *Brief Communications* 2: 225.
- Dowling, D. F. (1949) Problems of the transplantation of fertilized ova. *J. Agric. Sci.* 39: 374-396.
- Dufau, M. L., Tsuruhara, T., Horner, K., Podesta, E., and Catt, K. J. (1977) Evidence for the intermediate action of cyclic AMP and protein kinase in the steroidogenic action of gonadotropin in isolated Leydig cells. *Proc. Natl. Acad. Sci.* 74: 3419-3423.
- Duffy-Barbe, L., Franchimont, P., and Faure, J. M. A. (1973) Time-courses of LH and FSH release after mating in the female rabbit. *Endocrinology* 92: 1318-1321.
- Ellis, S. (1958) A scheme for the separation of pituitary proteins. *J. Biol. Chem.* 233: 63-68.

- Evans, H. M., and Simpson, M. E. (1940) Experimental superfecundity with pituitary gonadotrophins. *Endocrinology* 27: 305-308.
- Fevold, H. L., Hisaw, F. L., and Leonard, S. L. (1931) The gonad-stimulating and luteinizing hormones of the anterior lobe of the hypophysis. *Am. J. Physiol.* 97: 291-301.
- Fisher, B. (1987) Development retardation in cultured preimplantation rabbit embryos. *J. Reprod. Fertil.* 79: 115-123.
- Fisher, B., and Meuser-Odenkirchen, G. (1988) A two year follow up of effects of biotechniques on reproduction in the domestic rabbit. *Lab. Anim.* 22: 5-15.
- Flowers, B., and Day, B. N. (1990) Alterations in gonadotrophin secretion and ovarian function in prepubertal gilts by elevated environmental temperature. *Biol. Reprod.* 42: 465-471.
- Foster, D. L., Lemons, J. A., Jaffe, R. B., and Niswender, G. D. (1975) Sequential patterns of circulating luteinizing hormone and follicle-stimulating hormone in female sheep from early postnatal life through the first estrous cycles. *Endocrinology* 97: 985-994.
- Foster, M. A., and Fevold, H. L. (1938) The interrelationship of the pituitary gonadotrophic hormones in follicular development and ovulation of the juvenile rabbit. *Amer. J. Physiol.* 121: 625-632.
- Fugimoto, S., Pahlavan, N., and Dukelow, W. R. (1974) Chromosome abnormalities in rabbit preimplantation blastocysts induced by superovulation. *J. Reprod. Fert.* 40: 177-181.
- Gaupp, V., and Spatz, H. (1955) Hypophysenstiieldurchtrennung und Geschlechtsreifung, über regeneration-serscheinungen an der suprasellaren hypophyse. *Acta Neuroveg.* 12: 285-328.
- Glasier, A. F., Baird, D. T., and Hillier, S. G. (1989) FSH and the control of follicular growth. *J. Steroid Biochem.* 32:167-170.
- Goding, J. R., Catt, K. J., Brown, J. A., Kaltenback, C.C., Cumming, I. A., and Mole, B. J. (1969) Radioimmunoassay for ovine luteinizing hormone: secretion of luteinizing hormone during estrus and following estrogen administration in the sheep. *Endocrinology* 85: 133-142.
- Greenwald, G. S. (1961) A study of the transport of ova through the rabbit oviduct. *Fertil. Steril.* 12: 80-95.

- Greep, R. O., and Barnett, R. J. (1951) Effect of pituitary stalk-transection on reproductive organs of female rats. *Endocrinology* 49: 172-184.
- Greep, R. O., van Dyke, H. B., and Chow, B. F. (1942) Gonadotropins of swine pituitary. I. Various biological effects of purified thylakentrin (FSH) and pure metakentrin (ICSH). *Endocrinology* 30: 635-649.
- Grimek, H. J., Gorski, J., and Wentworth, B. C. (1979) Purification and characterization of bovine follicle-stimulating hormone: comparison with ovine follicle-stimulating hormone. *Endocrinology* 104: 140-147.
- Gwynne, J. T., and Strauss, J. F. (1982) The role of lipoproteins in steroidogenesis and cholesterol metabolism in steroidogenic glands. *Endocr. Rev.* 3: 299-329.
- Hackett, A. J., and Hafs, H. D. (1969) Pituitary and hypothalamic endocrine changes during bovine estrous cycle. *J. Anim. Sci.* 28: 531-536.
- Hall, L. L., DeLopez, O. H., Roberts, A., and Smith, F. A. (1974) Procedures for chronic intravenous catheterization in the rabbit. *Lab. Anim. Sci.* 24: 79-83.
- Hammond, J., Jr. (1949) Induced twins ovulations and multiple pregnancy in cattle. *J. Agric. Sci.* 39: 222-225.
- Hammond, J., Jr., Hammond, J., and Parkes, A. S. (1942) Hormonal augmentation of fertility in sheep. I. Induction of ovulation, superovulation, and heat in sheep. *J. Agric. Sci.* 32: 308-323.
- Hartman, C. G. (1930) Anterior lobe of the pig and the monkey ovary. *Proc. Soc. Exper. Biol. Med.* 27: 338-340.
- Hernandez, E. R., Hurwitz, A., Vera, A., Pellicer, A., Adashi, E. Y., LeRoith, D., and Roberts, C. R., Jr. (1992) Expression of genes encoding insulin-like growth factors and their receptors in the human ovary. *J. Clin. Endocrinol. Metab.* 74: 419-425.
- Hertz, R., and Hisaw, F. L. (1934) Effects of follicle stimulating and luteinizing pituitary extracts on the ovaries of the infantile and juvenile rabbit. *Amer. J. Physiol.* 108: 1-13.
- Hickey, G. J., Chen, S. A., Besman, M. J., Shively, J. E., Hall, P. F., Gaddy-Kurten, D., and Richards, J. S. (1988) Hormonal regulation, tissue distribution, and content of aromatase cytochrome P450 messenger ribonucleic acid and enzyme in rat ovarian follicles and corpora lutea: relationship to estradiol biosynthesis. *Endocrinology* 122: 1426-1436.
- Hilliard, J., Hayward, J. N., and Sawyer, C. H. (1964) Postcoital patterns of secretion of pituitary gonadotropin and ovarian progesterin in the rabbit. *Endocrinology* 75: 957-963.

- Hirshfield, A. N. (1991) Development of follicles in the mammalian ovary. *International Review of Cytology* 124: 43-101.
- Hisaw, F. L. (1947) Development of the Graafian follicle and ovulation. *Physiol. Rev.* 27: 95-119.
- Hodgen, G. D. (1989) Biological basis of follicle growth. *Hum. Reprod.* 4: 37-46.
- Hofeditz, C., Magoffin, D. A., and Erickson, G. F. (1988) Evidence for protein kinase C regulation of ovarian theca-interstitial cell androgen biosynthesis. *Biol. Reprod.* 39: 873-881.
- Jaffe, R. B. (1978) The endocrinology of pregnancy. *In: Reproductive Physiology.* Yen, S. S. C. and Jaffe, R. B. (eds), Philadelphia, W. B. Saunders Co. pp. 521-536.
- Kamolpatana, K. (1995) Development and viability of rabbit embryos after exposure to an electromagnetic field of 1.5 Gauss at 60 Hz during *in vitro* culture and after transfer of exposed embryos to recipient does. M.S. Thesis, Iowa State University, Ames, Iowa. pp. 25-26.
- Kennelly, J. J., and Foote, R. H. (1965) Superovulatory response of pre and post pubertal rabbits to commercially available gonadotrophins. *J. Reprod. Fertil.* 9: 177-188.
- King, J. M. (1965) Gonadotropins: Physico-chemical and Immunological Properties. Ciba Foundation Study Group 22: 63.
- Kumar, A. S. K. (1987) The effect of temperature and humidity stress on plasma progesterone, luteinizing hormone and cortisol profiles following cloprostenol injection in cycling Holstein cows in controlled environment. *Dissertation Abstracts International* 47: 3265-3266.
- Leung, P. C. K., and Steele, G. L. (1992) Intracellular signaling in the gonads. *Endocr. Rev.* 13: 476-498.
- Li, C. H., Simpson, M. E., and Evans, H. M. (1949) Isolation of pituitary follicle-stimulating hormone (FSH). *Science* 109: 445-446.
- Lublin, A., Wolfenson, D., and Berman, A. (1984) Circulatory adaptations to heat stress and their relationship to fertility. 35th Annual Meeting of the EAAP, The Hague, Netherlands C3b: 2-3.
- Madan, M. L., and Johnson, H. D. (1973) Environmental heat effects on bovine luteinizing hormone. *J. Dairy Sci.* 56: 1420-1423.

- Mandl, A. M., and Zuckerman, S. (1952) Growth of oocyte and follicle in adult rat. *J. Endocrinol.* 8: 126-132.
- Marden, W. G. R. (1952) The hormone control of ovulation in the calf. *Endocrinology* 50: 456-461.
- Matzuk, M. M., Kornmeier, C. M., Whitfield, G. K., Kourides, I. A., and Boime, I. (1988) The glycoprotein α -subunit is critical for secretion and stability of the human thyrotropin β -subunit. *Mol. Endocrinol.* 2: 95-100.
- McNatty, K. P. (1978) Follicular determinants of corpus luteum function in the human ovary. *Proc. Workshop on Ovarian Follicular and Corpus Luteum Function.*, Americana Hotel, Miami Beach, FL. June 11-13, 1978.
- Miller, H. L., and Alliston, C. W. (1974) Influence of programmed circadian temperature changes upon levels of luteinizing hormone in the bovine. *Biol. Reprod.* 11: 187-190.
- Moon, H. D., and Li, C. H. (1952) Effect of follicle-stimulating hormones on gonads of immature C57 black mice. *Proc. Soc. Exper. Biol. Med.* 79: 505-507.
- Niswender, G. D., Nett, T. M., and Akbar, A. M. (1974) The hormones of reproduction. *In: Reproduction in Farm Animals*, 3rd ed. Hafez, E. S. E. (ed), Philadelphia, PA. Lea & Febiger pp. 57-81.
- Niswender, G. D., Reimers, T. J., Diekman, M. A., and Nett, T. M. (1976) Blood flow: a mediator of ovarian function. *Biol. Reprod.* 14: 64-81.
- Ny, T., Bjersing, L., Hsueh, A. J. W., and Loskutoff, D. J. (1985) Cultured granulosa cells produce two plasminogen activators and an antiactivator, each regulated differently by gonadotropins. *Endocrinology* 116: 1666-1668.
- O'Connell, M. L., Canipari, R., and Strickland, S. (1987) Hormonal regulation of tissue plasminogen activator secretion and mRNA levels in rat granulosa cells. *J. Biol. Chem.* 262: 2339-2444.
- Ogasa, A., Miyajima, H., Iwamura, S., Domeki, I., Kawakami, E., and Tsutsui, T. (1989) Effect of high temperature and humidity in the periovulatory phase on swine ovarian function. *Nippon Juigaku Zasshi* 51: 627-629.
- Oliver, J. E., Aitman, T. J., Powell, J. F., Wilson, C. A., and Clayton, R. N. (1987) Insulin-like growth factor I gene expression in the rat ovary is confined to the granulosa cells of developing follicles. *Endocrinology* 224: 2671-2679.

- Orstead, K. M., Hess, D. L., and Spies, H. G. (1988) Pulsatile patterns of gonadotropins and ovarian steroids during estrus and pseudopregnancy in the rabbit. *Biol. Reprod.* 38: 733-743.
- Papkoff, H., Ryan, R. J., and Ward, D. N. (1977) The gonadotropic hormones, LH (ICSH) and FSH. *In: Frontiers in Reproduction and Fertility Control*. Greep, R. O. and Koblinsky, M. A. (eds), Cambridge, Mass., The MIT Press pp. 1-10.
- Parkes, A. S. (1942) Induction of superovulation and superfecundation in rabbits. *J. Endocr.* 3: 268-279.
- Parlow, A. F., Anderson, L. L., and Melampy, R. M. (1964) Pituitary follicle-stimulating hormone and luteinizing hormone concentrations in relation to reproductive stages in the pig. *Endocrinology* 75: 365-376.
- Pierce, J. G., and Parsons, T. F. (1981) Glycoprotein hormones: structure and function. *An. Rev. Biochem.* 50: 465-495.
- Pincus, G. (1940) Superovulation in Rabbit. *Anat. Rec.* 77: 1-8.
- Reichert, F. L. (1928) The results of a replacement therapy in a hypophysectomized puppy: four months of treatment with daily pituitary heterotransplants. *Endocrinology* 12: 451-467.
- Richards, J. S., Jahnsen, T., Hedin, L., Lifka, J., Ratoosh, S. L., Durica, J. M., and Goldring, N. B. (1987) Ovarian follicular development: from physiology to molecular biology. *Recent Prog. Horm. Res.* 43: 231-270.
- Richards, J. S., and Midgley, A. R., Jr. (1976) Protein hormone action: a key to understanding ovarian follicular and luteal cell development. *Biol. Reprod.* 14: 82-94.
- Sairam, M. R., and Bhargavi, G. N. (1985) A role for glycosylation of the α subunit in transduction of biological signal in glycoproteins hormones. *Science* 229: 65-67.
- Sairam, M. R., and Papkoff, H. (1974) Chemistry of pituitary gonadotrophins. *In: Handbook of Physiology*. Knobil, E. and Saywer, W. H. (Vol eds), sec. 7: *Endocrinology, Vol IV: The Pituitary Gland and its Neuroendocrine Control, part 2*. Baltimore, American Physiology Society (distributed by Williams & Wilkins Co.) pp. 111-131.
- SAS Institute Inc. (1989) *SAS Language and Procedures: Usage, Version 6 (1st ed.)*. Cary, NC.

- Scaramuzzi, R. J., Blake, C. A., Papkoff, H., Hilliard, J., and Saywer, C. H. (1972) Radioimmunoassay of rabbit luteinizing hormone: Serum levels during various reproductive states. *Endocrinology* 90: 1285-1291.
- Segaloff, D. L., Wang, H., and Richards, J. S. (1990) Hormonal regulation of luteinizing hormone/chorionic gonadotropin receptor mRNA in rat ovarian cells during follicular development and luteinization. *Mol. Endocrinol.* 4: 1856-1865.
- Sherwood, O. D., and McShan, W. H. (1977) Gonadotropins. *In: Reproduction in Domestic Animals*, 3rd ed. Cole, H. H. and Cupps, P. T. (eds), New York, Academic Press pp. 17-47.
- Short, A. V. (1972) *In: Reproduction in Mammals*, Book 3. Austin and Short (eds.), Cambridge, Cambridge University Press pp. 42-72.
- Smith, P. E. (1926) Hastening development of female genital system by daily hypophyseal pituitary transplants. *Proc. Soc. Exper. Biol. Med.* 24: 131-132.
- Smith, P. E. (1927) The disabilities caused by hypophysectomy and their repair. *J. Am. Med. Assoc.* 88: 158-161.
- Smith, P. E., and Engle, E. T. (1927) Experimental evidence regarding the role of the anterior pituitary in the development and regulation of the genital system. *Amer. J. Anat.* 40: 159-217.
- Smith, P. L., and Baenziger, J. U. (1988) A pituitary N-acetylgalactosamine transferase that specifically recognizes glycoprotein hormones. *Science* 242: 930-932.
- Starling, E. H. (1905) The chemical correlation of the functions of the body. *Lancet* 1: 340-341.
- Steel, R. G. D., and Torrie, J. H. (1980) *Principles and Procedures of Statistics: A Biometrical Approach* (2nd ed.). McGraw-Hill Inc., NY. pp. 86-121, 195-233 and 469-476.
- Steinkampf, M. P., Mendelson, C. R., and Simpson, E. R. (1988) Effects of epidermal growth factor and insulin-like growth factor I on the levels of mRNA encoding aromatase P-450 of human ovarian granulosa cells. *Mol. Cell. Endocrinol.* 59: 93-99.
- Sutherland, E. W., and Rall, T. W. (1960) The relation of adenosine-3', 5'-phosphate and phosphorylase to the actions of catecholamines and other hormones. *Pharm. Rev.* 12: 265.

- Ulberg, L. C., and Sheean, L. A. (1973) Early development of mammalian embryos in elevated ambient temperatures. *J. Reprod. Fertil. (Suppl.)* 19: 155-161.
- Voss, A. K., Sandmoller, A., Suske, G., Strojek, R. M., Beato, M., and Hahn, J. (1990) A comparison of mouse and rabbit embryos for the production of transgenic animals by pronuclear microinjection. *Theriogenology* 34: 813-824.
- Voutilainen, R., and Miller, W. L. (1987) Coordinate tropic hormone regulation of mRNAs for insulin-like growth factor II and the cholesterol side-chain cleavage enzyme, P450scc, in human steroidogenic tissues. *Proc. Natl. Acad. Sci. USA* 84: 1590-1594.
- Ward, D. N., Reichert, L. E., Jr., Liu, W. K., Nahm, H. S., Hsia, J., Lamkin, W. M., and Jones, N. S. (1973) Chemical studies of luteinizing hormone from human and ovine pituitaries. *Recent Prog. Horm. Res.* 29: 533-561.
- Wheeler, M. B., and Veldhuis, J. (1989) Facilitative actions of the protein kinase-C effector system on hormonally stimulated adenosine 3',5'-monophosphate production by swine luteal cells. *Endocrinology* 125: 2414-2420.
- Wilson, E. D., and Zarrow, M. X. (1962) Comparison of superovulation in the immature mouse and rat. *J. Reprod. Fertil.* 3: 148-158.
- Wolfenson, D., and Blum, O. (1988) Embryonic development, conception rate, ovarian function and structure in pregnant rabbits heat-stressed before and during implantation. *Anim. Reprod. Sci.* 17: 259-270.
- Ying, S.Y. (1988) Inhibins, activins, and follistatins: gonadal proteins modulating the secretion of follicle-stimulating hormone. *Endocr. Rev.* 9: 267-293.
- Zawadowsky, M. M. (1941) Hormonal stimulation of multiple birth in sheep. Cited by J. Hammond in *Marshall's Physiology of Reproduction*, A. S. Parkes, Ed. Vol. II, Ch. 21, 1952. London: Longmans, Green and Company pp. 648-669.
- Zondek, B. (1927) Hypophysenvorderlappen und ovarium. Beziehungen der endokrinen drüsen zur ovarialfunktion. *Arch. Gynaekol.* 130: 1-45.
- Zondek, B. (1930) Über die hormone des hypophysenvorderlappens. II. Follikelreifungshormon Prolan A-klimakterium-kastration. *Klin. Wochschr.* 9: 393-396.
- Zondek, B., and Aschheim, S. (1926) Über die funktion des ovariums. *Z. Geburtshilfe Gynaekol.* 90: 372-376.
- Zondek, B., and Aschheim, S. (1927) Das hormone des hypophysenvorderlappens. *Klin. Wochschr.* 6: 248-252.

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