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THE EFFECTS OF ACUTE  
RESTRAINT STRESS ON OVULATION  
AND SERUM HORMONE LEVELS IN  
THE LABORATORY RAT

Thesis for the Degree of M. S.  
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DONALD W. McKAY

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## ABSTRACT

### THE EFFECT OF ACUTE RESTRAINT STRESS ON OVULATION AND SERUM HORMONE LEVELS IN THE LABORATORY RAT

by Donald W. McKay

Effects of acute restraint stress administered on the afternoon of proestrus on ovulation and serum LH and prolactin concentrations were measured in cycling rats. Initially several restraint periods were checked for effects on ovulation, and it was found that restraint periods including the period of 3-5 p.m. were most effective for blocking ovulation. Secondly, intact and adrenalectomized rats were randomly assigned to one of the following restraint groups: 3-5 p.m., 4-6 p.m., 2-6 p.m., or control. Blood samples were taken under light ether anesthesia prior to stress, immediately following the restraint interval, and 90 minutes post-stress. It was found that restraint stress could block ovulation as effectively in adrenalectomized rats as intact, with the 2-6 p.m. restraint period most effective. A very high correlation was found between measured levels of serum LH and PRL prior to stress and the presence of eggs in the oviducts on the expected day of estrus.

The Effects of Acute  
Restraint Stress on Ovulation  
and Serum Hormone Levels in  
the Laboratory Rat

By

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## REVIEW OF THE LITERATURE

### Introduction

When Selye (1936) discovered a common denominator among sick people in the hospital, he called it the General Adaptation Syndrome or G.A.S. The G.A.S. may also be described as a general stress response, which when classically considered, dealt with the pituitary-adrenal axis. However, the effects of this generalized response are by no means limited to a simple increase in adrenocorticotrophic hormone (ACTH) leading to a rise in adrenal corticoids (Mason, 1968). Scientists and clinicians have examined many stimuli (stressors) that elicited a 'stress' response, and have attempted to quantify and qualify the effects.

### A Review of Stressors and Responses

Prolonged starvation and its effect on the uterine environment have been observed in the pig (Anderson, 1975) and in the rat (Henricks and Bailey, 1976). Subsequent refeeding caused acute cardiac accidents as well as permanent damage to the myocardium, arteries and arterioles in the pig (Johnson, 1966). In humans this most notably

occurred after the unsuccessful siege of Leningrad by the Germans when the link between the townspeople and the food supply was severed and suddenly reunited causing a series of cardiac arrests among the citizenry.

Teague (1970) noticed that heat stress caused a general decrease in food consumption in swine. In the ewe, heat stress has been associated with fetal dwarfing (Shelton and Huston, 1958), but this effect was not related to under-nutrition (Cartwright and Thwaites, 1976). Kamal and Johnson (1971) have used the loss of total body solids as a measure of heat stress in cattle. They suggested that an increased water turnover acted to prevent drastic and perhaps fatal increases in rectal temperature.

Heat stress has also been shown to cause profound alterations in blood flow (Hales, 1973). However, the response of the cardiovascular system varies from species to species. Hales and Dampney (1976) found that Merino sheep could tolerate quite large increases in heat simply by the redistribution of cardiac output. Greyhounds, which were known to be less heat tolerant responded with the redistribution as well as increased cardiac output.

Heat stress has been related to adrenal function when it was demonstrated that increases in body temperature caused embryonic degeneration in intact females while the same

stimulus elicited no change in adrenalectomized pregnant rats (Fernando-Cano, 1958) and ewes (Tilton, Hoffman, Berg, Light, and Buchanan, 1972).

Environment can affect stress reactions. For example, shipment of animals to market has often been the blame for manifestation of the PSE Syndrome in swine (Marple, Aberle, Forrest, Blake, and Judge, 1972). In the laboratory, the order of animal sacrifice is known to cause severe hormonal alterations (Seggie and Brown, 1976). In the classic experiments by Bruce (1968), the introduction of a strange male to a group of female mice may initiate and/or synchronize estrous cycles, as well as induce abortion at certain stages of pregnancy. Other noxious stimuli such as leg break, electric shock (Blake, 1975) as well as pharmacological agents, such as ether, biogenic amines and neural blocking drugs are responsible for the alteration of hormone production and secretion especially in tissues directly influenced by pituitary tropic hormones (Valverde-R, Chieffo, and Reichlin, 1973).

### Stress and the Adrenal Cortex

Since stress effects are so wide ranging and for a given stimulus quite repeatable, stress as a tool for the study of control mechanisms carries recognized credence.

Acute stress and its relationship to the hypothalamo-pituitary-adrenocortical axis have undergone extensive investigation. The impact of stress upon increased adrenal glucocorticoid secretion is generally accepted (Riegler, 1973; Brown, Uhlir, Seggie, Schally, and Kastin, 1970). This response is typified by an almost immediate pituitary release of ACTH followed by a rapid increase in serum corticosterone (Euler, Meites, and Riegler, 1975).

The ether stress induced increase in serum corticosterone followed a classical negative feedback type of control as demonstrated by Riegler and Hess (1972) when daily administration of dexamethasone, a synthetic steroid, reduced the adrenocortical response in a dose related manner. These data also showed age related differences in the degree of dexamethasone suppression of the aforementioned response. Aged animals exhibited less feedback inhibition in both chronic and acute experiments. This information coupled with earlier work which demonstrated decreased adrenal secretion to ACTH injection (Hess and Riegler, 1972) suggested an alteration in the sensitivity of the control mechanism perhaps at more than one level. Sex differences also added another dimension to the control of this system. Kitay (1963) used gonadectomized rats, and noticed distinct changes prior to and following ether stress between the

neutered sexes when contrasted with intact animals.

Ovariectomized females exhibited decreased resting and post ether stress corticosterone concentrations, while castrated males were not affected. Estrogen when added to adrenal slices in vitro elicited an increased corticosterone production which indicated a direct effect of estrogen upon the adrenals.

Other factors that have influenced the control system included the type of stress and the physiological status of the animal. Rabbits subjected to a heat stress of 40° C for one hour were able to resist plasma osmolality and pH changes when injected with dexamethasone (Chowers, Conforti, and Superstine; 1975). Intact animals that survived the heat stress were not able to reduce their body temperature to pre-stress temperatures, suggestive of an alteration in neural temperature control mechanisms. Regularly cycling rats exhibited a two-fold greater increase in corticosterone after 30 minutes than did lactating rats exposed to the same ether stress (Stern and Voogt, 1973/74). In the same report, pregnant rats were exposed to ether stress (a systemic stressor) or to suckling stimulus (a neurogenic stressor). The ether was more effective in elevating corticosterone levels, while it was less effective than suckling in altering prolactin concentrations.

The phenomena responsible for the release of ACTH are not so well understood (Smelik, 1969). Harris and co-workers (Harris and Jacobsohn, 1952; Green and Harris, 1947) disclosed the intimate relationship between the hypothalamus and pituitary. To elucidate further, Brodich (1963) placed lesions in the hypothalamus. When the animals were challenged by ether stress, a decreased corticosterone release was directly related to the decreased amount of intact hypothalamus. Subsequent work revealed a small amount of pituitary combined with an intact hypothalamus was capable of a greater stress response than the reverse situation. Evidence has been brought forth to illustrate two different corticosteroid receptor mechanisms in the hypothalamus for the feedback control of adrenocorticotropin (Jones, Tiptaft, Brush, Fergusson, and Neame, 1974). The possibility of the existence of two corticotropin-releasing factors in the swine hypothalamus was explored (Schally, Anderson, Lipscomb, Lang, and Guilleman, 1960). Moriarty and Moriarty (1975) have presented data that supported the presence of bioactive ACTH in the intermediate as well as the anterior lobe of the pituitary of the rat. Exposure to a neurogenic stressor (flashing lights), but not a systemic stressor (ether fumes) elicited a release of the intermediate lobe ACTH. In contrast to anterior

pituitary ACTH, dexamethasone did not block the stimulated release of intermediate lobe ACTH which suggested two separate control mechanisms of ACTH regulation.

Biogenic amines have been implicated with the release of ACTH, and the generalized stress response. The introduction of various catecholamines intraventricularly brought forth an increased ACTH secretion (Abe and Hiroshige, 1974) in the rat, however treatment with reserpine or monoamine oxidase inhibitor to either decrease or increase brain catecholamine content did not alter the diurnal pattern of corticosterone release, nor its stress response. The introduction of 6-hydroxydopamine (6-OHDA) decreased brain norepinephrine and dopamine content, but had little or no effect on circadian periodicity and no effect on stress responsiveness (Kaplanski, van Delft, Nyakas, Stoof, and Smelik, 1974). Additionally, the implantation of Phenoxybenzamine (an  $\alpha$ -adrenergic blocker) into the lateral ventricle elicited an augmented response to ether stress, whereas norepinephrine and dopamine did not (Eisenberg, 1975). This augmented response was blocked by dexamethasone. In the adrenal medulla, where similar catecholaminergic pathways exist, stress was associated with increased tyrosine hydroxylase activity which led to increased norepinephrine and epinephrine synthesis from tyrosine,

but not from dopamine (Kvetnansky and Weise, 1971). Depressed levels of dopamine following stress suggested that dopamine- $\beta$ -hydroxylase became the rate limiting enzyme at a time when dopamine formation is accelerated. It is interesting to note that steroids activate the enzyme phenethanolamine-N-methyl transferase while dopamine and high concentrations of norepinephrine inhibit this same enzyme which is responsible for the conversion of norepinephrine to epinephrine (Ciaranello, Barchas, Byers, Stemmler, and Barchas, 1969).

#### Stress Effects Involving LH and Ovulation

Several experiments have intimated that stress might interfere with ovulation. Hagino, Watanabe, and Goldzieher, (1969) injected ACTH into PMS treated infantile rats, and succeeded in blocking the expected ovulation. They also injected corticosterone or dexamethasone into similarly primed animals. Dexamethasone was more effective than corticosterone in the inhibition of ovulation. In the sow, Liptrap (1970) injected ACTH 24 hours prior to estrus. Ovulation did not occur in two out of three animals, and in each case estrus was shortened. The injection of corticosteroids mimicked the effect by also shortening estrus, but did not interfere with ovulation. In the mature rat,



Baldwin and Sawyer (1974) injected dexamethasone on different days of the four day estrus cycle. Dexamethasone when given during diestrus caused a 24 hour delay in ovulation by extending diestrus one day. If administered at late diestrus or early proestrus, the ovulation was again delayed, but an additional day of vaginal cornification occurred. The dexamethasone effect was said to have exerted itself at the pituitary level as the expected afternoon proestrous surge of luteinizing hormone (LH) was not detected. Additionally, when dexamethasone primed animals were challenged with various doses of luteinizing hormone-releasing hormone (LH-RH) they exhibited a depressed LH release. Restraint stress in the rat also has been implicated with a possible decrease in ovulation. Euker and Riegler (1973) after having administered restraint stress for two hours daily, three days prior to mating noticed that the expected cornified vaginal smear of estrus was delayed an average of three days, which suggested the blockage of gonadotropins necessary for estrogen secretion as well as ovulation. Litter size was not significantly altered by the treatment once ovulation and mating occurred. However, gilts subjected to increased temperatures prior to ovulation showed a decreased ovulation rate (Teague, Roller, and Grifo, 1968; Warnick, Wallace, Palmer, Sosa, Duerre, and

Caldwell, 1965). Nequin and Schwartz (1971) have illustrated the significance of adrenals in the normal timing of mating behavior in the rat, and that stress activation of the adrenals altered both mating behavior and LH release in the proestrous rat. When animals were subjected to sham ovariectomy, lordosis was advanced. Adrenalectomy alone delayed lordosis, while combined adrenalectomy and ovariectomy blocked lordosis from occurring at all in many animals. The latter procedure in combination with exogenous progesterone elicited lordosis in the five day cyclic rats. Several authors (Freeman, Dupke, and Croteau, 1976; Krey, Tyrey, and Everett, 1973; Ying and Greep, 1972) mentioned the role of progesterone facilitation of the estrogen-induced LH release, but only Nequin, Alvarez, and Schwartz (1975) concerned themselves with steroidal contributions from the adrenal. In that study, the timing of events in the rat estrous cycle: ballooned uteri, vaginal cornification, mating behavior, LH release, and ovulation were correlated with gonadotropin and steroidal profiles taken every two hours around the clock (in four and five day cyclic rats). Because estrogen and LH patterns were identical for both four and five day cyclers, estrogen, though an important determinant in reproductive phenomena, can be excluded as the trigger for LH release. Progesterone however,

displayed a distinctly different profile between the two types, and uterine ballooning was associated with the fall in progesterone, rather than an increase in estrogen, as was commonly thought. The exact role of adrenal participation remains obscure, but must be considered in the events responsible for the occurrence of either a four or five day estrous cycle in the rat. Ovarian rhythmicity superimposed upon that of the adrenal may well explain the ostensibly random nature of cycle length.

The relationship of pituitary release of LH by direction from the hypothalamus has been reviewed (McCann, Watanabe, and Dhariwal, 1967). Normal circulating levels of gonadotropins have been established in many species including the rat (Butcher, Collins, and Fugo, 1974), bovine (Miller and Alliston, 1974), porcine (Guthrie, Henricks, and Handlin, 1972), ovine (Cunningham, Symons, and Saba, 1975), and canine (Concannon, Hansel, and Visek, 1975).

While an interaction between steroids and the release of LH has been established, no exact mechanism has been proposed. The precise timing of progesterone action, and the following LH activity in rats was examined by Redmond (1968). Dependent upon the time of progesterone administration, gonadotropin release and ovulation appeared normally, partially, or were completely blocked. In the

same experiment, high doses of progesterone led to a decreased ovarian responsiveness to LH, while low doses necessitated only minimal LH to induce ovulation. In ovariectomized females pretreated with testosterone (to reduce existing high LH secretion), progesterone stimulated increased LH release after three hours (Jackson, 1973), and progesterone in combination with estrogen has been linked with increased pituitary stores of LH (Blake, Norman, and Sawyer, 1972). Progesterone also has been implicated with facilitation of ovulating hormone in sodium-pentobarbital blocked female rats (Kobayashi, Hara, and Miyake, 1973), but once again timing was an important factor. The administration of  $\alpha$ -adrenergic blockers or other drugs that depressed norepinephrine synthesis inhibited this progesterone effect such that no gonadotropin surge appeared (Kalra, Kalra, Krulich, Fawcett, and McCann, 1972). When dihydroxyphenylserine (DOPS), a precursor to norepinephrine, was given simultaneously with the blocker, the gonadotropin surge was restored.

Stress alterations of gonadotropin secretion have not been as consistent, nor as well understood as those of glucocorticoid release. Immobilization stress suppressed the spontaneous pulsatile LH release in ovariectomized rats while other stressors had no effect (Blake, 1975).

Ajika et al (1972) reported that ether stress induced increases in plasma levels of prolactin (PRL), LH, and follicle stimulating hormone (FSH) in ovariectomized rats, while Nembutal blocked the stress induced increases in LH and prolactin but not FSH. Nequin, Alvarez, and Campbell (1975) worked with ovariectomized five day cyclic rats on the morning of proestrus, and observed increased adrenal estrogen and progesterone with the advancement of the release of FSH prior to LH following surgical stress. In a clinical study, however, no difference was observed in human LH nor FSH levels during surgery (Charters, Odell, and Thompson, 1969). This last observation may be affected by anesthesia and/or altered baseline values due to emotional stress preceding surgery. In the bovine species, Miller and Alliston (1974) showed that an increased ambient temperature was associated with decreased peak and basal LH values in the blood. Such was not the case in swine (Riggs, Alliston, and Wilson, 1974). With an increased environmental temperature, peak LH values were increased by stress in gilts. Riegler and Meites (1976) were able to demonstrate a difference between LH responsiveness in young and aged male rats. The young males showed an immediate rise in serum LH, while no demonstrable change occurred in the aged group with the sample period.

### Stress and Prolactin

Although prolactin is released by the pituitary under the control of the hypothalamus, the predominant force of the hypothalamus in this regard is inhibition of PRL release (Meites, 1967). Serum PRL levels have been characterized in rats (Amenomori, Chen and Meites, 1970) and cattle (Koprowski, Tucker, and Convey, 1972). The dependency of prolactin secretion on sex and age has been demonstrated in the rat (Shaar, Euker, Riegler, and Meites, 1975). Male rats showed no cyclic changes in serum PRL concentration (Amenomori et al, 1970), while females characteristically showed two peaks of PRL release which occurred during the afternoons of proestrus and estrus (Butcher et al, 1974). Endocrinologists have agreed that suckling induces increased prolactin secretion (Tucker, 1971; Voogt and Carr, 1975), but the control of this release has been confounded by many conflicting reports. Biogenic amines, especially dopamine, have been implicated as the probable prolactin inhibiting factor (PIF) which is released by the hypothalamus (Shaar, 1975; Blake, 1976). Thyrotropin releasing hormone has been shown to contain a significant amount of prolactin releasing ability (Tucker and Wettemann, 1976). The effects of various steroids on PRL secretion have been examined by many investigators. Treatment with estradiol benzoate or testosterone propo-

nate into castrate female and male rats, respectively, elicited a dose related increase in serum prolactin levels (Shaar, Euker, Riegler, and Meites, 1975). In another study with ovariectomized rats, large doses of progesterone led to increased plasma PRL concentrations (Kalra, Fawcett, Krulich, and McCann, 1973). In the same study, estradiol benzoate injection on the morning of estrus but not on the morning of diestrus I was followed by increased plasma PRL concentrations. Injections of progesterone into intact cycling female rats on the morning of proestrus advanced the release of LH and PRL and was followed by a "supersecretion" of progesterone (Uchida, Kadowaki, Miyake, and Wakabayashi, 1972). Estrogen antibodies injected during diestrus day II inhibited increases in LH or PRL during proestrus (Neill, Freeman, and Tillson, 1971). Manifestations of uterine ballooning, vaginal cornification, and ovulation were also decreased. Simultaneous treatment with diethylstilbestrol reversed the anti-estrogen effects.

Control mechanisms which mediate prolactin release in response to a stressor have been the subject of intense investigation. Neill (1970) advised caution in collection of blood samples, as response by prolactin to noxious stimuli varied with the physiological status of the animal. Riegler and Meites (1976) demonstrated that the intensity of the

stressor and the physiological status of the animal determined the direction and magnitude of the subsequent PRL release. At times when serum prolactin was elevated, such as during proestrus, estrus, pregnancy, and lactation, stress served to lower serum PRL concentrations. Conversely, stress administered at times when initial serum concentrations were low, such as diestrus, caused serum PRL levels to become elevated. Harms, Langlier, and McCann (1975) substantiated the possible role for adrenal alteration of prolactin secretion by demonstrating potentiation of stress induced PRL release following adrenalectomy and a reduced response after treatment with dexamethasone.

Because of the failure of reserpine to block stress mediated increases of prolactin secretion, Valverde-R, Chieffo, and Reichlin (1973) maintained that this secretion was not due to inhibition of PIF secretion; rather they claimed a prolactin releasing substance mediated this acute secretory response.

A variety of anesthetics have been implicated with alterations in blood PRL levels (Wakabayashi, Arimura, and Schally, 1971; Lawson and Gala, 1974; Bellinger and Mendel, 1975), but Riegler and Meites (1976) warned that interpretation of these results must take into consideration the intensity of the stressor as well as the physiological



status of the animal.

When hyper-prolactin secretion was induced by daily injections of Perphanazine, unstressed rats exhibited larger serum progesterone concentrations than those animals subjected to prior ether stress (Chatterton, Chien, Ward, and Miller, 1975).

Stress effects on reproductive function have been clearly demonstrated in a variety of species. Before the control mechanisms involved in mediating these responses can be fully understood, more precise information must be gathered concerning the specific factors that affect reproduction. This study addresses the effects of acute stress administered on the afternoon of proestrus on serum LH and PRL levels and ovulation in adrenalectomized and intact cycling rats.

## MATERIALS AND METHODS

### Experimental Animals

Female rats of the Long-Evans variety and of similar genetic stock were nurtured in a temperature controlled room (22° C) and exposed to a 12 hour light/dark cycle with lights on from 6:00 a.m. until 6:00 p.m. Daily vaginal lavage and subsequent cytological examination were utilized to determine each rat's status with respect to the estrous cycle. All rats received commercial laboratory rat diet and water ad libitum; rats following adrenalectomy received 1.0% saline in lieu of tap water.

### Adrenalectomy

Regularly cycling virgin rats were randomly selected for adrenalectomy. The operation was performed under ether anesthesia and two incisions were made on the animal's ventral side just posterior to the last rib. After excision of the adrenals, the animal was administered antibiotic topically as the incisions were closed. The animals were allowed to recuperate, and regular estrous cyclicity was reestablished prior to further experimentation. At that time, thoroughness of adrenalectomy was determined by

visual inspection of the tissues removed.

### Treatments

The first experiment considered the effect of restraint stress administered on the afternoon of proestrus on ovulation. Separate groups of rats received restraint stress treatments between 2-4 p.m., 4-6 p.m., 3-5 p.m., 2-6 p.m., 4-8 p.m., and 12-6 p.m. Restraint stress was achieved by physical restraint in a supine position on the laboratory counter top. All animals were anesthetized at the onset of the restraint period to facilitate handling. Ovulation was measured as the number of eggs recovered from the oviduct on the following day. Between 11 a.m. and 1 p.m. on the day of estrus, the oviducts were removed and the eggs were teased from the ballooned portion of the oviduct and counted using a dissecting microscope.

In the second experiment similar groups of intact and adrenalectomized rats received restraint stress treatments between 3-5 p.m., 4-6 p.m., or 2-6 p.m. Separate control groups, intact and adrenalectomized, did not receive the restraint treatments. All animals subjected to restraint stress in this experiment had blood samples taken prior to the initiation of the restraint, immediately after the stress period and 90 minutes following the end of the

particular restraint stress regime. Oocytes were located as before, however, the left oviduct was initially examined. If ova were found, the right oviduct was then removed and a total ovulation number was recorded. If no ova were found in the left oviduct, the rat was sutured and 24 hours later the right oviduct was removed and examined to determine whether the stress treatments had delayed rather than blocked ovulation.

#### Blood Collection

Prior to most treatments, animals under light ether anesthesia donated approximately 1 ml. of whole blood by suborbital sinus puncture via a heparanized capillary tube. This technique, which allowed collection within 60 seconds of initial cage disturbance has been demonstrated to cause negligible stress effects on serum hormone concentrations in that specimen (Euker, Meites, and Riegler, 1975). Other sampling by the same technique occurred at selected times during or after restraint stress. The resulting whole blood was allowed to clot at room temperature for 30-60 minutes, refrigerated overnight and centrifuged for 10 minutes to facilitate the harvest of serum. The samples were then frozen until assays were performed.

### Radioimmunoassay for Serum LH and PRL

The double antibody radioimmunoassay procedures for the measurement of LH and prolactin are those of Monroe et al (1969) and Niswender et al (1969) respectively, and in routine use in the laboratory of Dr. J. Meites, Michigan St. Univ. Radioactive antigen using purified rat prolactin (H-10-10-B Prolactin) or LH (LER 1056 LH) and  $^{125}\text{I}$  was prepared and diluted with 0.1% gelatin phosphate buffered saline (PBS) to a working solution of approximately 30,000 cpm/100 $\mu\text{l}$  as detected by an automatic gamma well counter (Searle, Inc.).

Anti-ovine LH antiserum (Niswender) prepared by immunization of rabbits with ovine pituitary LH was diluted to a 1:28,000 concentration.

Anti-rat prolactin antiserum prepared likewise was diluted to a 1:5,000 working concentration. Ovine-anti-rabbit gamma globulin antiserum served to precipitate the antigen-antibody complexes.

With the exception of specific antigens and antisera, the radioimmunoassay procedures for LH and prolactin determination are identical. Selected aliquots of serum were pipetted into test tubes and diluted to a volume of 0.5 ml. with 0.1% gelatin PBS. After addition of 200 $\mu\text{l}$  of the appropriate antiserum, tubes were vortexed and allowed to

incubate at 4° C for 24 hours allowing time for endogenous hormone to bind to the antibody. At that time, 100µl of the radioiodinated antigen were added, vortexed and allowed to incubate for another 24 hours. This time enabled the radioactive hormone adequate time to fill any vacant receptor sites. The second antibody (200µl of ovine anti-rabbit gamma globulin) was incubated with the preceding for 72 hours, and during that time bound with the previously established antigen-antibody complex. Three ml of cold PBS were added to each sample tube and immediately centrifuged for 20 minutes at 2200 rpm. The supernatant was decanted, and the bound fraction remaining in the tubes was counted in a gamma well counter.

#### Corticosterone Fluorometric Assay

To insure the validity of the adrenalectomies, the pre- and post-stress serum samples were subjected to a fluorometric corticosterone assay (De Moor and Steno, 1963).

A Turner model 110 fluorometer was equipped with appropriate filters (excitation light of 470 nm and emission at 530 nm). Serum samples were pipetted into glass stoppered centrifuge tubes containing 5 ml of methylene chloride. The sample volume was brought up to 1.0 ml with distilled water. The tubes were subsequently stoppered and slowly rotated for three minutes and centrifuged.

After the aqueous layer was aspirated and discarded, partial extract purification was achieved by subjecting the sample to a cold 0.1 N NaOH wash which removed excess lipids and acids. Four ml of the now hormone containing methylene chloride was transferred to a clean tube. Five ml of a 3:1 mixture of concentrated sulfuric acid and absolute ethanol were added to each tube as a fluorescing agent. By again shaking the tubes vigorously, corticosterone was extracted into the fluorescing reagent. Centrifugation facilitated the separation of solvent layers, and the methylene chloride layer was discarded.

Unknown corticosterone was compared with known standards and a reagent blank of distilled water was carried through the procedure to account for background fluorescence.

### Statistical Analysis

Initial plasma concentrations of LH and prolactin were analyzed for effects of the factors: ovulatory status, adrenal status, restraint period, and cycle length by analysis of variance performed with the aid of the ANOVA subprogram of the Statistical Package for the Social Sciences (SPSS) on the CDC 6500 computer (Nie et al, 1975). Frequency of ovulation was analyzed by the subprogram CROSSTABS of SPSS, controlling for cycle length, adrenal

status, and restraint group. Split-plot analyses of corticosterone concentrations by restraint groups and by ovulatory status were run on PROFILE, a program administered by the College of Education, Michigan St. Univ. Ovulation rate between intact and adrenalectomized rats was examined using the Student's t test.



## RESULTS

Results of the preliminary experiments with intact female rats indicated that restraint stress administered on the afternoon of proestrus influenced the number of rats that subsequently ovulated (See Table 1). All control and restraint stress treated rats showed normal vaginal cornification of the predicted day of estrus. Furthermore, animals restrained during mid-afternoon (i.e. restraint intervals that included the hours of 3 p.m. through 5 p.m.) were more susceptible to blockage of ovulation than rats restrained early or late in the afternoon. Restraint stress treatments did not affect the number of ova recovered from rats which ovulated.

The second experiment which included both intact and adrenalectomized rats showed similar effects of restraint stress on ovulation. As in the preliminary experiment, all rats showed normal vaginal cornification on the expected day of estrus such that there were no detectable differences in vaginal cytology on the day of estrus with respect to animals that did or did not ovulate. Of those animals in which ovulation was not detected the first day and were subsequently re-examined in the remaining

Table 1. Effect of Restraint Stress Administered During Proestrus on Ovulation

Treatment	no. rats	% Ovulating	Mean no. Ova	% Not Ovulating
Control	10	100	11.9	0
2-4 p.m. Restraint	10	80	11.9	20
4-6 p.m. Restraint	10	80	10.6	20
3-5 p.m. Restraint	14	64	11.6	36
2-6 p.m. Restraint	18	44	9.5	56
12-6 p.m. Restraint	9	44	7.8*	56
4-8 p.m. Restraint	8	100	12.0	0

\*One rat in this group had only two ova.

oviduct 24 hours later, 16 out of 28 rats exhibited a cornified vaginal smear instead of a more typical leukocytic smear. Ova were recovered on the second day following the stress treatment from only one rat, an adrenalectomized rat in the 4-6 p.m. stress group that displayed a double cornified smear.

The greatest percent inhibition of ovulation in both the intact and adrenalectomized rats was found in the 2-6 p.m. stress groups (See Table 2). Adrenalectomy did not significantly affect the ovulatory rate ( $p < .05$ ) nor the number of animals ovulating per restraint group when compared with intact animals. However, two animals from the 2-6 p.m. adrenalectomized restraint group did not survive until the check for ova was completed, and the one rat that was perceived to have ovulated did so at a low rate and only from one ovary.

The levels of serum LH in the rats from the second experiment are shown in Figure 1. Both intact and adrenalectomized rats that ovulated had significantly ( $p < .001$ ) higher initial serum LH levels than non-ovulating rats in all of the treatment intervals tested. Only two of the rats with measured serum LH concentrations at or below 200 ng/ml at any sampling time ovulated. It is possible that our experimental intervals were not sufficient to

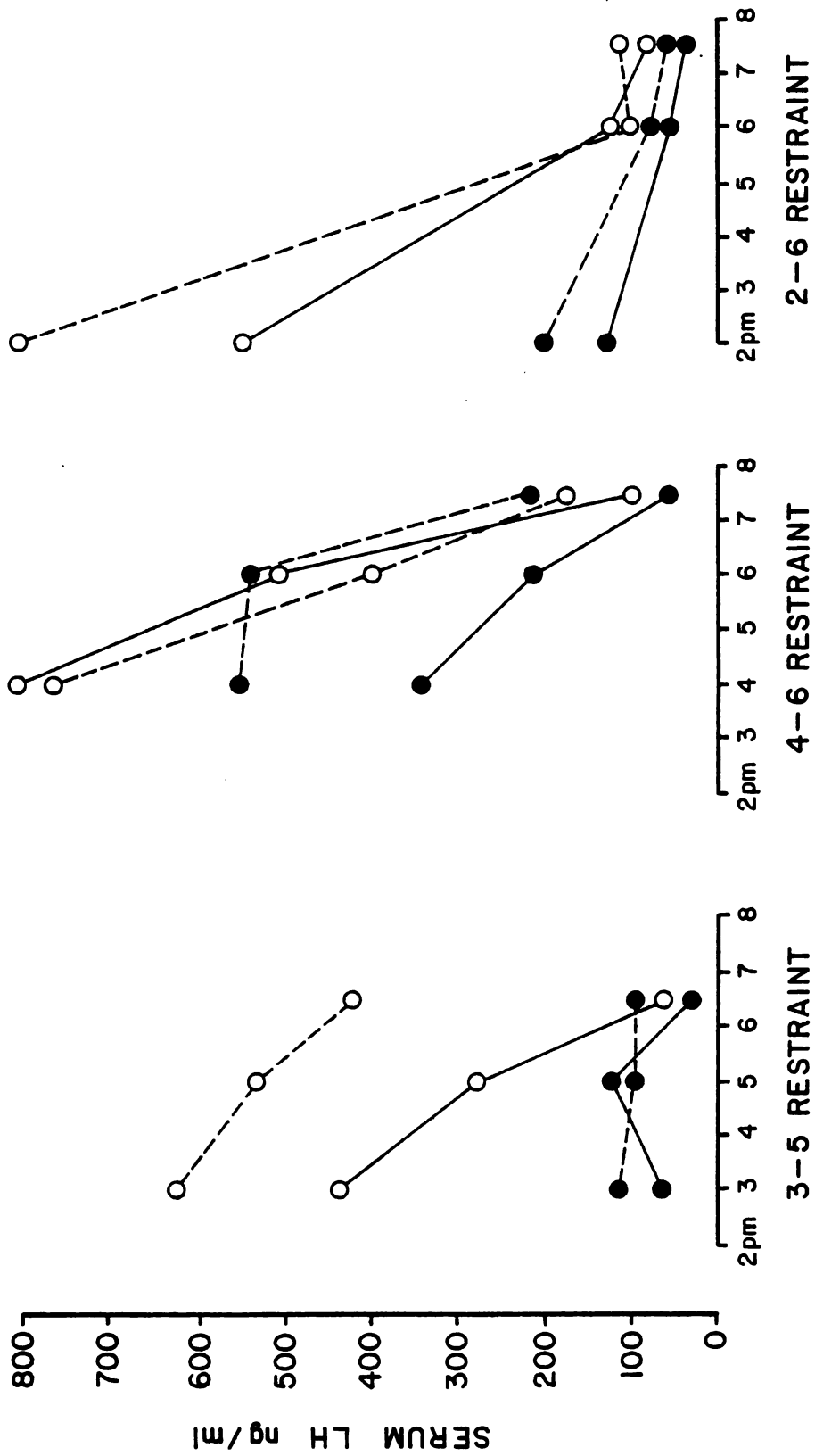
Table 2. Effect of Restraint Stress Administered During Proestrus on Ovulation in Intact and Adrenalectomized Rats.

Treatment	no. Rats	% Ovulating	Mean no. Ova	% Not Ovulating
Control				
Intact	10	100	11.9	0
Adrenalectomized	8	100	11.6	0
3-5 p.m. Restraint				
Intact	10	60	12.2	40
Adrenalectomized	10	40	9.8	60
4-6 p.m. Restraint				
Intact	10	70	11.9	30
Adrenalectomized	10	70	11.6	30
2-6 p.m. Restraint				
Intact	10	40	13.8	60
Adrenalectomized	7*	14	2.0	86

\*Two additional rats from this group died during the treatment

Figure 1. The effect of restraint stress on serum LH in intact and adrenalectomized cycling rats.

Restraint treatments were administered between 3-5 p.m., 4-6 p.m., or 2-6 p.m. on the afternoon of proestrus. Mean serum LH levels are plotted from ovulating (o) and non-ovulating (●) rats as well as intact (—) and adrenalectomized (---) animals. Blood samples were taken before the stress, at the end of the stress, and 90 minutes after the end of the stress treatment.



detect the proestrous LH surge in these two rats.

In our colony, the proestrous LH surge has been established to occur in a range from 2:00 p.m. until 6:00 p.m. (See Appendix A). The analysis indicated a significant ( $p < .002$ ) main effect of the restraint period on the initial level of LH, reflecting the different starting times of restraint stress.

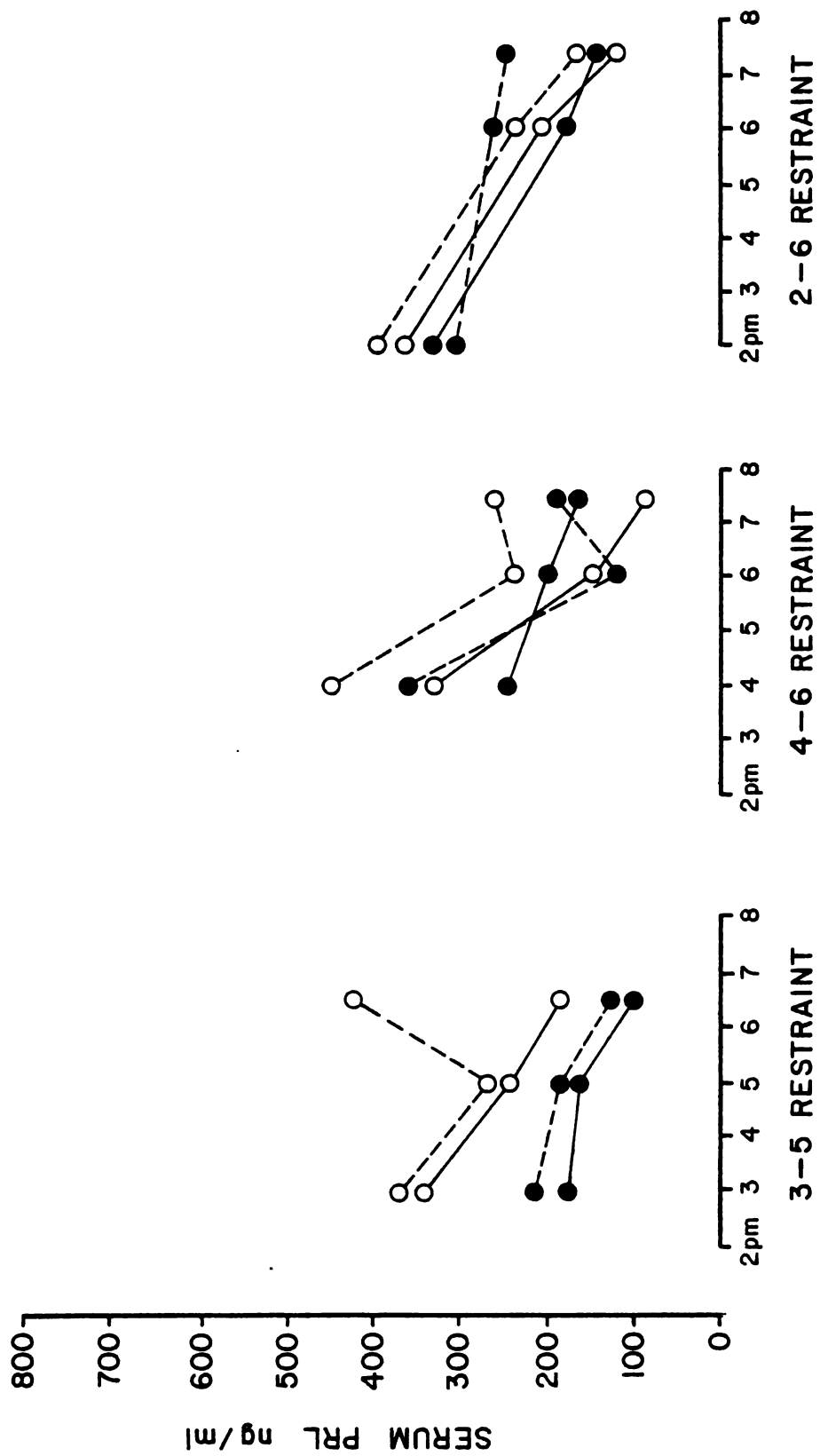
It was expected and demonstrated that more rats experienced the afternoon surge by 4:00 p.m. than by 2:00 p.m. Neither adrenal status nor cycle length were found to have significant effects on the initial LH concentration. Following restraint stress, LH values were generally depressed from the pre-stress sample, and at 90 minutes post-stress, all groups had remained lower than the initial values.

Concentrations of serum prolactin from rats in the second experiment are depicted in Figure 2. Although the effects of restraint stress on prolactin did not appear to be as drastic as those of LH, serum prolactin concentrations in adrenalectomized and intact rats were significantly ( $p < .001$ ) linked to the ovulatory status of the animals. Higher prolactin levels were seen in rats that had ovulated and lower values were associated with those that had not ovulated. The initial prolactin levels were not significantly ( $p < .06$ ) related to the time of stress initiation

Figure 2. The effect of restraint stress on serum PRL in intact and adrenalectomized cycling rats.

Restraint treatments were administered between 3-5 p.m., 4-6 p.m., or 2-6 p.m. on the afternoon of proestrus. Mean serum PRL levels are plotted from ovulating (o) and non-ovulating (●) rats as well as intact (—) and adrenalectomized (---) animals. Blood samples were taken before the stress, at the end of the stress, and 90 minutes after the end of the stress treatment.





despite an elapsed time of two hours from the initiation of the earliest restraint (2:00 p.m.) to the latest (4:00 p.m.). This could possibly have been a reflection of the broad prolactin peak that has been demonstrated on the afternoon of proestrus (See Appendix A). As was the case with serum LH, adrenal status and cycle length were not significant factors influencing the pre-stress concentration of serum prolactin.

Serum prolactin levels were lowered at the end of the restraint interval in all groups. However, the 90 minute post-stress sampling for intact ovulators in the 3-5 p.m. restraint group indicated higher concentrations than the pre-stress sampling.

Serum corticosterone was measured in the adrenalectomized rats to insure the thoroughness of the adrenalectomy. Serum from all adrenalectomized rats had measureable corticosterone levels (See Table 3). While no difference was found between restraint groups, a significant ( $p < .001$ ) increase in serum corticosterone occurred following restraint stress. This increase when contrasted with intact rats subjected to similar restraint stress is negligible (See Appendix B). No significant difference in corticosterone levels was detected between those rats that did ovulate, and those that did not (See Figure 3).

Crosstabulation of cycle length (4 or 5 day) by ovulatory status on the day of estrus failed to demonstrate

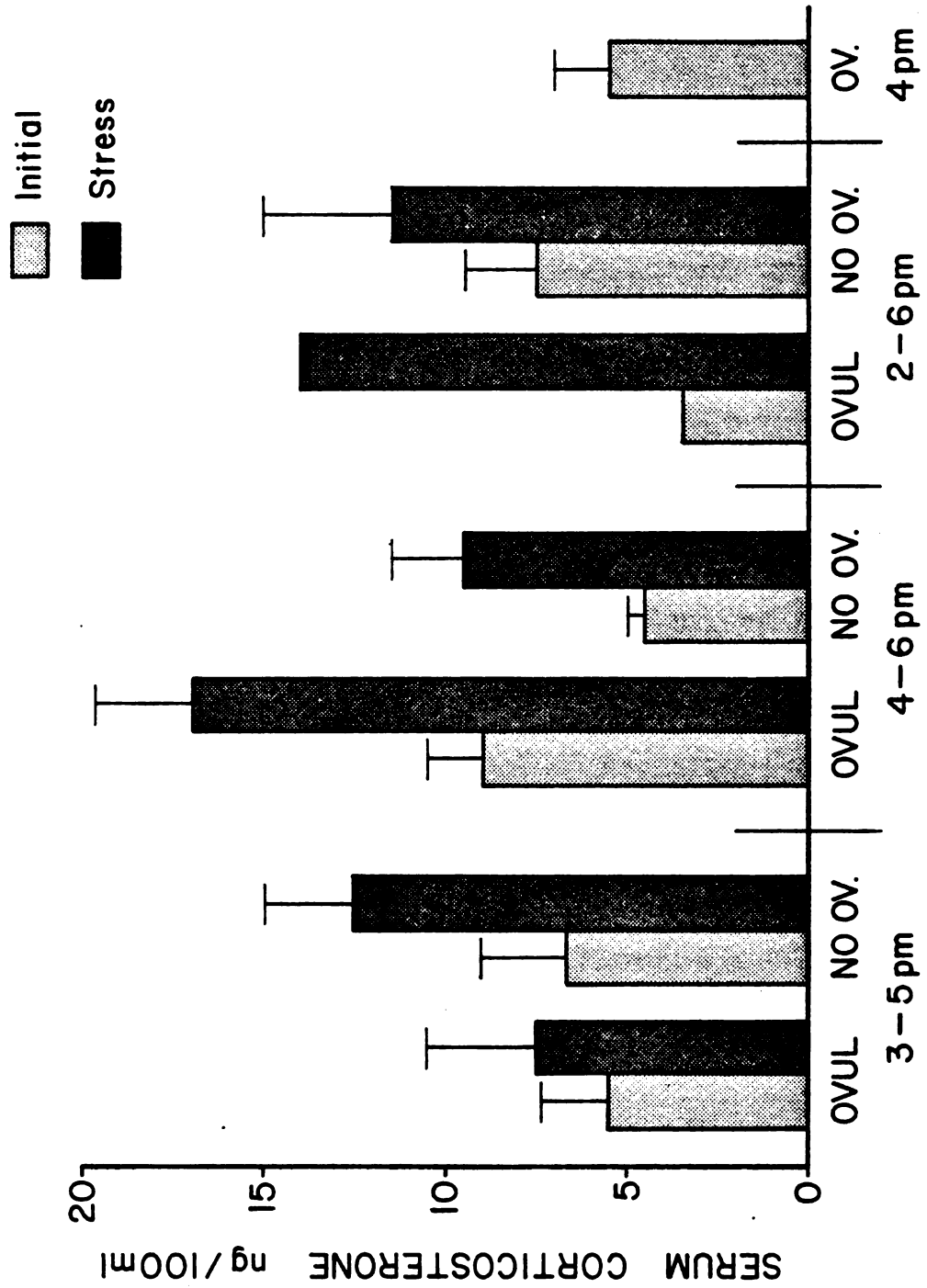
Table 3. Serum Corticosterone: Pre- and Post-Stress Samples from Adrenalectomized Female Rats

Restraint Group	Pre-Stress		Post-Stress	
3-5 p.m.	6.08	3.08	10.88	5.14*
4-6 p.m.	7.04	3.79	13.63	6.89*
<u>2-6 p.m.</u>	<u>6.90</u>	<u>4.41</u>	<u>11.91</u>	<u>7.91*</u>
<u>Total</u>	<u>6.65</u>	<u>3.72</u>	<u>12.17</u>	<u>6.91*</u>

\*p<.001 increase above pre-stress

Figure 3. Serum corticosterone levels before and after restraint stress treatments in adrenalectomized rats.

Group mean corticosterone levels and the standard error of the mean are plotted for rats that ovulated and those that did not ovulate in each treatment group. The OV. 4 pm group represents the serum corticosterone levels of the non-stressed adrenalectomized control group.



statistically significant results. Additionally, adrenalectomy did not appear to cause a shift in cycle length, as four rats switched from a four day to a five day cycle, while six animals did the opposite and went to a four day cycle prior to restraint stress.

## DISCUSSION

These results demonstrated that restraint stress administered to rats on the afternoon of proestrus can block ovulation. A very high correlation was found between measured levels of serum LH and PRL prior to stress and the presence of eggs in the oviducts on the expected day of estrus in intact and adrenalectomized rats. The results suggested that the mechanisms of stress inhibition of ovulation involved reduced gonadotropin secretion which was independent of stress-induced increases in secretions of the adrenal glands.

The most critical time interval for stress effects on serum LH levels was in the late afternoon of proestrus. Preliminary work showed that individual rats from our colony had proestrus serum LH and PRL peaks that occurred between 2:00 and 6:00 p.m. Exposure to restraint stress treatments within this interval significantly reduced the number of rats that ovulated. Stress treatments initiated prior to this critical late afternoon time were less effective and restraint stress regimes administered after the proestrus LH surge were totally ineffective in blocking ovulation. The most effective treatment intervals were those that

included the period from 3:00 p.m. through 5:00 p.m. The results of these experiments suggested that most individual rats with high serum LH levels in their pretreatment blood samples ovulated normally. Restraint stress blocked pituitary LH release and ovulation in those rats that did not have high initial LH levels. Euker, Meites, and Riegler, (1973) reported that stress can reduce the elevated serum LH in proestrous rats. While stress reduced serum LH concentrations in individual rats with elevated pre-stress serum LH levels in this study, the presence of normal numbers of ova in the oviducts on the day of estrus suggests that sufficient LH had been released for normal ovarian stimulation. The ability of restraint stress to block the LH surge was implied in a different experiment when diestrus I female rats were subjected to similar restraint on three consecutive days (Euker and Riegler, 1973). While subsequent litter size was not affected, the expected estrus period was delayed on an average of three days. Blake (1975) found similar results when testing the effects of several stressors on ovariectomized rats. Only immobilization stress was able to interrupt the pulsatile LH surges that are typical of this condition.

Although the data suggested that stress can block LH secretion, the mechanism remains obscure. Wood (1976)



demonstrated that stress does not interfere with pituitary LH secretion following LH-RH challenge in male or female rats. This observation demonstrated that the primary effect of stress on blocking LH secretion occurred at a higher level than the pituitary, implying inhibition of hypothalamic gonadotropin releasing hormone control mechanisms, rather than alteration of pituitary response to releasing hormone regulation. The efficacy of these acute stress treatments suggested that hypothalamic LH releasing hormone content would not be the limiting factor in pituitary stimulation. However, stress effects on neurotransmitters affecting secretion of hypothalamic LH releasing hormone could relate to these data.

Norepinephrine has been reported to be the primary hypothalamic amine which stimulates LH secretion (Cocchi, Fraschini, Jalanbo, and Muller, 1974). Palkovits, Kobayashi, Kizer, Jacobowitz, and Kopin (1975) showed that acute stressors could reduce arcuate nucleus content of both dopamine and norepinephrine.

Adrenocortical secretions have been implicated in mechanisms controlling proestrous gonadotropin secretion. Nequin, Alvarez, and Campbell (1975) showed that surgical stress on the morning of proestrus stimulated adrenal estrogen and progesterone release which induced an early

gonadotropin surge on the afternoon of proestrus. However, Baldwin and Sawyer (1974) reported that a large dose of dexamethasone (200  $\mu$ g) injected into proestrous rats at 9:00 a.m. blocked the proestrous surge, and Blake (1976) found that an intravenous infusion of epinephrine could block the proestrous secretion of LH in rats. These results implied that adrenal secretion of steroids and epinephrine may be the mechanism of stress induced alterations in reproductive function. The present study showed detectable serum corticosterone levels in our adrenalectomized rats which were increased following the stress treatments. It is assumed that this corticosterone was secreted by accessory adrenal tissue and possibly the ovaries. However, the increase in corticosterone following stress was not out of the range of normal intact rat serum corticosterone and represented only a fraction of the corticosterone present in the blood of intact rats receiving similar stress treatments (Riegler, 1973). For this reason it is doubtful that adrenal responses that caused embryonic mortality in heat stressed pregnant rats (Fernando-Cano, 1958) share a similar mechanism as reported in this study as similar results were obtained with intact and adrenalectomized rats.

Other workers have altered the catecholaminergic environment within the brain and have measured stress

responsiveness. The implantation of an  $\alpha$ -adrenergic blocker (Phenoxybenzamine) into the lateral ventricle resulted in an augmented response to ether stress that could be blocked by dexamethasone (Eisenberg, 1975). Abe and Hiroshige (1974) were able to show increased ACTH secretion by administration of norepinephrine, dopamine, serotonin, and carbachol. Following depletion of brain amines by reserpine, or a general increase of amines induced by a MAO inhibitor, no change in diurnal pattern nor stress response of corticosterone was noted. Similarly, the addition of 6-OHDA into the lateral ventricle which caused a depletion of brain norepinephrine and dopamine, elicited no changes of plasma corticosterone diurnal periodicity nor stress responsiveness (Kaplanski et al, 1974). The above experiments suggested an involvement of catecholamines in stress responsiveness, yet general depletion or repletion of catecholamines did not alter diurnal periodicity nor stress responsiveness. A possible mechanism may involve other neurotransmitters that might impose themselves upon the catecholaminergic pathway to alter relative quantities of the catecholamines. Such a molecule might be serotonin (5-HT) known for its anti-dopaminergic effects. If serotonin is the substance responsible for release of corticotropin releasing factor (CRF), the following schema may be appropriate for the

proestrous rat: Stress would activate both catecholaminergic and serotonergic systems, and cause increases in dopamine and serotonin. Serotonin could act to 1) cause release of CRF, 2) cause activation of Phenylethanolamine-N-Methyl-Transferase (PMNT) causing increased epinephrine which may inhibit Dopamine- $\beta$ -Hydroxylase causing a buildup of dopamine and a depletion of norepinephrine. The CRF would cause pituitary release of ACTH, while the decreased norepinephrine may inhibit LH-RH release and subsequently depress LH. The increased dopamine would cause a drop in pituitary prolactin release.

If the rat was diestrus, when endogenous dopamine levels are high and norepinephrine low, the 5-HT activation of PMNT would not cause as great an increase in epinephrine as its substrate, norepinephrine, would not be as abundant. Since dopamine is at maximal or high levels already, the increased 5-HT may act to impair its function, while dopamine conversion to norepinephrine would decrease its concentration. The end result would be increased PRL and LH secretion from the pituitary. Additionally, the stress induced 5-HT would stimulate CRF as in the proestrous rat.

The aforementioned plan also explains the dexamethasone suppression of stress induced ACTH and PRL. If 5-HT is sensitive to negative feedback, then adrenal glucocorticoids

would depress 5-HT which would shut off CRF release and ACTH. The lowered 5-HT would also not inhibit dopamine activity which would allow increased dopamine effects and lower PRL release.

Another possible mechanism of stress interference may involve altered feedback from the ovary. Intact animals have been shown to react to progesterone injections by producing gonadotropin surges (Kalra et al, 1973). The administration of  $\alpha$ -adrenergic blockers inhibited these progesterone induced responses, but simultaneous administration of DOPS, a precursor to norepinephrine restored the animals' ability to respond (Kalra et al, 1972). Prolactin has been shown to cause the release of progesterone from the adrenal (Piva et al, 1973). The results of this study indicated that prolactin was depressed following stress, and that ovulation was associated with higher levels of PRL prior to stress. A possible mechanism for the blockage of ovulation may initially involve decreased prolactin release that would result in inhibition of progesterone release from the ovary and/or the adrenal. The lack of a progesterone effect at the hypothalamus would then explain the absence of the LH surge.

The results of this experiment suggest that stress effects on the ovulatory mechanism of the rat do not require

stress-induced stimulation of adrenal steroid or catecholamine secretions. Adrenalectomy did not affect ovarian cyclicity, LH secretion, nor ovulatory rate. There were also no differences in serum LH levels, percentage of animals ovulating or number of ova ovulated between intact and adrenalectomized rats receiving the various stress regimes.

These experiments lead us to believe that stress of sufficient intensity can interfere with reproduction by blocking the release mechanism for LH releasing hormone from the hypothalamus. These data indicate that stress-induced alterations in adrenal steroid or catecholamine secretion are not required for stress inhibition of pituitary LH or PRL release or ovulation.

## SUMMARY

The effect of restraint stress on proestrous serum LH, prolactin, and ovulation was determined in intact and adrenalectomized rats. Separate groups of rats were subjected to restraint stress treatments at intervals between 12:00 noon and 8:00 p.m. on the afternoon of proestrus. The effect of the stress was measured in terms of serum LH and prolactin concentrations in blood samples taken before and after the stress and the numbers of ova recovered from the oviducts on the expected day of estrus. A high correlation was found between serum LH and prolactin levels and the presence of ova in the oviducts. Restraint stress blocked the proestrous LH surge in rats that did not have high LH levels in their pre-treatment blood sample. Although restraint stress reduced serum LH as well as prolactin, most rats with these high initial LH levels ovulated normally. Stress did not affect the numbers of ova recovered in rats that ovulated following the stress treatments. There were no differences between stress effects on serum LH or prolactin and ovulation in intact and adrenalectomized rats. Additionally, cycle length had no effect on the stress treatment. These data indicate that stress-induced alterations in adrenal steroid or catecholamine secretion are not required for stress inhibition of pituitary LH release or ovulation.

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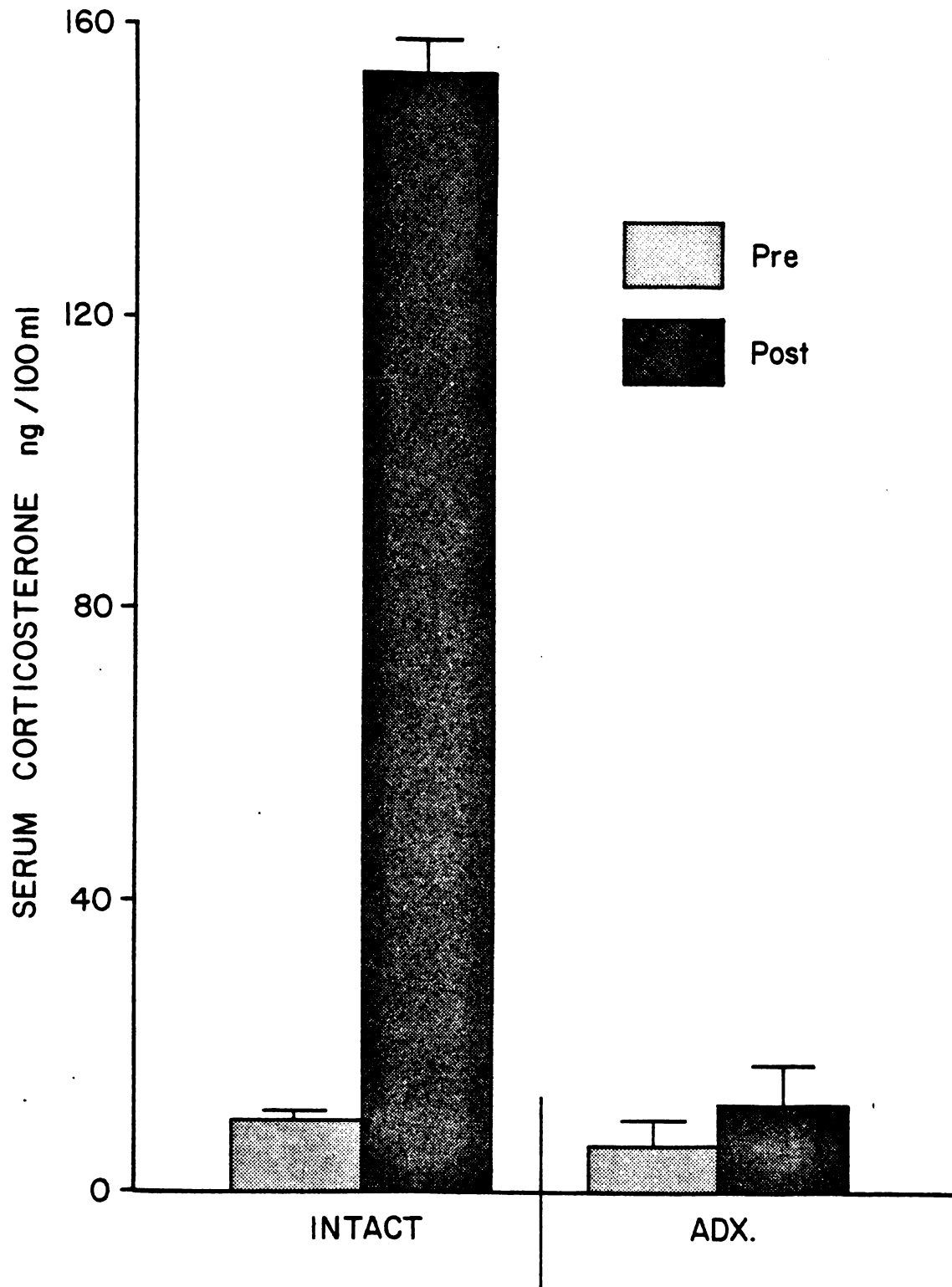
## Appendix A

Serum LH and PRL Collected Every Two Hours on the Afternoon  
of Proestrus from the Endocrine Research Unit Rat Colony.

<u>Time</u>	<u>Mean LH <math>\pm</math> S.E. ng/ml</u>	<u>Mean PRL <math>\pm</math> S.E. ng/ml</u>
2:00 p.m.	46.2 $\pm$ 9	124.6 $\pm$ 58
4:00 p.m.	737.4 $\pm$ 180	214.0 $\pm$ 46
6:00 p.m.	344.9 $\pm$ 143	186.6 $\pm$ 18

Appendix B. Serum corticosterone levels before and after restraint stress treatments in intact and adrenalectomized rats.

Group Mean corticosterone levels and the standard error of the mean are plotted. The data for the intact animals was abstracted from Riegler (1973) and Riegler and Hess (1972).



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