THE INFLUENCE OF THE PINEAL GLAND ON PUBERTY IN THE NEONATAL FEMALE RAT

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FEMALE RAT

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Figure Page

CHAPTER I

INTRODUCTION

Pineal studies have been divided into four historical periods. The first is that period of superstitious notions including both the Pre-Cartesian and the Post-Cartesian periods. The second is the period of preliminary studies. The third period covers the time when most of the studies on internal pineal function occurred and the fourth and last period includes the studies of effects of pineal function on the rest of the body.

Herophilus and Erasistratus, during the Pre-Cartesian superstitious period of pineal studies, around 300 B.C., first described the organ. It was regarded as the memory valve that controlled the flow of memories, stored in the rear of the brain ventricle and forward to the consciousness-serving part of the brain. Some authorities agreed with this idea, but the majority agreed with Galen who rejected the idea.

Descartes was known to be responsible for the next period of pineal superstition with his hypothesis. Descartes, before his death in 1650, gave a pleasing account of his psychological and physiological reasons for reviewing the pineal gland which he called the gland conarienne, as the "seat of the soul", "whence les esprits animaux" might diffuse themselves. As with every other hypothesis, some supported the notion and others disapproved.

These Cartesian superstitions persisted because leading practitioners such as Theophile Bonet of Geneva supported it. The first report of pineal disease in a patient with a mental disorder was published in 1686 by Dr. Edmund King. His patient suffered form schizophrenia and upon post mortem examination was found to have a petrified pineal gland. Bonet in 1700, published a book which discussed cases of pineal calcification in mental disorders. Arnold in 1786, Crichton in 1798, and Haslam in 1809 all wrote that some patients with mental disease had abnormally calcified pineal glands despite the insistence of anatomists such as van Soemmering and Baillie that pineal calcification was a normal finding. By 1830, careful statistical studies permanently disproved Bonet's idea. For centuries thereafter the pineal was thought to have no function.

Up to the beginning of this century, the pineal's possible function as a gland of internal secretion was entirely unsuspected. As early as 1900, there were published histological studies of the pineal, many of which were inaccurate. However, by 1923, F. K. Walter described a number of long club-shaped processes which he believed to be parenchymal cells of the pineal along with neuroglia cells. About the same time it was published that the sympathetic nervous system supplies its vessels.

In 1905, an extensive comparative study was done and reported that the pineal is only an evolutionary vestigium of a parietal eye still observable in certain reptilia (Berkeley, 1926). Based upon studies in 1921, on the cat and man, Tilney and Warren stated that the pineal follows the general lines of glandular differentiation, is a glandular structure and is in some way necessary to metabolism.

In all vertebrates the pineal originates as a neuroepithelial evagination protruding from the roof of the diencephalon. In man, this pineal anlage is first observed at the start of the second fetal month. The pineal in the adult human is a conical organ that lies within the posterior border of the corpus callosum and between the superior colliculi. It weighs about 120 mg and is mostly enveloped by pia mater from which blood vessels, unmyelinated nerve fibers and stroma of connective tissue penetrate the organ. The mammalian pineal is innervated almost solely by autonomic postganglionic fibers originating in the superior cervical ganglia. Postganglionic sympathetic fibers reach the pineal along its blood vessels or by coalescing into two unmyelinated nerves, the nervi conarii, which penetrates the distal pole of the organ. The sympathetic fibers terminate near the pineal parenchymal cells, and some actually form synapses. These nerve terminals generally exhibit the 400 Å. granular vesicles characteristic of sympathetic neurons elsewhere in the body (Williams, 1975).

Another peculiarity of the human pineal is its tendency to calcify. Shortly after birth, human pinealocytes begin to produce and secrete a ground substance of unknown chemical composition which serves as the matrix for subsequent calcification. The composition of the calcium salts deposited does not differ from those found in bone and tooth (Williams, 1975).

In 1920, Berblinger and Uemara found that the gland, if it had a secretion, is more important in the prepubertal period than later in life.

A few of the early theories on the function of the pineal gland were quite different and seemed humorous. W. Timme (Berkeley, 1926) offered evidence to prove that progressive muscular distrophy was a disease due to insufficiency of pineal secretions. Majendie's ancient

supposition that the pineal gland acts as a valve on the aqueduct of Sylvius, thereby regulating fluid pressure in the brain, was updated by Walter and Kolmer and Lowry. These three men affirmed the pineal to be a regulatory apparatus for the secretion of the liquor cerebrospinalis. Marburg suggested that the pineal gland was concerned with heat regulation (Berkeley, 1926).

In 1926, Berkeley published a book in which he drew the following conclusions concerning the pineal gland. He reaffirmed that the pineal gland is a gland of internal secretions; that in childhood and adolescence its secretions promote mental, bodily and sexual precocity; that it is not essential to life at any age, but it probably has some useful metabolic function throughout life. It bleaches the skin of frogs, acting like epinephrine and unlike pituitrin, in this regard (Berkeley, 1926).

There are two cardinal means of demonstrating glandular function: (1) by producing a deficiency state by excising the gland, or (2) by exaggerating the functional state by administering an extract of glands from the same or different animals. When these procedures were applied about a half century ago, the results were mainly inconclusive. Not until 1954 when Kitay and Altschule made their critical appraisal of the early work, did a renewed effort to unfold the mysteries of the pineal restart.

The second period, that of preliminary pineal studies began when the ablative approach became popular in endocrine research. The mortality rate was tremendous in such studies, therefore, the observations reported were based on small numbers. Nevertheless, there was vast literature by 1954 and an analysis of this material was made by Kitay and Altschule.

The third period, that of the study of internal pineal functions, has been made possible because of recent advances in biochemical methodology. This area appears to yield much information in the future. Wurtman, Axelrod, and Kelly (1968) summarized the present information on internal pineal function.

The fourth period involving evaluation of the biological role of the pineal gland has developed slowly. Now in the mid 1970's, indesputable evidence has been obtained. The pineal is a neuroendocrine transducer, it responds to photic stimuli, it biosynthesizes serotonin, melatonin, and melanotropic factors, it influences the pituitary-gonadal axis, it exhibits marked diurnal fluctuations in enzyme and indoleamine content, and most important, it appears to be the seat not of the soul but of a timing mechanism whereby the body can tell the time of day (Altschule, 1975). It is with the neuroendocrine aspect of the pineal and how this function relates to the onset of puberty which this paper deals.

CHAPTER II

LITERATURE REVIEW

As has been mentioned, Kitay and Altschule concluded in 1954 that, of the many investigations on pineal function so far performed, those showing an influence on sexual development and function were statistically most sound. Since then much work has been done in this field. The following review is divided into two sections. The first deals with the normal hormonal aspects of puberty in the female rat and the second portion discusses the influence of the pineal gland on reproductive physiology.

Hormonal Aspects of Puberty

It has been assumed since the early work of Price and Ortiz (1944) that the ovaries produce little or no steroids in the neonatal period. Recently, it has been possible to measure plasma estradiol (Meijs-Roelofs et al., 1973b; Weisz and Gunsalus, 1973; Ojeda et al., 1975) and more recently progesterone (Meijs-Roelofs et al., 1975; Dohler and Wuttke, 1974; Ramaley et al., 1975). During the neonatal period plasma progesterone begins to rise after day 10 (1 ng/ml) and reaches a peak value on day 29 (3 ng/ml) in rats. It then declines to intermediate levels through day 35 when it gradually, but significantly increases up to puberty. It appears that in the female rat, the gonads fully develop only after they have been exposed to relatively high titers of gonado-

tropins in the very early days of life. The pre-pubertal pattern of plasma LH is quite similar in both male and female in that the initial high levels at 10-12 days (400 ng/ml) begin to decline at about 15 days to minimal values (150 ng/ml) and remain low until the time of puberty when it reaches about 1500 ng/ml (Ojeda and Ramirez, 1972; Dohler and Wuttke, 1974; Ojeda et al., 1975). The peaks in the gonadotrophin levels seen at 12-13 days of age are of considerable interest. The nature of the triggering mechanism of these surges is not entirely clear. It is of interest to note that the peaks occurring at 12-13 days of age correspond well with the time of initial eye-opening. It could be suggested that light is the trigger of these surges acting through the pineal gland at this age. The pineal effect may be coupled with the negative feed-back effect of the gonadal steroids also released at this time (Eneroth et al., 1975).

Prolactin is low during development in the female rat (less than 50 ng/ml) (Voogt et al., 1970; Ojeda and McCann, 1974) but afternoon elevations appear shortly before vaginal opening and are maximal on the first proestrus day, at the time of the preovulatory surge of gonadotropins (200 ng/ml) (Ojeda and McCann, 1974; Dohler and Wuttke, 1974).

In the female, responsiveness to negative feedback of estrogen is present at an early age, but the responsiveness is less evident than that observed after the third week of life (Ojeda adn Ramirez, 1973; Ojeda et al., 1975). Whether there is a decrease in responsiveness around the time of puberty based on earlier studies (Donovan and Van Der Werff Ten Bosch, 1965; Ramirez and McCann, 1963) remains to be proven. The rate of metabolism of estradiol increases with development (Ojeda et al., 1975) and this could account for the apparent increase in threshold

for negative feedback around the time of puberty. It has been suggested that the preovulatory discharge of gonadotropin which occurs on the afternoon prior to the first ovulation in the pubertal female rat is brought about by an enhanced release of either FSH-RF and LH-RH (McCann, 1976). The fact that puberty does not develop in the female rat for an additional two weeks after ovulation induced by gonadotropins suggests that the ovary is not the primary site of the changes which bring about puberty (McCann, 1976). The priming action of LH-RH first observed around the time of proestrus in pubertal animals may be induced by endogenous estrogen which may magnify the preovulatory discharge of gonadotropins. It is possible that at about day 15, the high titers of progesterone act together with estrogen to suppress gonadotropin secretion (Meijs-Roelofs et al., 1975). Other possiblities are loss of the high affinity binding protein which would elevate free estradiol and thereby suppress gonadotropins by augmented negative feedback and lastly, that the sensitivity of the negative feedback increases at this stage (Ojeda and Ramirez, 1973; Ojeda et al., 1975). The decline in estrogen titers could be related to the switch of steroid production which takes place around 15-21 days of age. Frisch (1974) states that it is quite clear that puberty is more closely related to the attainment of the critical weight than to the chronological age of the animal.

However, Ojeda (1976a) found that the onset of puberty in the female rat is associated with an afternoon proestrus-like increase in circulating gonadotropin levels which preceeds the first ovulation. The surge in gonadotropin was accompanied by a similar striking increase in plasma prolactin titers. In 30 out of 33 rats these hormonal changes and the ovulatory process were closely related to vaginal opening, occurring in

the majority of the animals when the vaginal membrane had ruptured a few hours before. Beck et al. (1977) suggests that the steadily increasing prolactin levels inhibit pituitary LH release so that endogenous estradiol, which reach adult levels at this time cannot evoke an LH peak. Due to the limited duration shown in the present investigation, this inhibitory action of prolactin is probably no longer functional at the time of puberty. This desensitization of the inhibitory feedback action of prolactin thus, allow endogenous estrogens to induce a preovulatory LH surge. However, vaginal opening was not always preceeded by a gonadotropin discharge, suggesting that a more sensitive vaginal epithelium will result in an early vaginal opening which then will preceed the gonadotropin discharge.

Even though there was a dramatic increase in FSH and LH on the afternoon of proestrus, which was presumably brought about by the release of LH-RH, there was no decrease in the hypothalamic content of the neurohormone. The close relationship between increased gonadotropin levels, ovulation and vaginal opening indicates that gonadotropin release does not gradually rise as puberty approaches, but increases abruptly during a period of a few hours. This may be the reason that some authors fail to detect significant changes in plasma gonadotropin titers at the time of puberty.

The gradual elevation of circulating prolactin observed in the afternoons preceeding the preovulatory peak may be related to an enhanced sensitivity of the prolactin releasing apparatus to the stimulatory effect of estrogen (Ojeda and McCann, 1974) secreted in increased amounts by the ovaries and perhaps by the adrenals (Gorski and Lawton, 1973; Shaikh and Shaikh, 1975).

Since there is a gradual increase in estrogen secretion without a concomitant gradual increase in LH or FSH titers, it appears that the main factor responsible for triggering gonadotropin release in the pubertal rat is an activation of the positive feedback of estrogen and not a decreased sensitivity of the hypothalamic-pituitary unit to negative feedback action of this steroid.

Although a change in the threshold for the negative feedback of gonadal steroids has been seen, it is also apparent that the metabolic clearance of estrogen increases with development (DeHertogh et al., 1970) and could, in part, if not totally, explain the apparent change in the set point for feedback observed during the pubertal process.

Gonadal maturation may be a key factor in determining the initiation of puberty. The elevated plasma FSH titers present during the first three weeks of life (Ojeda and Ramirez, 1972) may act to sensitize the ovary to the small amount of circulating LH. An augmented ovarian responsiveness to gonadotropin stimulation would then result in follicullar development and increase estrogen release which would trigger the preovulatory surge of gonadotropins (Ojeda et al., 1976b). Also, interestingly enough, a decreased sensitivity of the hypothalamic gonadostat to ovarian steroids appears to be one of the predominant mechanisms that initiates puberty in the human female (Ojeda et al, 1976b).

The Influence of the Pineal on Reproductive Physiology

During the past 10-15 years many fascinating investigations have been performed on the structure, innervation, biochemistry and function of the mammalian pineal gland. As far as pineal function is concerned,

our present knowledge is not very detailed although it can be stated that the pineal gland is most active organ synthesizing a number of compounds. Its secretory products when released into the systemic circulation exert an effect on several endocrine organs probably via the hypothalamo-pituitary system. Pineal antigonadotropic function has been studied most extensively.

Removal of the organ, pinealectomy, under normal conditions is the most common way to establish the function of an endocrine organ, has not consistently furnished unequivocal results. In prepuberal rats exposed to normal conditions of illumination, pinealectomy resulted in an increase in the weight of the ovaries and the uterus while precocious opening of the vagina was observed (Simonnet et al., 1951; Kitay, 1954; Wurtman et al., 1959; Kincl and Benagiano, 1967; Reiter et al., 1970). Wragg (1967) criticizing the results obtained after pinealectomy, concludes that only within a restricted age range, i.e., operation 26 to 30 days and sacrifice at 50 to 54 days can reasonable evidence be obtained that pinealectomy induced ovarian hypertrophy. Sullens and Overholser (1941) also found no significant differences in the rate of development of pinealectomized rats as indicated by the time of hair appearance, eye opening, and time of sexual maturity.

It is now known that the amount of light to which an animal is subjected is related to pineal function, as it is to the development and function of the sex organs. The effect of long periods of either light or darkness on the primary and secondary sex organs of mammals was observed early. More recently, they could be related to pineal function. Prolongation of daily photoperiod or continuous light causes an increase in ovarian weight and precocious estrus in the rat (Fiske, 1941; Wurtman

et al., 1961; Fiske, et al., 1962). Pinealectomy in the prepuberal rat produces several alterations in gonad function which are similar to those observed in animals exposed to continuous lighting (eg. ovarian enlargement; and increased incidence of daily vaginal smears showing estrous phases). That the effects of light and pinealectomy are not additive suggests that both operate by reducing the availability of the same inhibitory compound (Wurtman et al., 1961). Under continuous lighting, pineal serotonin content, lipid content and pineal weight were found to be decreased (Fiske et al., 1960; Wurtman et al., 1961; Quay, 1961, 1962). It can be concluded that pineal function is generally inhibited in rats exposed to long periods of light, which evidently reaches the organ by way of the eyes, while the sex organs are stimulated. If immature albino rats are either deprived of light or rendered anosmic gonadal maturation is retarded slightly. Conversely, if weanling male or female rats (21 to 23 days old) have their eyes and their olfactory bulbs removed the reproductive organs remain significantly smaller than normal until the animals are at least 78 days old. The restraining influence on gonadal growth of this dual sensory deprivation is counteracted by pinealectomy. Reiter et al., (1969a, b) also found that newborn rats must be exposed to alternating light dark cycles for the first 20 days of life in order for light deprivation to delay the onset of puberty in female rats. He proposed that the pineal gland requires alternating period of light and darkness in early life before it can eventually become inhibitory to the reproductive system, a sort of pineal priming. However, in adult animals photic radiation entering the nervous system via the eyes appears to be the primary means whereby it can modulate the activity of the pineal gland. The only known exception is

in newborn rats where an extraretinal pathway for the control of pineal activity. This route, if it does exist, becomes obsolete after three weeks of age raising the question as to whether nursing rats may receive information about the photoperiod from their mothers. The retinal pathways mediating vision and pineal activity are separated at some point behind the chiasm.

Permanent illumination may lead to permanent estrus and vaginal cornification, without ovulation. Vaginal opening occurred earlier in intact animals kept in constant light than in those kept in an alternating photoperiod of (32.1 vs 38.1) (Ramaley and Bartosik, 1975). Exposure to continuous light has been shown to significantly accelerate sexual maturation in female rats. It appears that constant light may change the sensitivity of the hypothalamic-pituitary axis to estrogen feedback. However, increased daily light exposure does not result in increased basal levels of serum FSH and LH in immature female rats (Piacsek and Streur, 1975).

Prolongation of daily dark periods or exposure of the animals to constant darkness shows opposite effects (Reiter and Hester, 1966). Blinding or placing an animal in darkness is known to retard growth and increase the number of pituitary acidophils. This inhibitory influence of darkness on bodily growth is reversed by removal of the pineal gland. Pinealectomy alone has been observed to accelerate growth. Growth was diminished and pituitary GH levels were lowered by either blinding or olfactory bulb removal. With combined sensory deprivation, poor growth was observed and pituitary growth hormone levels were reversed when the rats were pinealectomized (Sorrentino and Reiter, 1971). Henzl et al., (1970) suggests that the epiphysis cerebri slows body growth. Advanced

bone maturation seen in pinealectomized animals would tend to support this hypothesis but periodic measurements of growth hormone would be needed to establish this with certainty. It appears possible that several observations on the effects of pinealectomy reported previously, i.e., earlier vaginal introitus, increased weights of ovaries, testes, seminal vesicles and ventral prostate may be a reflection of earlier maturation and faster body growth. Pinealectomized females were found to be heavier than control animals. Additionally, the mean time of vaginal opening in control animals was 41 days while in the operated females this occurred at 34 days. Depriving rats of light before puberty delayed pubertal onset and gonadal maturation only in those animals which had not been pinealectomized (Reiter, 1972, 1973). It then can be concluded that long periods of darkness generally stimulate the function of the pineal by way of the visual system thereby modulating the function of the sex organs.

Fraschini (1969) observed a significant increase in both LH and FSH in the anterior pituitary of pinealectomized rats. In the 3 week old rat pinealectomy resulted in significantly greater pituitary prolactin concentrations and lower plasma prolactin concentrations, as compared with sham-operated animals. The results here are probably best explained by an ability of the pineal to inhibit PIF (Relkin et al., 1972a, 1972b).

Administration of pineal extracts to normal maturing rats resulted in a significant decrease in ovarian weight while opening of the vagina was delayed. That the pineal gland probably exerts its antigonadotropic influence via the anterior pituitary was shown by Wurtman, et al., (1961) by administering pineal extracts to hypophysectomized rats in which normal ovarian size was maintained by injections of FSH. In these animals

the administration of pineal extracts did not show any effect while it did in non-hypophysectomized control animals.

Melatonin and 5-methoxytryptophol, the two methoxyindoles of pineal origin, when injected into the lateral ventricle of the brain of immature female rats resulted in significant delay of vaginal opening. In particular, melatonin has been shown to delay puberty in immature females, depress ovarian weights, and reduce the occurrence of estrus smears both in cycling and in constant estrous rats. Other pineal indoles such as serotonin, 5-methoxytryptophol, and 5-hydroxytryptophol are reported also to possess antigonadotropic activity (Reiter and Fraschini, 1969b).

Since previous experiments had shown that melatonin specifically inhibits secretion of LH and that 5-methoxytryptophol specifically blocks release of FSH, this supports the hypothesis that the onset of sexual maturation needs a balanced secretion of both gonadotropins (Collu, et al., 1971). When the secretion of either gonadotropin is inhibited puberty is delayed. Using ovarian growth and vaginal introitus as indices of puberty it was shown that pinealectomy advanced pubertal onset in female rats (Reiter, 1974; Kincl and Benagiano, 1967). It is also known that the onset of puberty is assisted by an adjustment of the hypothalamic set point to a higher level, thus, making it less sensitive to hormonal influences (Reiter, 1974).

As far as the probable regulation of the function of the anterior pituitary by the pineal gland and therefore possible pineal regulation of hypophyseotropic hypothalamic centers is concerned Clementi, et al., (1966) and Fraschini, et al., (1968) studied changes in the ultrastructure and gonadotropin content in the anterior pituitary after pinealectomy as well as the median eminence of the hypothalamus and into the midbrain

reticular formation. Their results seem to indicate that the pineal gland would exert an inhibiting influence on the hypophyseal gonadotropic cells via hypothalamic and even mesencephalic centers. The changes observed in the fine structure of gonadotropic cells induced by implants of pineal tissue or of melatonin were identical with those obtained by inhibiton of the synthesis and release of LH-RH and FSH-RH (Kappers, 1976). It is generally considered that the pituitary-gonadal axis of the maturing rat is more easily suppressed by the pineal or by pineal substances than is this system in adult animals. This difference is possibly due to the greater sensitivity of the hypothalamic feedback centers of immature animals to circulating hormones, both gonadal and extra-gonadal (Reiter and Sorrentino, 1971).

Recently serotonin containing nerve cells have been observed by Smith and Kappers (1975) in the nucleus arcuatus and in part of the nucleus ventromedialis of the hypothalamus which are known to be specially involved in the production of LH-RH. It was found that pinealectomy reduced the number of serotonin containing neurons in these nuclei, while their normal number was restored by administration of pineal extract.

Arginine vasotocin (AVT), an active octapeptide, has been demonstrated to be synthesized in the fetal pineal gland and perhaps the subcommisural organ, and that it is secreted from the pineal gland by ependymal secretory cells into the third ventricle. AVT concentration decreases with development which correlates with the regression of pineal secretory ependymal cells during development. Pavel et al., (1975) interpreted this to mean that AVT is most important in the fetal animal. He demonstrated that the stimulatory action of PMS on immature mice uteri and ovaries were inhibited by both pineal peptides and

and synthetic AVT, thus the inhibitory principle is identical.

Vaughan, et al., (1976) reported that single daily subcutaneous injections of AVT at day one throughout five after birth, however, resulted in significantly elevated testis and accessory organ weights at 30 and 60 days of age. By 90 days of age, the weights, gonads and accessory organs in the control animals were equivalent to that of AVT-treated groups. This was interpreted as indicating a possible early maturation of the hypothalamico-hypophysial-gonadal axis after AVT injection. It is felt that the action of AVT probably occurs at either the hypothalamus where it may modify Gn-RH release or at the pituitary where it may modulate synthesis and/or release of gonadotropins.

From bovine pineal extracts Benson, et al., (1971, 1972a, 1972b) isolated a pinal polypeptide showing a 60-70 times stronger antigonadotropic activity than melatonin. Two melatonin-free bovine pineal extracts, when administered to rats, inhibited compensatory ovarian hypertrophy and delayed vaginal opening time. Orts and Benson (1973) tested an aqueous extract of bovine pineal glands, partially purified by chromatography and ultrafiltration to exclude melatonin, for its ability to inhibit serum and pituitary LH in orchidectomized rats. The extract significantly inhibited the 24 and 96 hour postcastration rise of serum LH. Pituitary LH content and concentrations were reduced after 4 days of treatment with the extract.

Reiter (1976) presented a theory for cellular release of pineal polypeptides. In this scheme the pineal polypeptide hormones are exocytotically released from cells in conjunction with carrier proteins. The hormone is then exchanged for calcium resulting in the liberation of the hormone into the pineal capillaries and in the eventual deposition of calcium within the pineal gland.

CHAPTER III

MATERIALS AND METHODS

Animals

Male and female rats of the Sprague-Dawley strain were obtained from Charles River, Inc. These animals were mated at Oklahoma State University's facilities. When parturition occurred, the female neonates were randomly divided into the following treatment groups: Control (C), Constant Light (CL), Blinded (Bl), Pinealectomized (Px), Pinealectomized-Blinded (PxBl), and Sham-Pinealectomized (SPx).

Experimental Design

The experimental design used in this study is summarized in Figure 1. On day 0 (day of birth), the CL animals were placed in a room with constant fluorescent lighting and were maintained in this enviroment until sacrificed. On day 2, the Px and SPx groups were surgically treated. All groups were housed in an ambient temperature of 23 $^{\circ}$C and all except the CL groups were maintained under natural diurnal lighting of 14 hours of light and 10 hours of dark (14L:10D) with the dark phase of photoperiod beginning at 2000 hours and lasting until 0600 hours. All groups had free access to water and Purina Rat Chow. The experimental period extended from June to September. Each animal was weighed every five days and the weights recorded. The rats were weaned at 21

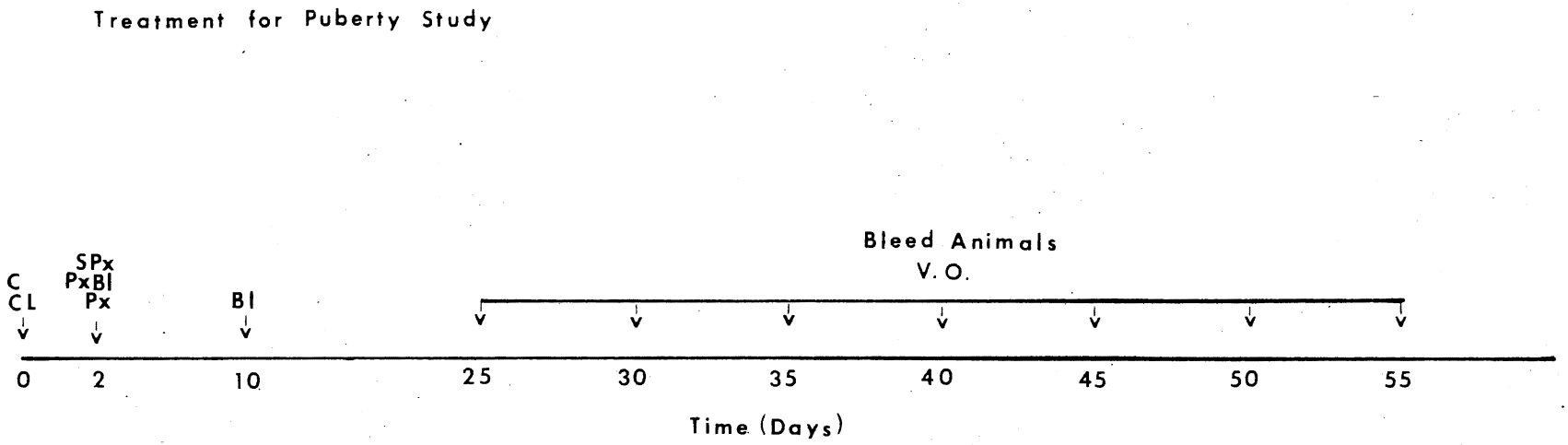

Figure 1. Experimental Design. Animals were placed in Constant Light (CL) on the day of birth (day 0), Sham-Pinealectomized (SPx), Pinealectomized-Blinded (PxBl) or Pinealectomized (Px) on day 2. Another group of animals were Blinded (Bl) on day 10, Control animals (C) and all others, except for the CL animals, were placed in a 14L:10D lighting regime. Animals from each group were observed for vaginal opening (VO), randomly sacrificed and bled every 5 days from day 25 through day 55.

days of age and placed 3-4/cage in a battery of cages. The animals were sacrificed at 5 day intervals from age 25 through 55 days at which time blood was collected by cardiac puncture. The blood was allowed to clot at room temperature and centrifuged. The serum was collected and sorted frozen at -20° C until the time of hormonal analysis. The animals were grossly inspected for completeness of pinealectomy when they were sacrificed.

Pinealectomy

At two days of age, the animals to be Px were anesthetized using hypothermia. The rats were placed individually in a glass container in a styrofoam chest containing dry ice until the animal was motionless. The operation site and the instruments were cleaned with alcohol. Two incisions were made forming a 45° angle, anterior to posterior, with the intersection at a point caudal to the confluence of sinuses on the dorsal aspect of the skull. At this point the pineal gland can easily be plucked from the area between the occipital poles of the cerebral hemisphere and the tectum of the midbrain. The simplicity of the operation is made possible because the pineal in the rodent is adherent to the undersurface of the confluence of sinuses and is attached to the epithalamus by only a tenuous stalk. An electrocautery knife was used to expose the area underlying the sinuses. A suction device was used to remove any tissue fluid or blood and to hold the flap of skin away from the exposed area. Hypothermia decreased the amount of bleeding. Using microforceps, the pineal body and stalk were gently removed. The skin was replaced over the exposed area, alcohol was applied to the surgical area and then several coats of diluted colloidon were applied

over the incision. This method proved to be more efficient than suturing and prevented the mother from opening the wound and destroying the neonate. The animals were warmed and returned to the nest. The sham operations were performed in the same manner with the exception that the glands were not removed. All the animals survived the surgery with the exception of one rat which was inadvertently left on the ice and two others were disposed of by the mother.

Optic Enucleation

At 10 days of age, the animals to be used for the Bl and PxBl groups were anesthetized using hypothermia as previously described. The eyelids were cut open with a scapel blade. Curved forceps were place behind the eyeball which was then gently removed from the orbit and the optic nerve then cut. The operation site was swabbed with alcohol to cleanse the area and to remove any blood. The animals were warmed and returned to the nest.

Vaginal Opening Time

Starting at day 25 and continuing through day 55, the females from each treatment group were randomly selected and observed for vaginal opening. This was the criteria used to determine the onset of puberty.

Hormone Assays

Serum samples were assayed for LH, prolactin and progesterone by radioimmunoassay (RIA). The RIA for LH and prolactin was performed in triplicate in 10 X 75 mm disposable culture tubes. The procedures utilized for the LH assay were those described by Niswender (1968) in which

an ovine: ovine double antibody system is used. Anti-ovine LH (AOLH) and purified LH for iodination was provided by Dr. Gordon Niswender and Dr. Leo Reichert, Jr., respectively. To each tube was added 300 µl of 1% BSA-PBS (pH 7.6), 200 µl AOLH (pH 7.6), 100 µl of LH-^{125}I and 200 µl anti-rabbit gamma globulin (ARGG). The tubes were incubated between each addition at 4°C and on the 6th day the tubes were centrifuged at 5°C at 2000 rpm for 20 min. The tubes were decanted and the tube containing the bound fraction was counted on an automatic gamma counter. The concentrations are expressed in terms of NIAMD Rat LH RP-1 having a biological potency of 0.03 X NIH LH-S1 ovarian ascorbic acid depletion (OAAD assay). Data points were plotted using a standard curve which was analyzed by a programmable Olivetti P652. The second antibody, anti-rabbit gamma globulin, was produced in sheep at Oklahoma State University.

The RIA for prolactin was analyzed using a double antibody technique similar to that of LH. Anti-rat prolactin, purified rat prolactin for iodination and reference preparation were all supplied by NIH. All values were expressed in terms of NIAMD Rat Prolactin RP-1. The same solutions used in the LH assay were added to each assay tube except that only 200 µl of 1% BSA-PBS, 100 µl of serum and 100 µl of Prl-^{125}I were added.

Progesterone was assayed using a modification of an extraction method described by Smith and Hafs (1973). Tritiated progesterone (3000 cpm) was added to the sample prior to extraction for recovery estimation. Progesterone was extracted twice from serum samples (100 µl-500 µl) with 2 ml of diethylether. The extract was applied to a Sephadex LH-20 column (0.9 g Sephadex LH-20 in a 10 ml syringe),

previously washed with benzene: methanol (90:10) and eluted with iso-octane:benzene:methanol (62:20:18). The first 4 ml were collected, evaporated and reconstituted with methanol. Five hundred microliter aliquots for recovery and duplicate aliquots of 100 µl and 200 µl were assayed for each serum sample. To these evaporated samples, 100 µl of progesterone antisera (provided by Dr. G. Abraham), 100 µl Prog-^{3}H (20,000 cpm) and 100 µl progesterone buffer were added. The tubes were incubated at 4°C for 18 hours. A dextran charcoal solution was added, incubated another 20 min. and then centrifuged for 10 min. at 5°C at 2000 rpm. The solutions were decanted into scintillation vials and 10 ml scintillation cocktail containing PPO and toluene were added to each vial. The bound fraction in each vial was then counted for 10 min. on a Packard Gamma Counter. The data was analyzed by the same method as the LH and prolactin samples. Each sample was then corrected for procedural losses by dividing the total recovery by the individual recovery.

Statistical Evaluation

The data were analyzed for statistical significance by analysis of variance, the Student's t-test and Duncan's new multiple range test, (Steele and Torrie, 1960).

CHAPTER IV

RESULTS

The results of this study, which was designed to test the possible role of the pineal gland in the control of the onset of puberty in the female rat, is presented in two sections, (see Figure 1 for experimental design). The first section deals with the effects of the pineal gland on vaginal opening time and body weight and in the second the data concerning the circulating levels of the hormones, LH, prolactin, and progesterone is presented. Experimental groups of animals are designated Control (C), Pinealectomized (Px), Pinealectomized-Blinded (PxBl), Constant Light (CL), Sham-Pinealectomized (SPx), and Blinded (Bl).

Effects of the Pineal on Body Weight and Vaginal Opening Time

Effects on Body Weight

After weighing each animal every five days, it was found by analysis of variance, that there were no significant differences in the comprehensive weights between the various treatments (Table I). The mean weight for the C animals was slightly higher because this was calculated starting on day 35 as opposed to day 25 when weighing of the treated groups commenced. However, Px animals had a decrease in overall weight at 45 days of age and the CL animals showed a drop in weight at 50 days

TABLE I

THE EFFECT OF TREATMENT ON BODY WEIGHT

Experiment	N	Mean ± S.E. (g)	F	P of greater value
C	12	144.89 ± 8.34	1.23	N.S.
Px	18	114.52 ± 11.99		
Bl	13	123.31 ± 10.55		
PxBl	7	133.34 ± 10.87		
CL	14	116.94 ± 10.94		

N.S. - no significance
(refer to text for legend of abbreviations)

of age. The other treatments caused a steady increase in body weight up to the time of sacrifice (Figure 2). An analysis of variance between treatments on each of the five day intervals showed a significant difference only on day 35 ($P < .05$). When a Duncan's New Multiple Range test was performed on this data, it was determined that there was a significant difference ($P < .05$) between Bl and CL animals; Bl and C animals; Px and CL animals; Px and C animals and between PxBl and CL animals (Table II).

Effects of Vaginal Opening Time

The vaginal opening (VO) times ranged from 34.1 days in the Px animals to 41 days in the Bl animals. The mean VO time for the control animals occurred on day 40.2. Bl animals had the least statistically significant ($P < .1$) vaginal opening time when compared with C animals and were the only animals to exhibit a delayed VO time when compared to C animals. Animals which were Px and those which were place in CL had the most significant VO times with a confidence level of 99.9% followed by PxBl animals as found by the Student's t-test ($P < .01$) (Table III and Figure 3). An anlysis of variance of the data from all groups indicated there was a significant difference ($P < .005$) between all groups. Performance of Duncan's New Multiple Range test on this data indicated a significant difference ($P < .05$) between Bl and Px animals; Bl and CL animals; C and Px animals; PxBl and Px animals; and between CL and Px animals (Table III).

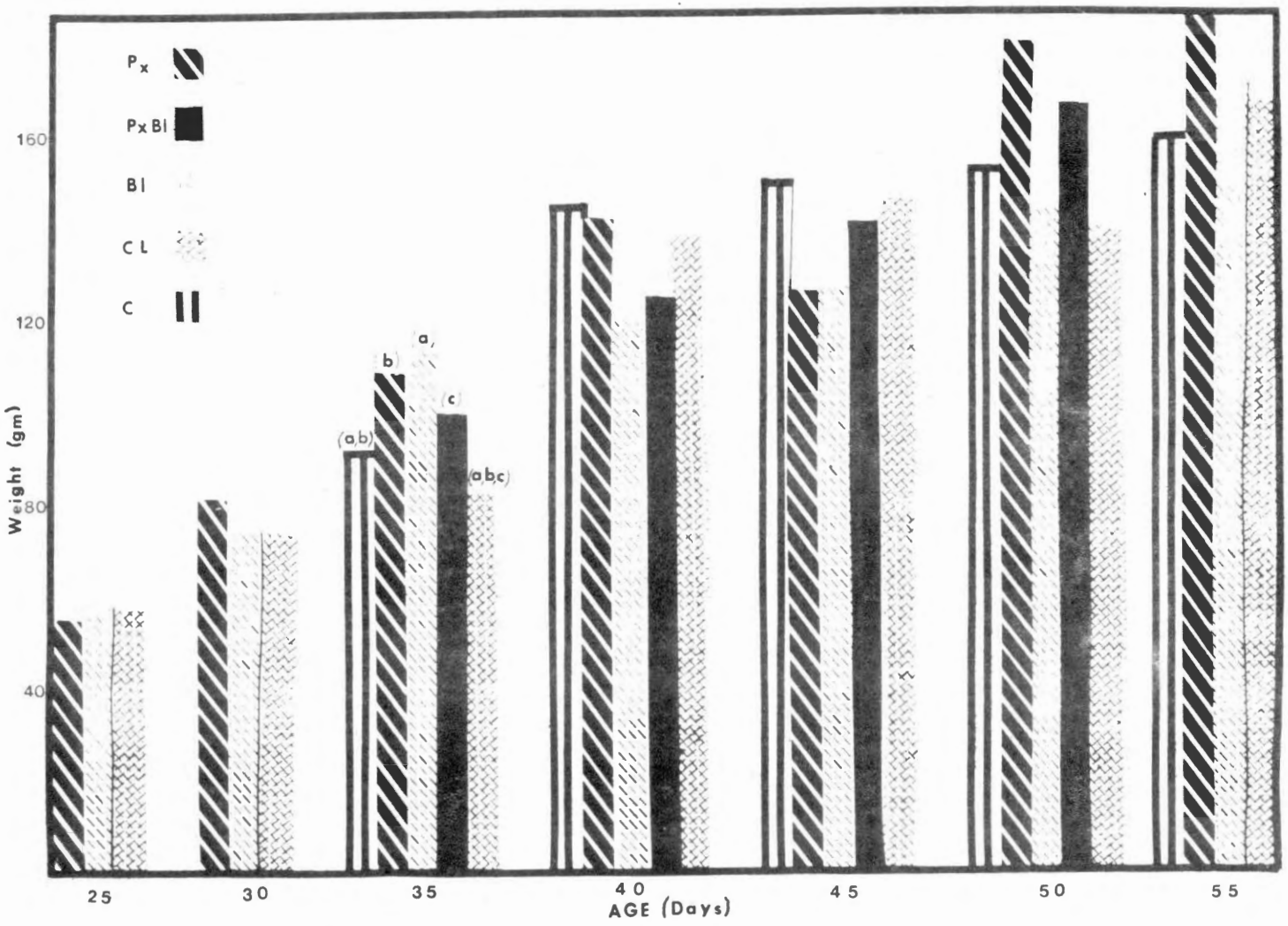

Figure 2. Body Weight Versus Age In Prepubertal Female Rats. The bars represent the mean body weight of each group over a 30-day period. On day 35, the like letters in parentheses represent groups which are statistically different (refer to text for legend of abbreviations).

TABLE II

INFLUENCE OF PINEAL ON BODY WEIGHT
IN PREPUBERTAL FEMALE RATS

Days of Age	n	Total SS	Treatment SS	Error SS	F Value	P
25	7	41.44	22.29	19.15	2.33	N.S.
30	7	185.45	64.17	121.28	1.06	N.S.
35	11	2253.83	1525.68	678.15	3.49	.05
40	11	3905.39	1137.91	2767.48	0.62	N.S.
45	9	804.45	584.09	220.35	2.65	N.S.
50	10	3967.39	1742.12	2225.27	0.98	N.S.
55	9	1963.63	733.28	1230.35	0.99	N.S.

CL	C	PxBl	Px	Bl
83.09	89.84	100.80	109.80	113.55 **

Values of $P_{.05}$	2	3	4	5
SSR	3.15	3.30	3.37	3.47
R_p = LSR	12.98	13.60	13.88	14.13

** Determined by Duncan's New Multiple Range Statistical Analysis where any two numbers underlined are not statistically significant
(refer to text for legend of abbreviations)

TABLE III

INFLUENCE OF PINEAL ON VAGINAL OPENING TIME
IN PREPUBERTAL FEMALE RATS

Experiment	n	Mean ± S.E. (Days)	F*	t**	P of larger value
C	30	40.25 ± 0.2780	67.58		.005
Px	67	34.10 ± 0.3990		12.65	.001
Bl	61	40.83 ± 0.24		1.70	.1
PxBl	38	39.00 ± 0.33		2.89	.01
CL	66	37.90 ± 0.33		5.45	.001

* F values determined by Analysis of Variance.
** t values determined by Student's t-test.

Px	CL	PxBl	C	Bl
34.10	37.90	39.00	40.35	40.88 *

Value of $P_{.05}$	2	3	4	5
SSR	2.80	2.95	3.05	3.12
R_p	2.60	2.75	2.85	2.91

* Determined by Duncan's Multiple Range Statistical Analysis where any two numbers underlined are not statistically significant.
(refer to te t for legend of abbreviations)

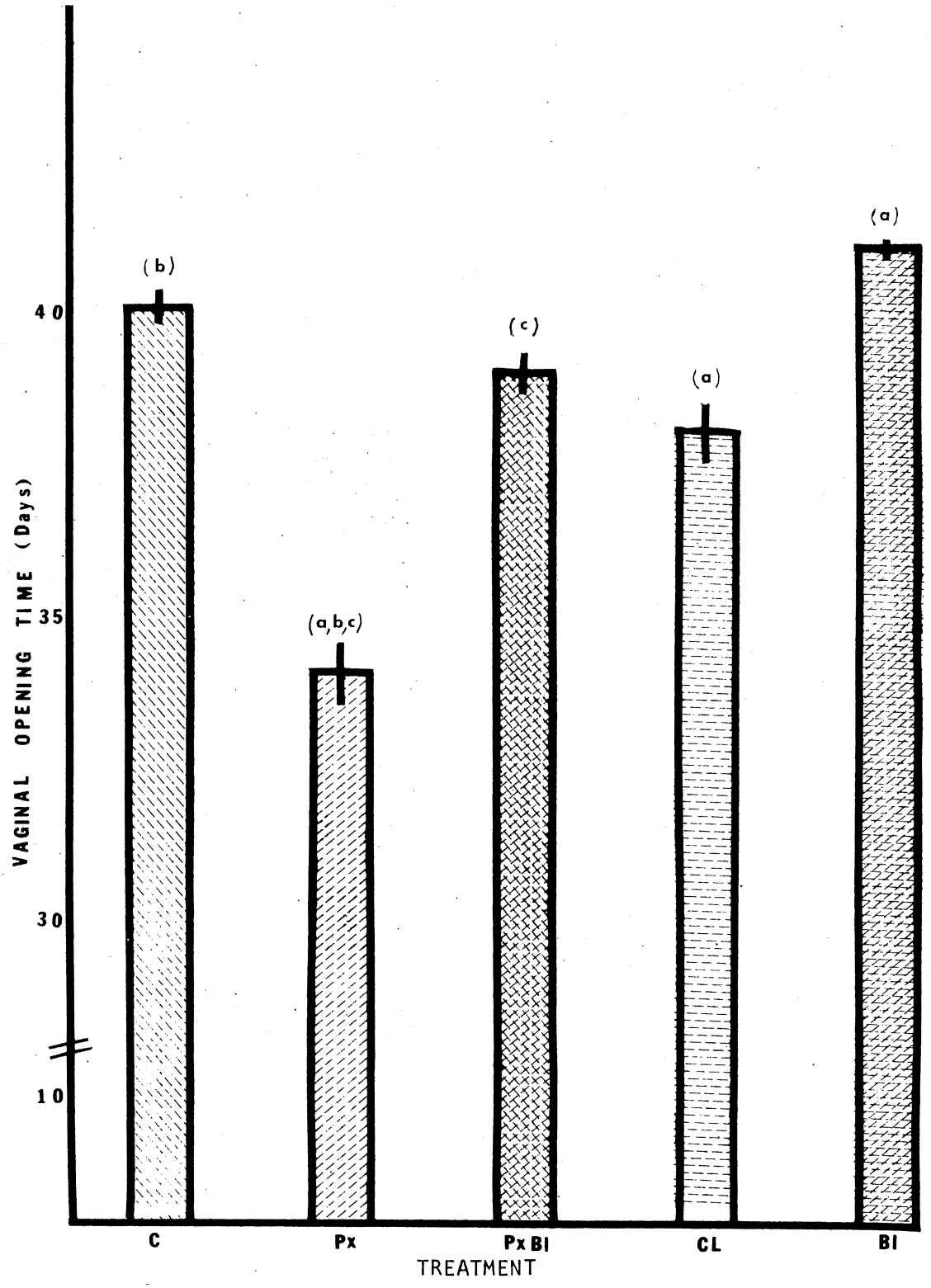

Figure 3. Vaginal Opening Times Versus Treatments. The like letters in parentheses represent significant differences between treatment and the unlike groups are not significant. The vertical lines indicate the standard errors. See text for explanation of abbreviations used for the treatments.

Effects of the Pineal on Circulating Levels of Gonadotropic Hormones

Effects of Treatment on LH

The effects of the various treatments on serum LH concentrations are summarized in Figures 4 and 5. There is a significant difference between the treatments ($P < .05$) whereas, there was no significant difference between the C group and SPx group of animals, (Figure 5, Table IV). It was found that the mean concentration of serum LH in the Px group of animals was significantly ($P < .05$) greater than that of the C animals. In C animals the serum LH peak occurred at day 40, coinciding with the day of vaginal opening time. Animals placed in CL or those which were Bl displayed a peak concentration of serum LH at day 35, whereas in Px and C animals this occurred at day 40. In PxBl animals, the peak LH concentration was delayed until day 45, (Figure 11). The SPx group of animals showed no distinct peak of LH concentration between days 25 and 55. There were significant differences in LH values between days within each group at the time of each peak, which are summarized in Table V. There was a 95% confidence level between groups on day 40 and on day 45. By day 50, there was a significant difference ($P < .005$) between, 1) Px and C animals, 2) Px and CL animals, 3) Px and PxBl animals and 4) Px and Bl animals (Table VI).

Effects of Treatment on Prolactin

The effects of the various treatments on circulating prolactin levels are depicted graphically in Figures 6 and 7. C animals displayed a peak prolactin concentration on day 40. A distinct peak in serum

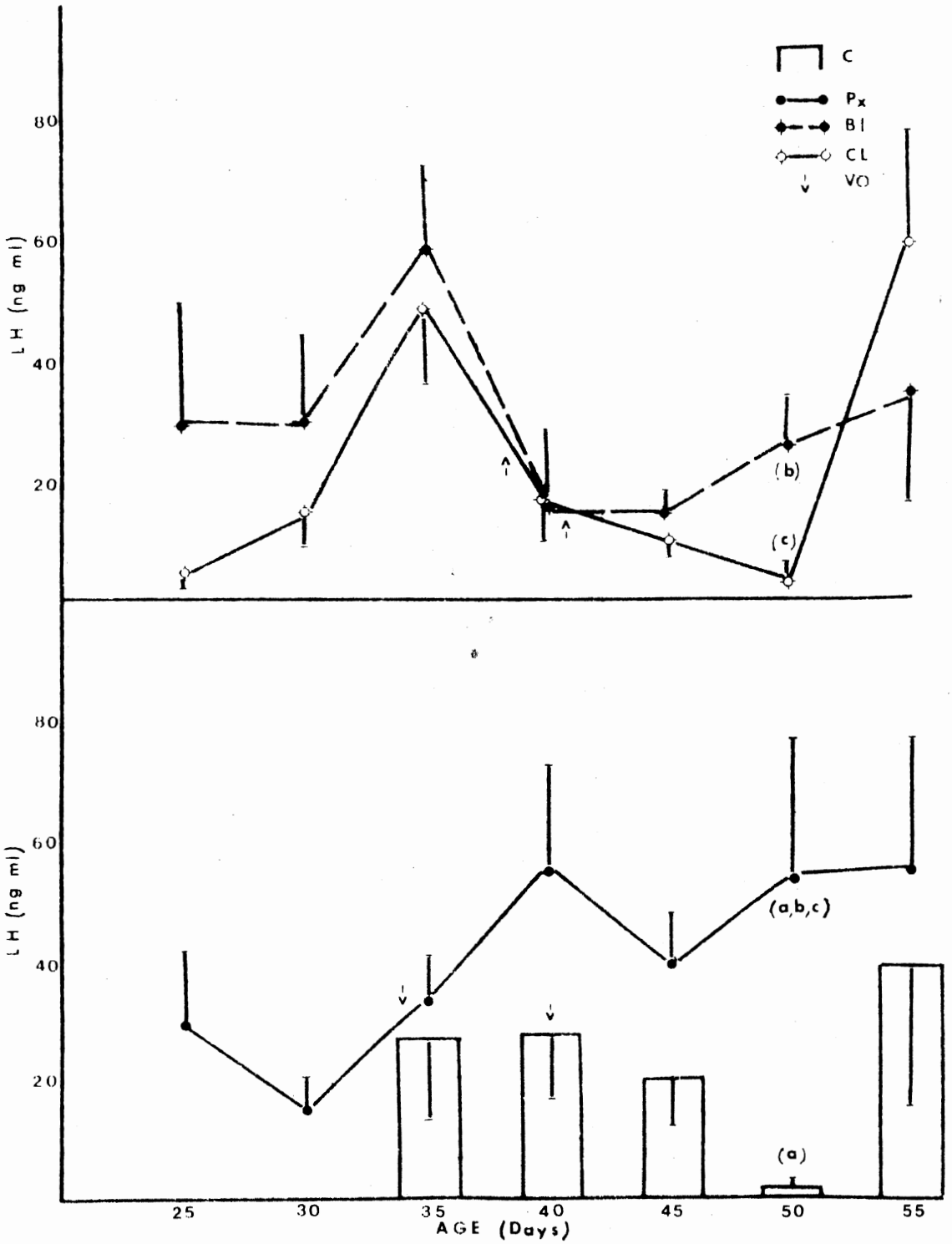

Figure 4. Changes of Serum LH Concentrations as A Function Of Age In Immature Female Rats. The like letters in parentheses represent significant differences in LH concentration, whereas unlike letters are not significantly different. The vertical lines indicate standard error. The arrows indicate vaginal opening time, (refer to text for legend of abbreviations.)

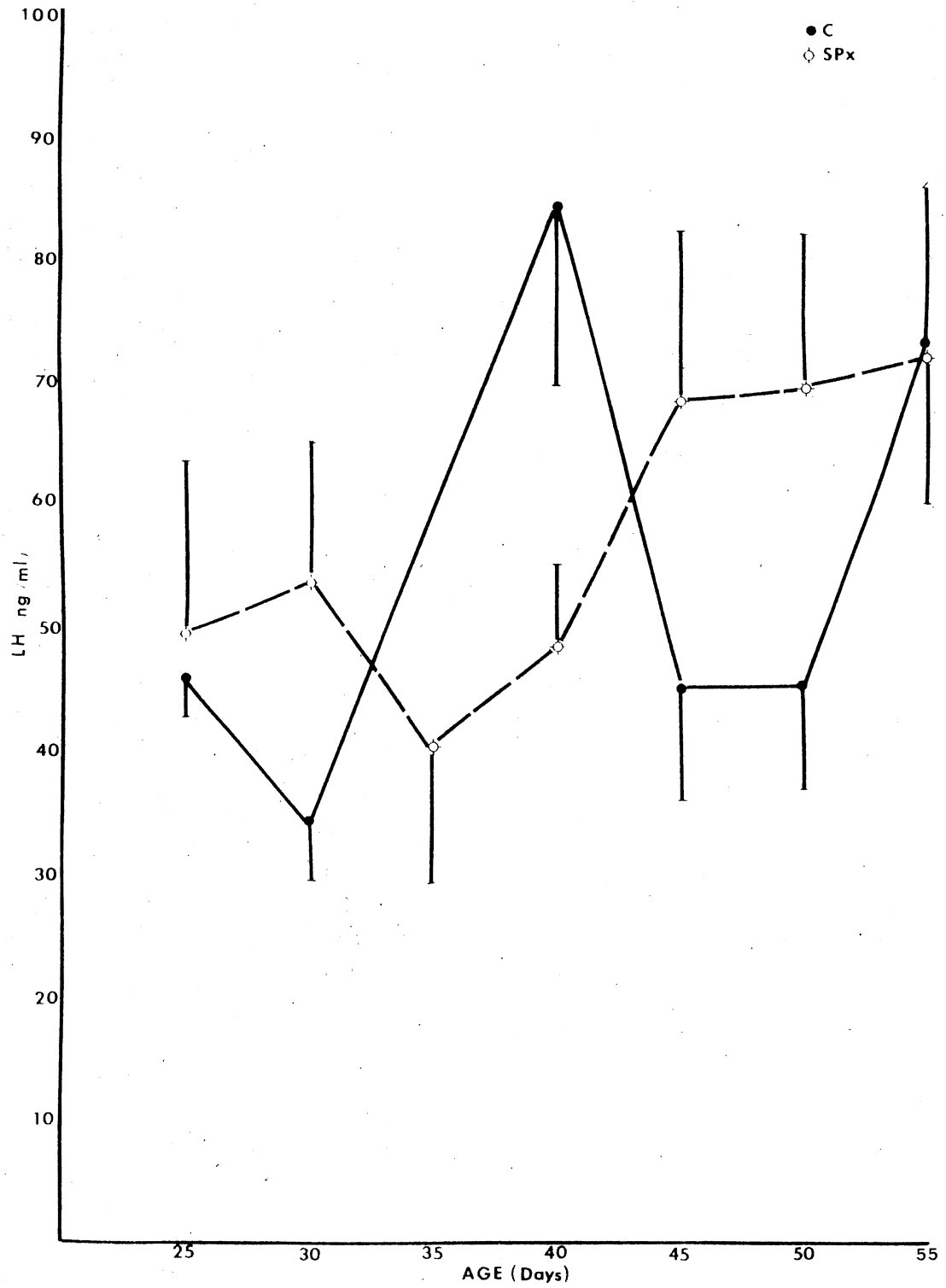

Figure 5. Changes In Serum LH Versus Age. The vertical lines represent standard error (refer to text for legend of abbreviations).

TABLE IV

INFLUENCE OF PINEAL ON SERUM LH IN PREPUBERTAL FEMALE RATS

Treatment	n	Means (ng/ml)	S.E.*	F**	P of larger values
Px	69	26.76	5.00	2.55	.05
Bl	60	27.42	11.90		
C_1	40	22.38	6.05		
PxBl	8	19.00	6.54		
CL	76	19.77	3.46		
C_2	80	57.09	5.63	0.07	N.S.
SPx	77	55.159	5.07		

C vs Px	n = 109	t = 1.8323	P < .05
C vs Bl	100	.3776	.5
C vs PxBl	48	.3796	.5
C vs CL	136	.3747	

* 30 day mean

** Among treatment F value as determined by Analysis of Variance. (refer to text for legend abbreviations)

TABLE V

IN TREATMENT VARIATIONS OF LH LEVELS BY t-test

Treatment	Age (days)					
	25-30	30-35	35-40	40-45	45-50	50-55
Px	0.9588 N.S.	2.0142 P < .05	1.2334 N.S.	0.8962 N.S.	0.6305 N.S.	0.0258 N.S.
Bl	0.0806 N.S.	1.6528 N.S.	4.1552 P < .001	0.3195 N.S.	1.7551 P < .1	0.3965 N.S.
C	1.5504 N.S.	3.0830 P < .01	0.0939 N.S.	2.174 P < .05	2.2365 P < .05	1.6775 N.S.
PxBl				0.3827 N.S.	1.0308 N.S.	
CL	1.6568 N.S.	2.6866 P < .02	1.9848 P < .1	0.6551 N.S.	1.9580 P < .1	2.8157 P < .01
SPx	0.2242 N.S.	0.7815 N.S.	0.6084 N.S.	0.6209 N.S.	0.0617 N.S.	0.1904 N.S.

N.S. - no significance
(refer to text for legend abbreviations)

TABLE VI

THE EFFECTS OF TREATMENT ON SERUM LH VALUES AT VARIOUS AGES

Age (days)	n	Total SS	Treatment SS	Error SS	F	P value
25	29	41,2205.93	4,127.21	37,078.72	1.45	N.S.
30	40	24,703.43	1,495.01	23,208.43	1.19	N.S.
35	43	76,139.64	7,283.05	68,856.59	1.38	N.S.
40	37	33,328.89	7,972.19	25,356.7	2.52	.05
45	45	23,326.25	5,075.51	18,250.74	2.78	.05
50	37	23,271.42	11,747.16	11,523.65	8.16	.005
55	21	49,870.93	2,034.77	47,836.16	0.24	N.S.

C	CL	PxBl	Bl	Px
1.25	4.66	8.25	27.92	55.14

*

Values of $P_{.05}$	2	3	4	5
SSR	2.89	3.04	3.12	3.20
R_p = LSR	25.87	27.21	27.92	28.64

N.S. - no significance (refer to text for legend of abbreviations)

* - Determined by Duncan's New Multiple Range Analysis where any two numbers underlined are not statistically significant.

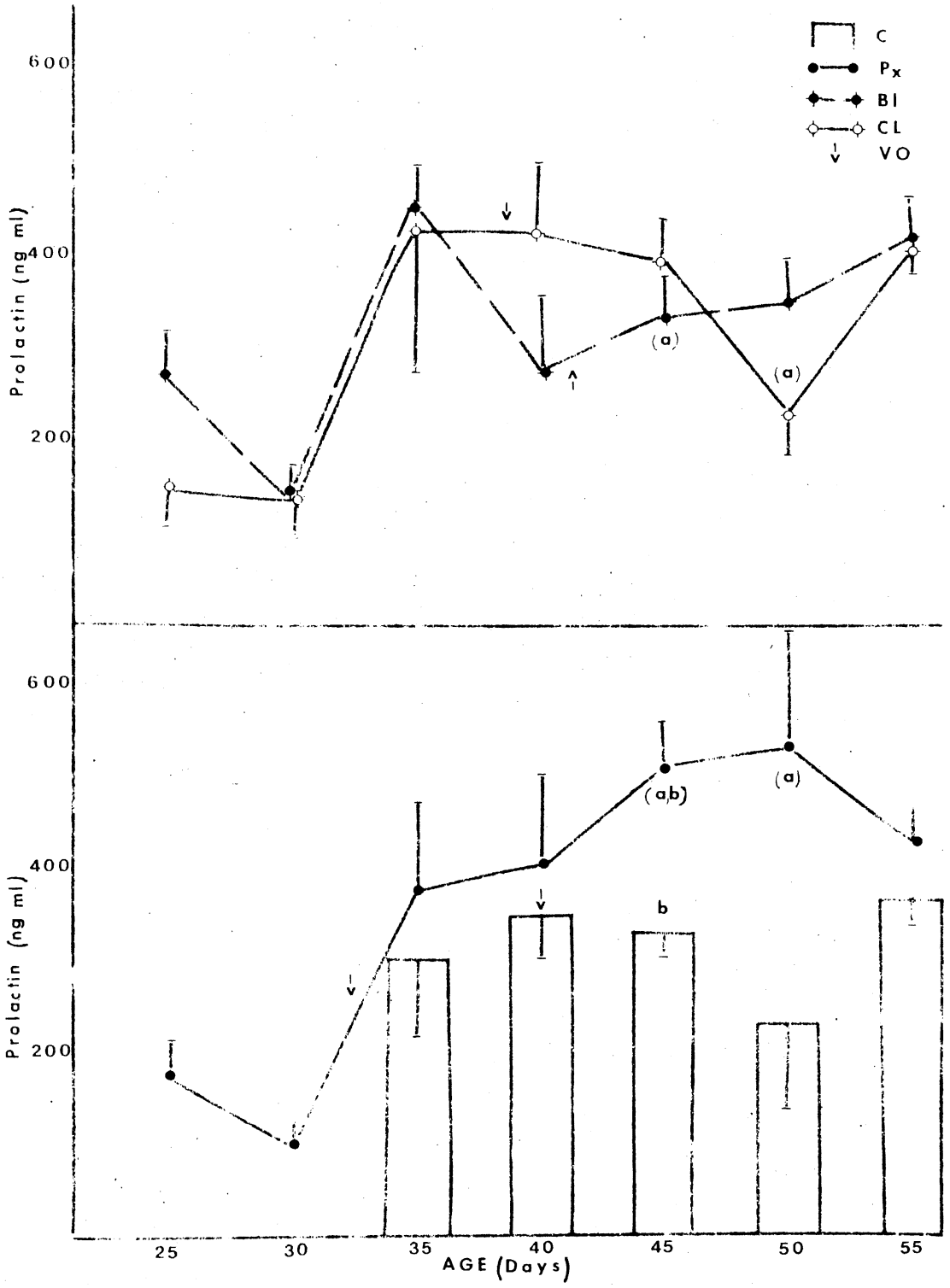

Figure 6. Changes in Serum Prolactin as a Function of Age In Immature Female Rats. The letters that are similar represent significant differences. The arrows indicate vaginal opening and the vertical lines are the standard errors. (refer to text for details).

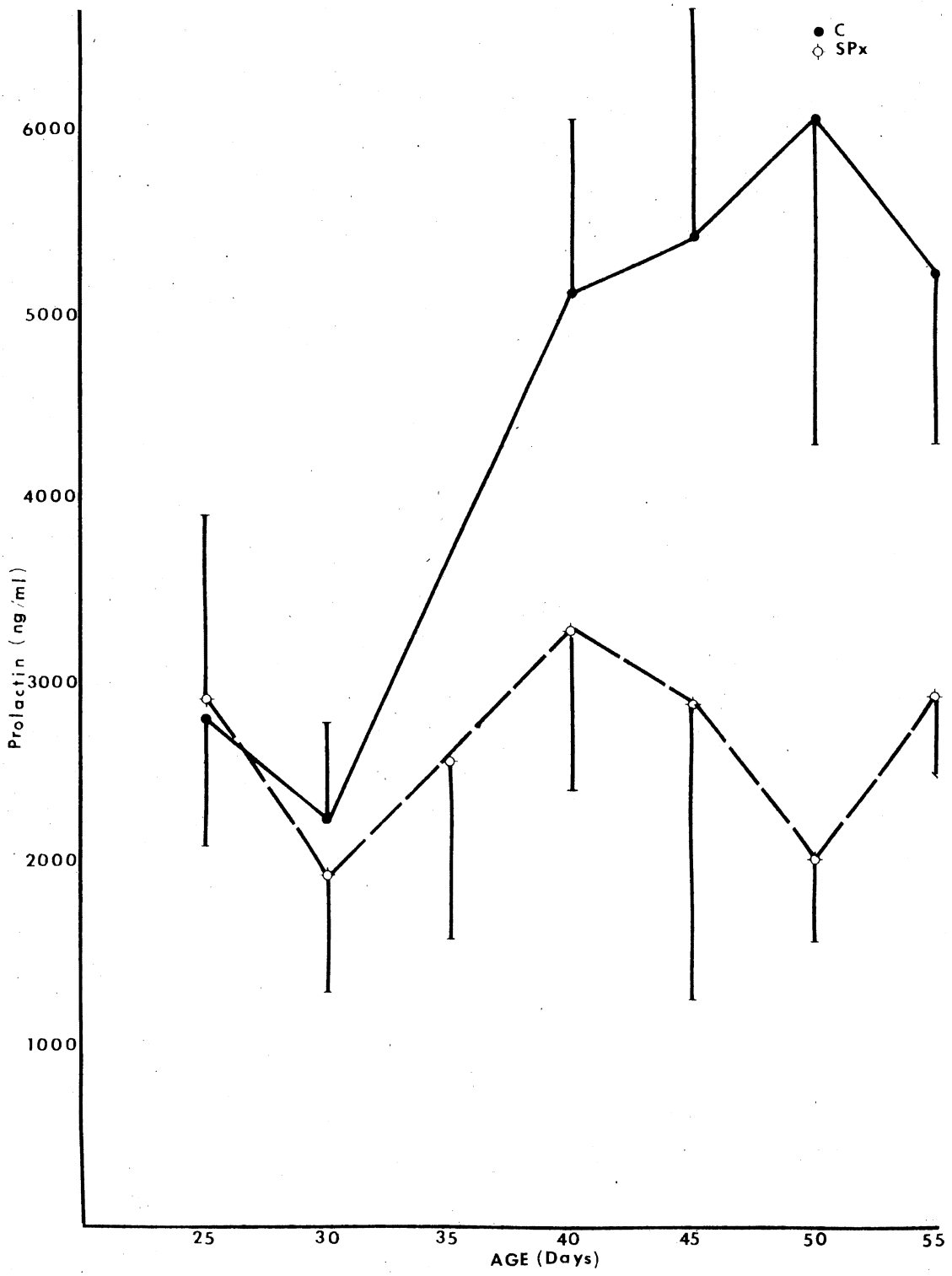

Figure 7. Changes in Serum Prolactin Versus Age In Immature Female Rats. The vertical lines represent standard error.

prolactin concentration was observed at day 35 in animals which were blinded. In the animals housed in CL there was a sharp rise in serum prolactin at day 35 and continued at this level for another 10 days, at which time the concentration began to decrease. Serum prolactin in the Px group sharply rose between days 30 and 35 and continued to rise until day 50. The PxBl group of animals had a prolactin peak on the same day as the LH peak, day 40. The PxBl group of animals was the only group which was significantly differenct from the C group, ($P < .02$) (Table VII, Figure 11). All of the groups, except SPx animals, exhibited a significant change in prolactin concentration over the 30 day interval, (Table VIII). All of these significant differences were observed around the time that prolactin reached its optimal concentration. As with LH concentrations, prolactin values in CL and Bl animals exhibited a peak at 35 days or 5 days earlier than the C animals. Prolactin concentrations displayed a significant difference among groups only on day 45 ($P < .01$), (Table IX). On day 45, the significant differences were between Px and CL animals; and C animals and Px; and CL animals had shown a significant difference on day 50 (Table IX).

Figures 8-13 represent the same data as described in preceeding paragraphs but depict the serum prolactin and LH concentrations in individual treatments so that the time at which the peak hormonal concentration occurs, may be compared. Serum prolactin and LH concentrations of C animals tend to be parallel throughout the observation period, each peaking at day 40 (Figure 8). The mean hormone values for the SPx group seem to be reciprocal to each other in-as-much-as when LH is at its optimum, prolactin is at a basal level, with the exception occurring between days 35-40 (Figure 9). Figure 10 illustrates

TABLE VII

INFLUENCE OF PINEAL GLAND ON SERUM PROLACTIN
IN PREPUBERTAL FEMALE RATS

Treatment	n	Means ± S.E.* (ng/ml)	F	P of larger values
Px	60	347.57 ± 28.05	2.67	.025
Bl	60	330.70 ± 21.57		
C_1	37	305.02 ± 35.31		
PxBl	7	422.54 ± 28.85		
CL	73	262.30 ± 20.66		
C_2	83	4062.81 ± 449.23	2.73	0.1
SPx	80	2660.25 ± 312.83		
C vs Px	n = 97	t = .9403	N.S.	
C vs Bl	97	.6181	N.S.	
C vs PxBl	42	2.5686	P < .02	
C vs CL	110	1.0399	N.S.	

N.S. - no significance (refer to text for details).
* 30 day mean

TABLE VIII

IN TREATMENT VARIATIONS OF PROLACTION LEVELS BY t-test

Treatment	Age (days) 25-30	30-35	35-40	40-45	45-50	50-55
Px	2.2155 P < .05	2.9905 P < .01	0.2114 N.S.	1.0800 N.S.	0.0546 N.S.	0.6584 N.S.
Bl	2.0824 P < .1	6.3710 P < .001	2.0212 P < .05	0.8639 N.S.	0.1607 N.S.	1.5259 N.S.
C	0.5855 N.S.	2.2589 N.S.	0.4658 P < .05	0.4537 N.S.	0.9784 N.S.	1.5132 N.S.
CL	0.1409 N.S.	1.7943 P < .1	0.0256 N.S.	0.3013 N.S.	4.7184 P < .001	7.4109 P < .001
PxBl	-	-	-	1.1223 N.S.	3.4759 P < .05	-
SPx	0.8929 N.S.	0.6029 N.S.	0.5889 N.S.	0.1762 N.S.	0.5085 N.S.	1.2685 N.S.

N.S. - no significance (refer to text for details).

TABLE IX

THE EFFECTS OF TREATMENT ON SERUM PROLACTIN VALUES AT VARIOUS AGES

Age (days)	n	Total SS	Treatment SS	Error SS	F	P values
25	21	208,515.94	45,339.64	163,176.31	2.50	0.1
30	32	270,319.81	8,343.89	261,975.92	0.46	N.S.
35	38	1,923,258.83	153,471.82	1,769,787.02	0.98	N.S.
40	38	1,801,500.99	145,599.94	1,725,901.05	0.70	N.S.
45	48	8,814,471.99	33,503.51	482,968.48	7.38	.005
50	40	1,369,695.49	417,635.70	951,059.80	3.84	.01
55	23	129,595.42	10,470.35	119,125.07	0.56	N.S.

Day 45

C	Bl	CL	PxBl	Px
322.76	344.30	399.80	490.00	512.00

*

Value of $P_{.05}$	2	3	4	5
SSR	2.86	3.01	3.10	3.17
R_P	143.4	150.95	155.46	158.97

Day 50

CL	C	Bl	PxBl	Px
218.20	235.30	352.45	406.27	520.43

Value of $P_{.05}$	1	3	4	5
SSR	2.86	3.01	3.10	3.17
R_P	223.30	235.02	242.04	247.51

N.S. no significance (refer to text for legend of abbreviations). * - Determined by Duncan's New Multiple Range Analysis and any two numbers underlined are not statistically significant.

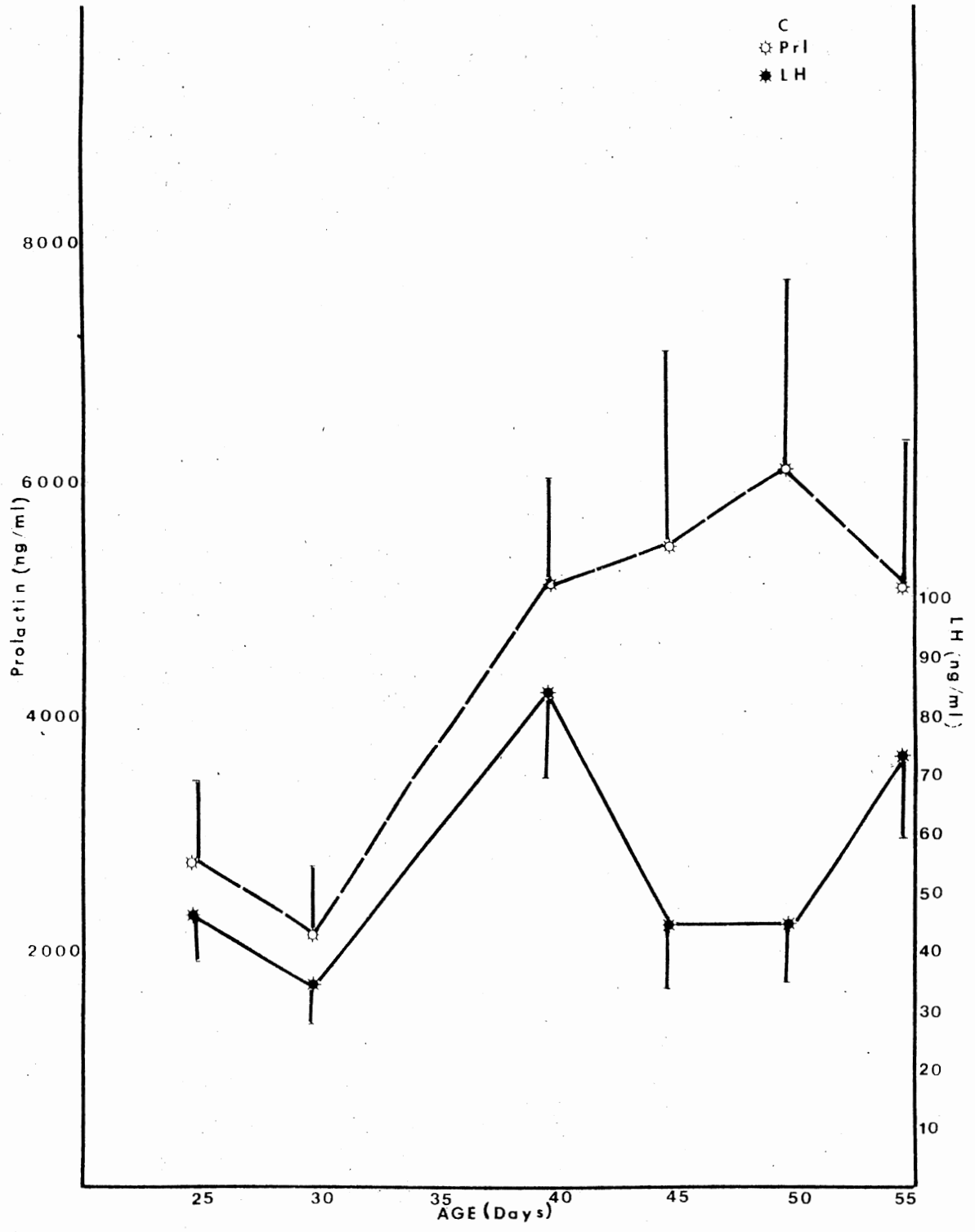

Figure 8. Changes in Serum Prolactin (left ordinate) and Serum LH (right ordinate) as a Function of Age in Immature Female Control Rats. The vertical lines represent standard error.

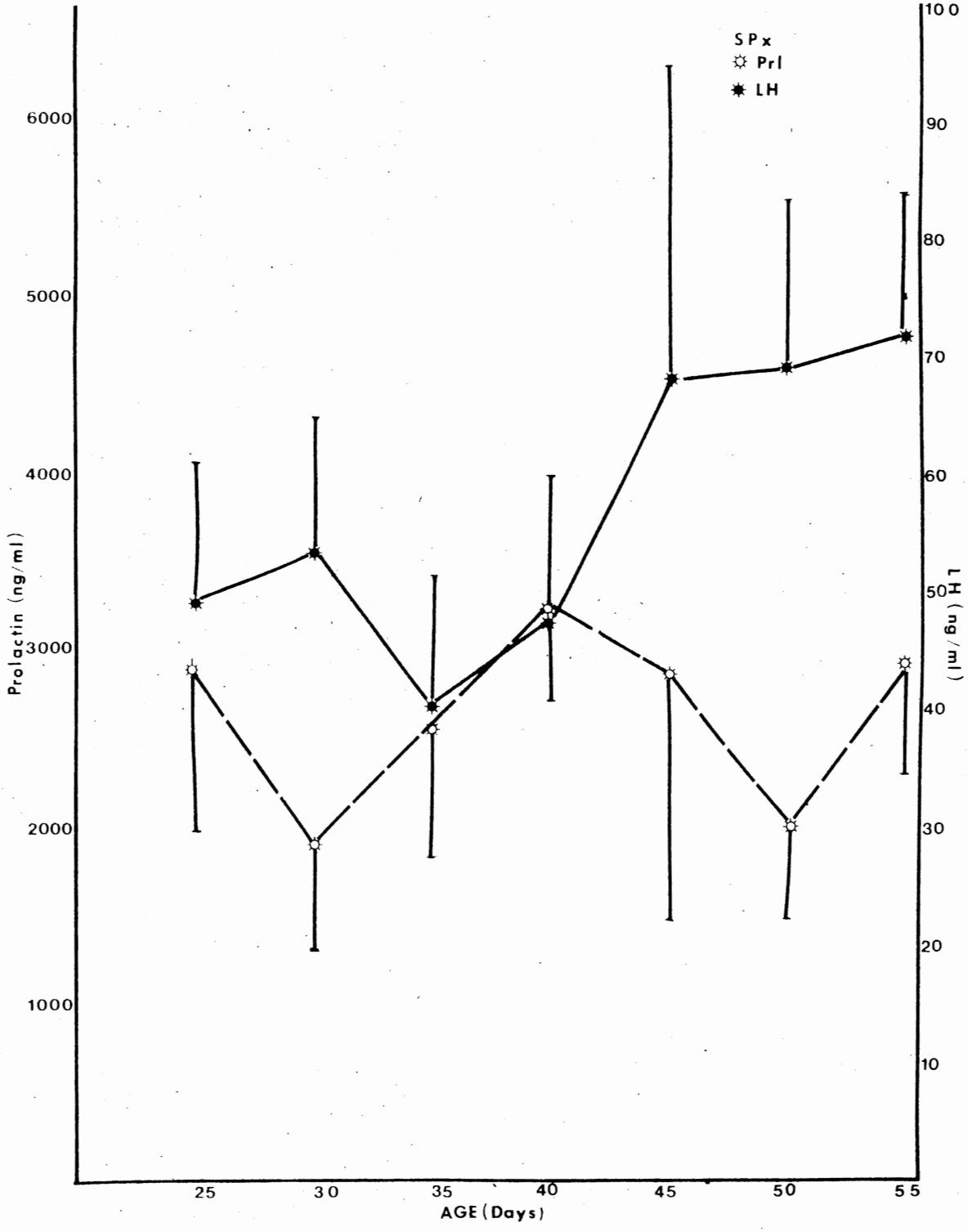

Figure 9. Changes In Serum Prolactin (left ordinate) and Serum LH (right ordinate) as a Function of Age In Immature Sham-Pinealectomized Female Rats. The vertical lines represent the standard error.

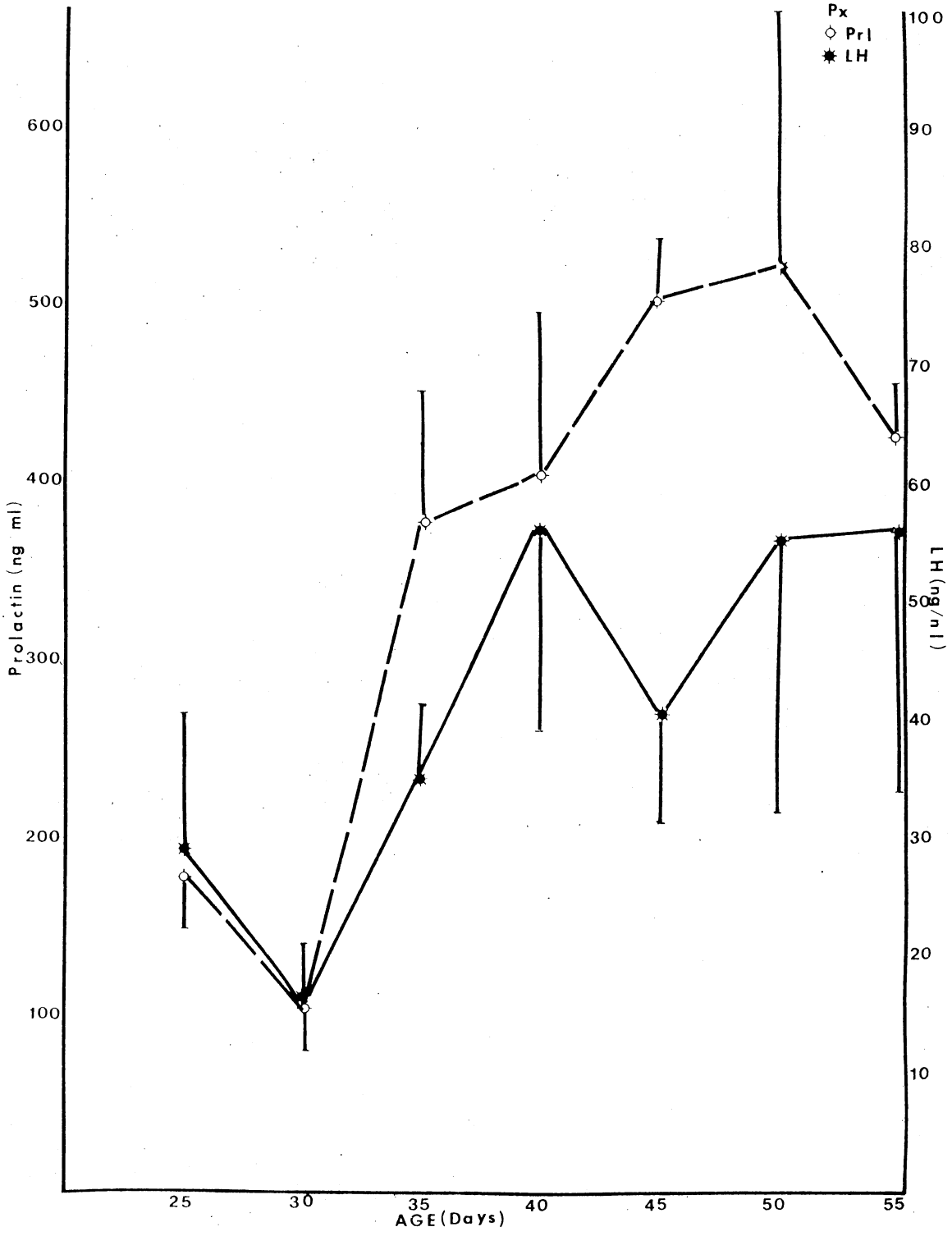

Figure 10. Changes in Serum Prolactin (left ordinate) and Serum LH (right ordinate) as a Function of Age In Immature Pinealectomized Female Rats. The vertical lines represent the standard error.

that the groups reached an optimum LH concentration at day 40 while prolactin sharply increased from day 30 to day 35 and then gradually increased until the animals reached 50 days of age. The PxBl animals (Figure 11) displayed LH and prolactin peaks on day 45, the only group in which hormonal peaks occurred after that of the C animals. The Bl animals showed characteristics similar to that of PxBl animals with the exception of the absolute values. In these animals, prolactin concentration does no rise as high nor decrease as sharply as LH (Figure 13).

Effects of Treatment on Progesterone

Progesterone levels were significantly ($P < .005$) different between all of the treatment groups (Table X). PxBl animals were the only group in which progesterone concentration was different from that of the C animals. As for the hormonal changes within the group, all of the groups with the exception of PxBl, had a confidence level of 95% or 99% (Table XI). The C and the PxBl groups had a peak value of progesterone on day 40 (Figure 14). The Px animals had a high value of progesterone on day 25 which decreased sharply to its nadir at day 35 while the SPx group showed a moderate but significant ($P < .05$) peak at 35 days (Figure 15). As with the Px animals, the Bl group had a high progesterone concentrations on day 25 which decreased to its nadir on day 35, then rose slightly to reach another significant, but smaller, peak by day 45 (Figure 16). The CL animals displayed a maximal progesterone value on 25 days of age which decreased to basal levels at day 40 and remained low throughout the remainder of the observation period (Figure 17).

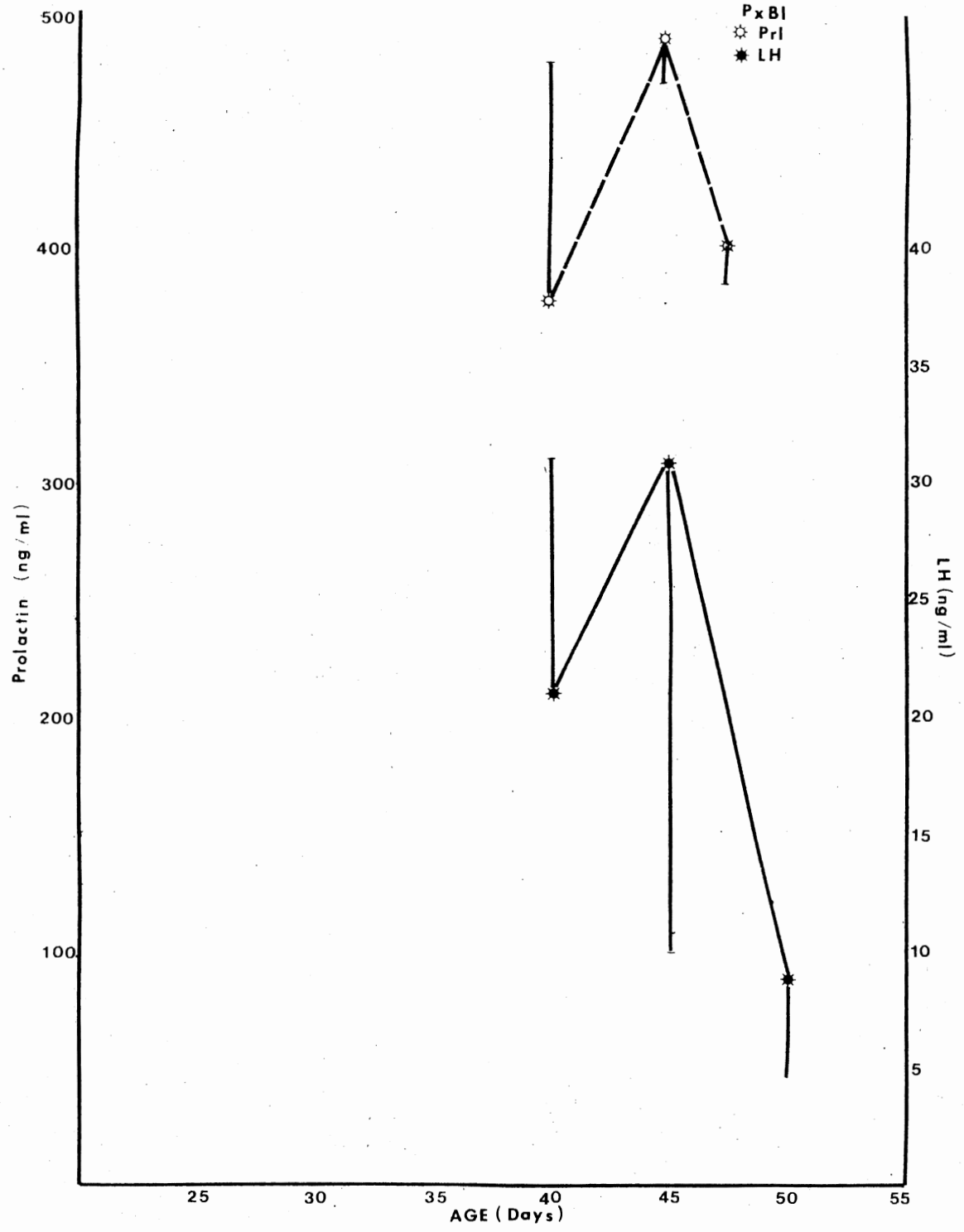

Figure 11. Changes in Serum Prolactin (left ordinate) and Serum LH (right ordinate) as a Function of Age in Immature Pinealectomized-Blinded Female Rats. The vertical lines represent standard error.

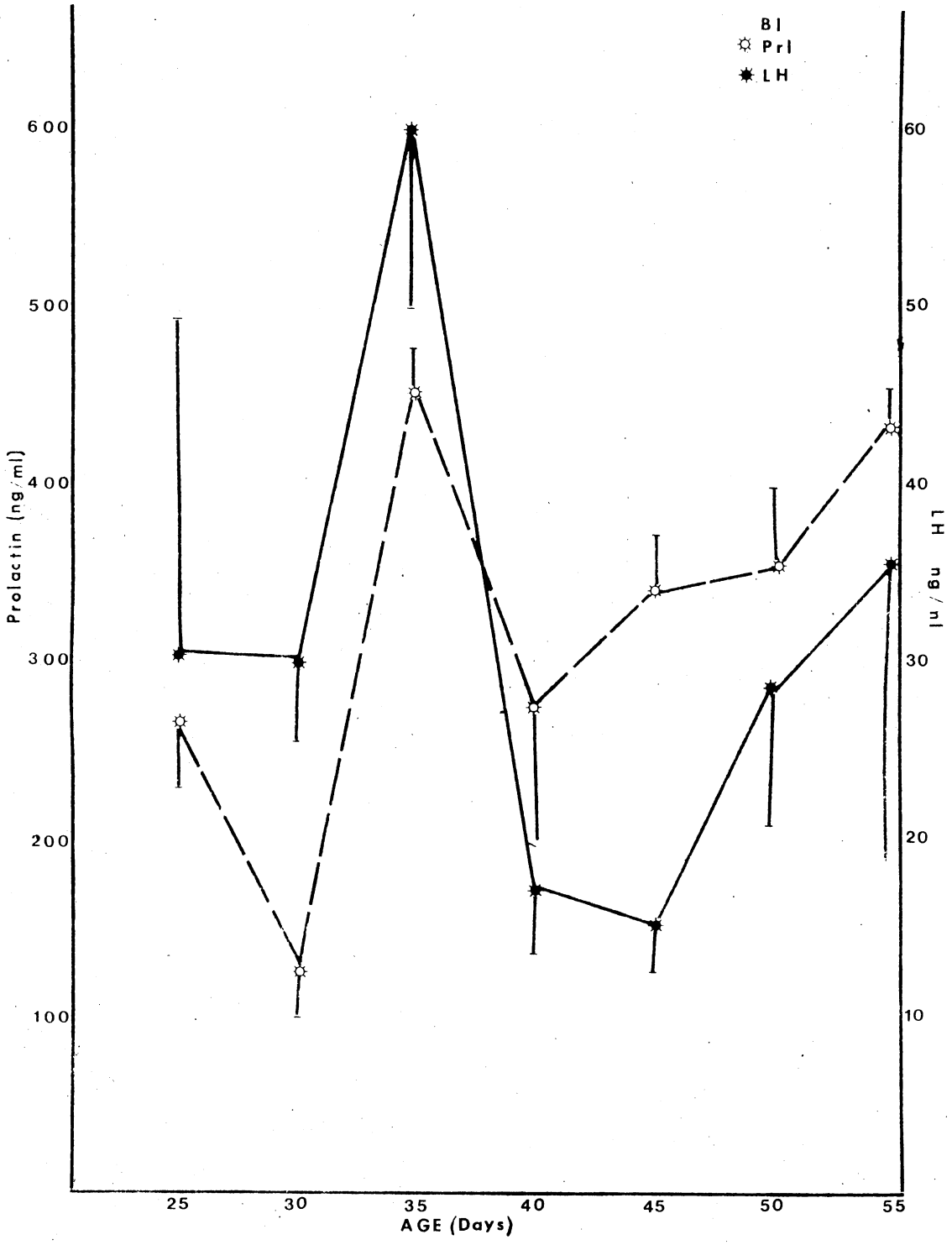

Figure 12. Changes in Serum Prolactin (left ordinate) and Serum LH (right ordinate) as a Function of Age In Immature Blinded Female Rats. The vertical lines represent standard error.

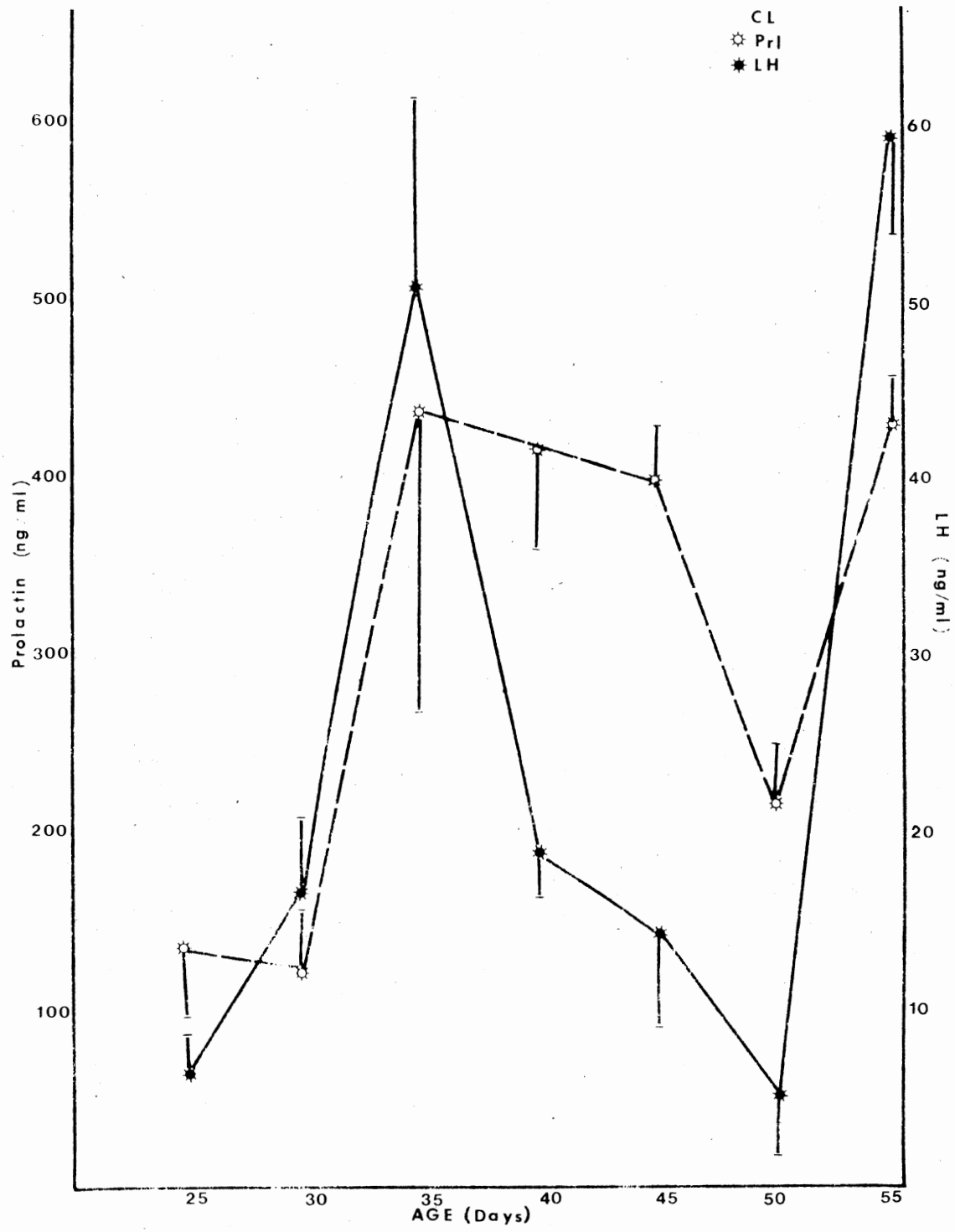

Figure 13. Changes in Serum Prolactin (left ordinate) and Serum LH (right ordinate) as a Function of Age in Immature Female Rats Housed in Constant Lighting. The vertical lines represent standard error.

TABLE X

THE EFFECTS OF TREATMENT ON PROGESTERONE

Experiment	n	Mean ± S.E.	F	P of larger value
C	35	41.28 ± 6.22 (pg/ml)	22.91	0.005
PxBI	7	51.28 ± 19.03		
CL	60	1279.56 ±151.43		
BI	53	169.95 ± 13.17 (ng/ml)	15.88	0.005
Px	47	246.61 ± 42.29		
C	61	136.30 ± 9.81		
SPx	64	251.12 ± 32.20		

C vs PxBI	n = 42	t = 0.5085	N.S.
C vs CL	95	8.1704	P < .001
SPx vs BI	117	2.3332	P < .02
SPx vs Px	111	0.0848	N.S.
C vs BI	114	2.0491	P < .02
C vs Px	108	2.5409	P < .01
C vs SPx	125	3.4110	P < .001

N.S. - no significance (refer to text for legend of abbreviations).

TABLE XI

IN TREATMENT VARIATIONS OF PROGESTERONE LEVELS BY t-test

Experiment	Age-days 25-30	30-35	35-40	40-45	45-50	50-55
C_1	1.2623* N.S.	1.1942* N.S.	2.1525* P < .05		1.3493* N.S.	0.5684* N.S.
C_2			0.9754 N.S.	1.9459 N.S.	0.4063 N.S.	0.6457 N.S.
PxBl				1.1049 N.S.	0.9758 N.S.	
CL	3.9050 P < .01	0.0882 N.S.	1.4454 N.S.	0.7278 N.S.	0.0790 N.S.	0.3611 N.S.
Px	4.2907 P < .01	2.4385 P < .05	0.2453 N.S.	2.3982 P < .05	0.5463 N.S.	0.2398 N.S.
SPx	0.0327 N.S.	2.3307 P < .05	2.1907 P < .05	0.0597 N.S.	0.6988 N.S.	1.1294 N.S.
Bl	2.7849 P < .05	2.3472 P < .05	1.2740 N.S.	1.8685 P < .1	0.2604 N.S.	2.2496 P < .05

N.S. - no significance (refer to text for legend of abbreviations).
* A group of control were assayed with the second assay.

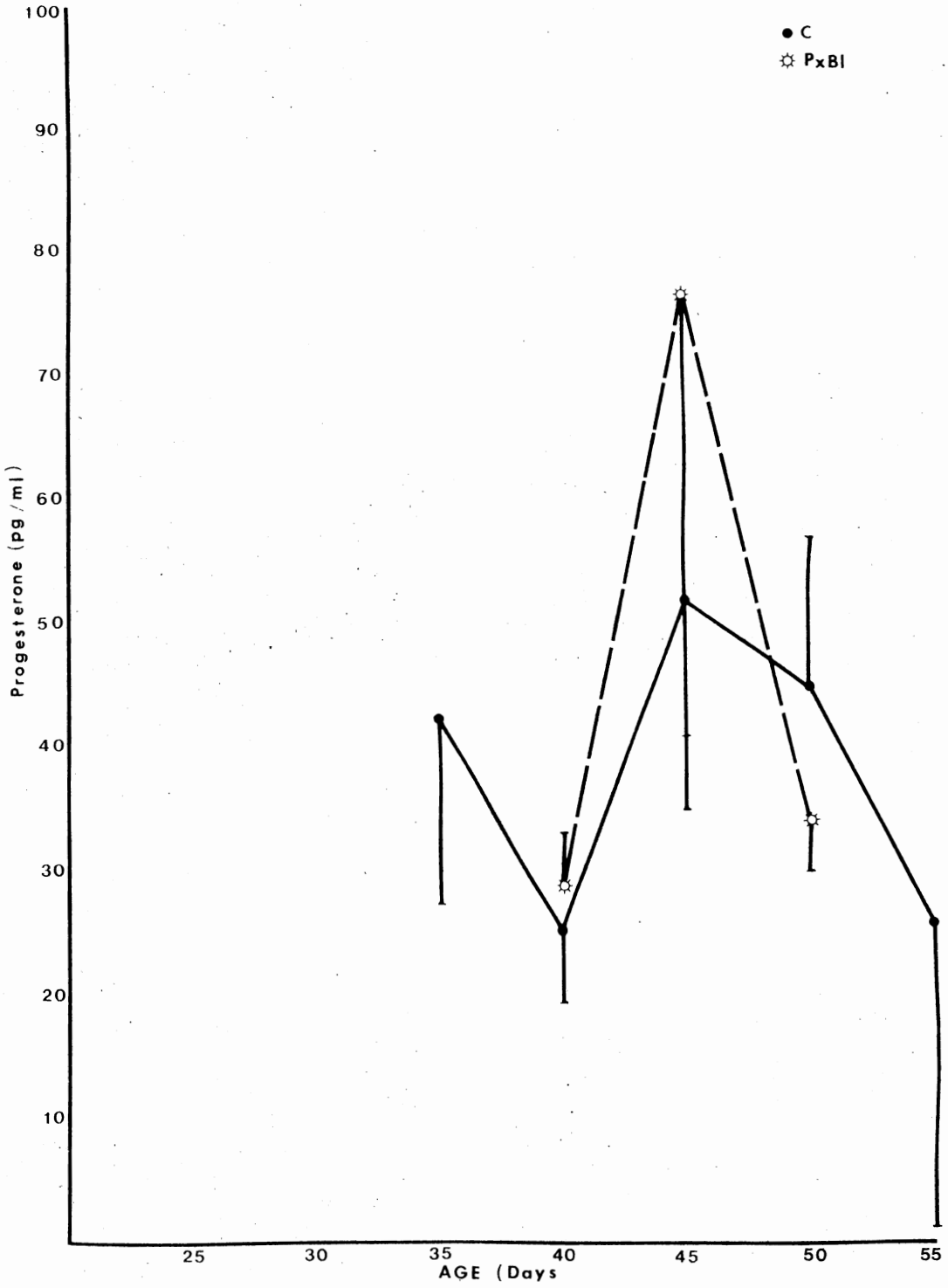

Figure 14. Changes in Serum Progesterone Versus Age In Immature Female Rats. The vertical lines represent standard error.

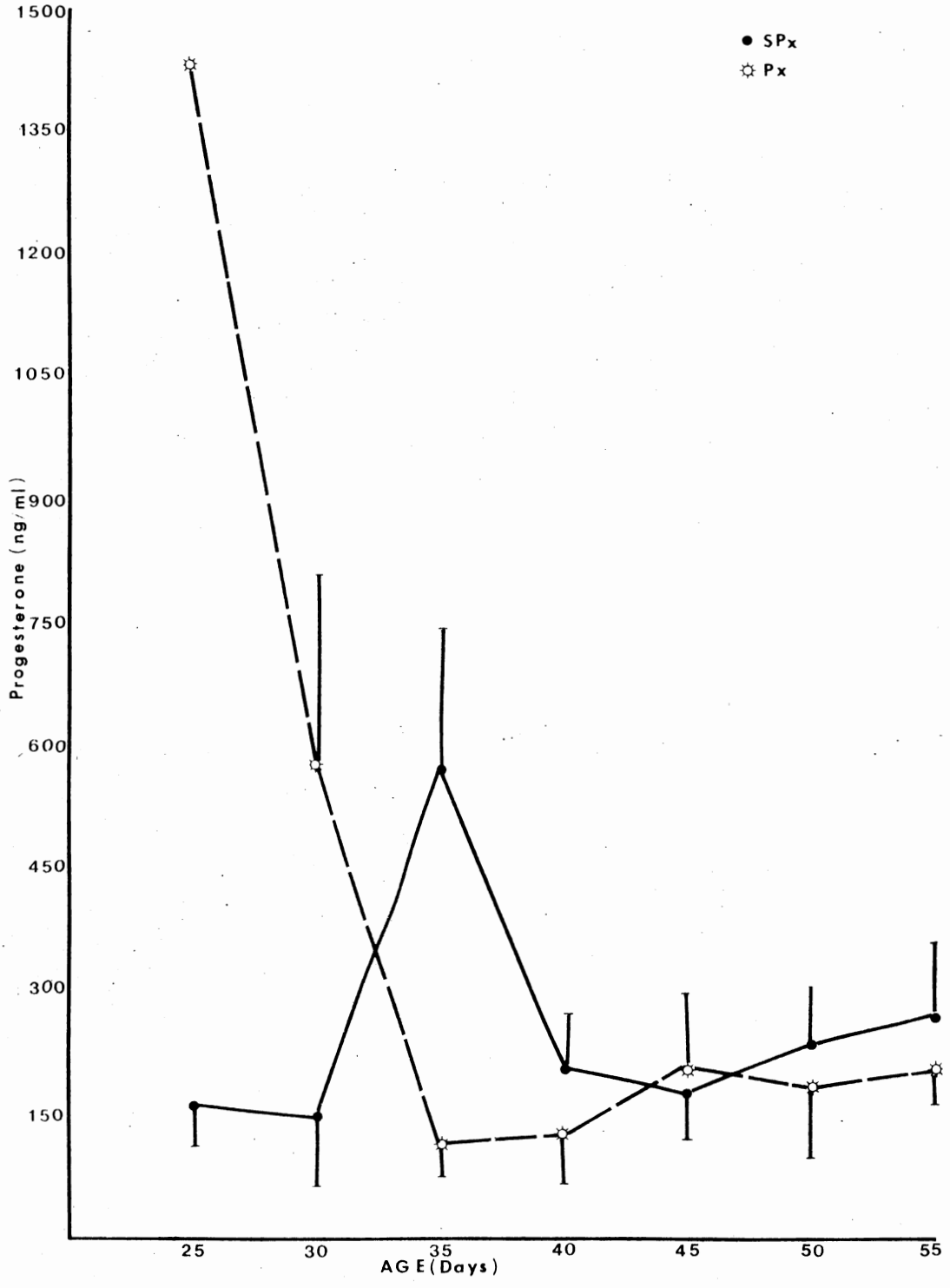

Figure 15. Changes in Serum Progesterone Versus Age In Immature Female Rats. The vertical lines represent standard error.

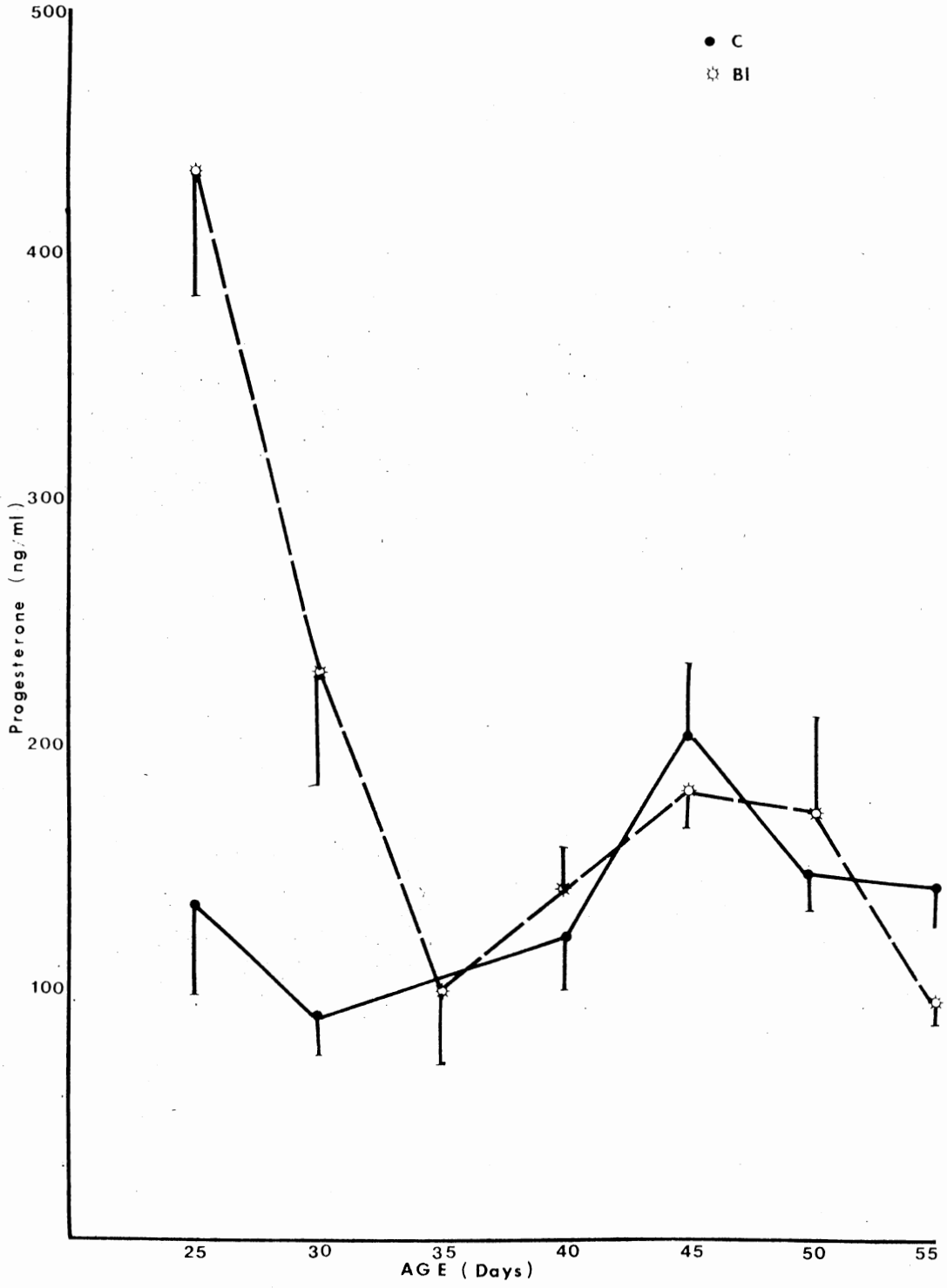

Figure 16. Changes In Serum Progesterone Versus Age In Immature Female Rats. The vertical lines represent standard error.

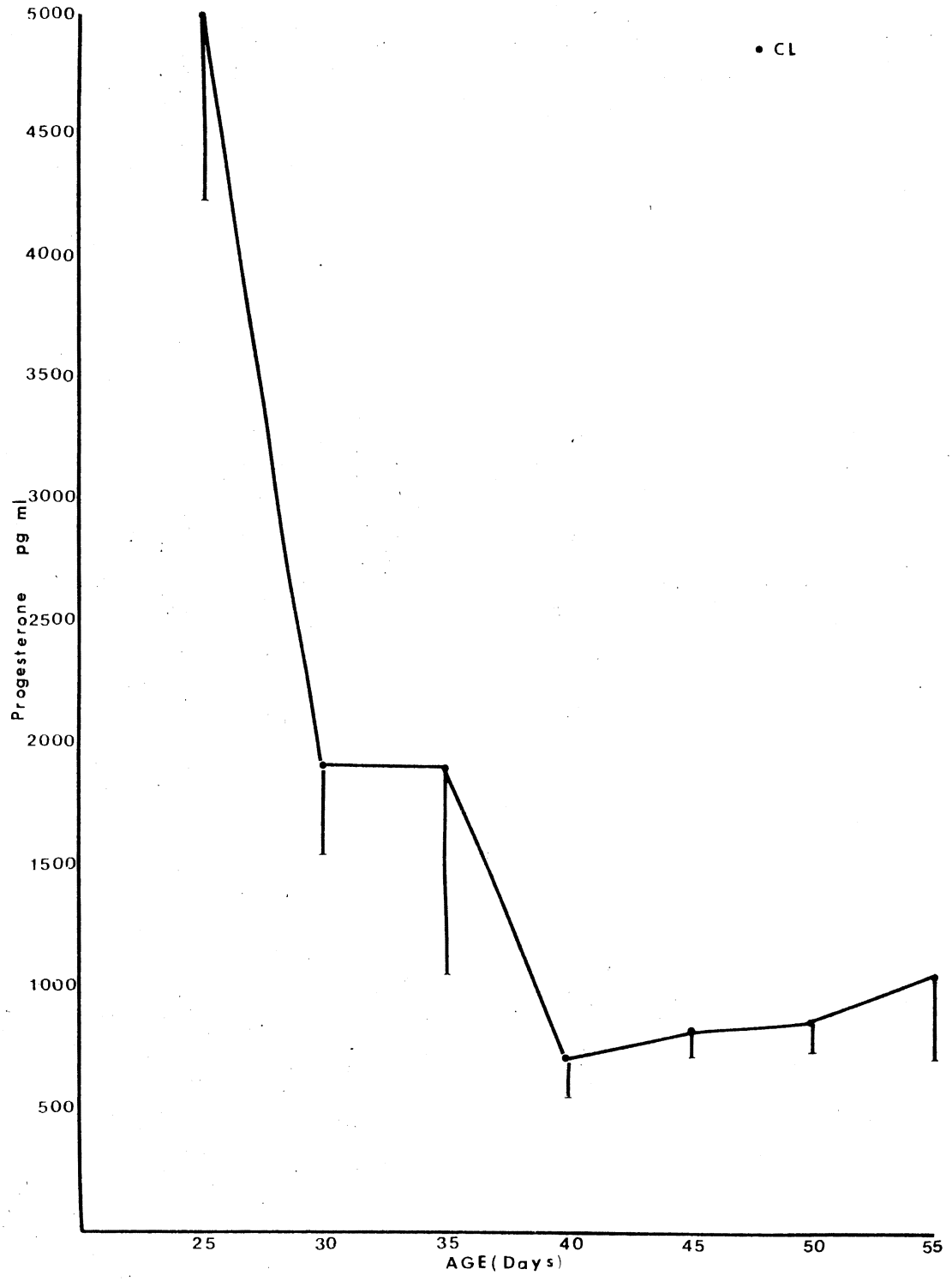

Figure 17. Changes in Serum Progesterone Versus Age In Immature Female Rats. The vertical lines represent standard error.

Two sets of C animals were utilized at separate times of the year. Thus, when the serum was assayed for progesterone concentrations, one C group was run with the PxBl animals and the CL animals while another C group was compared with the Px, SPx and Bl animals. Because of the difference in binding of the assays which were performed at two different times of the year, Figure 18 graphically illustrates the progesterone values as a percent of the control values with which they were assayed. This means of depicting the progesterone concentrations is meant to reduce the error in the assays.

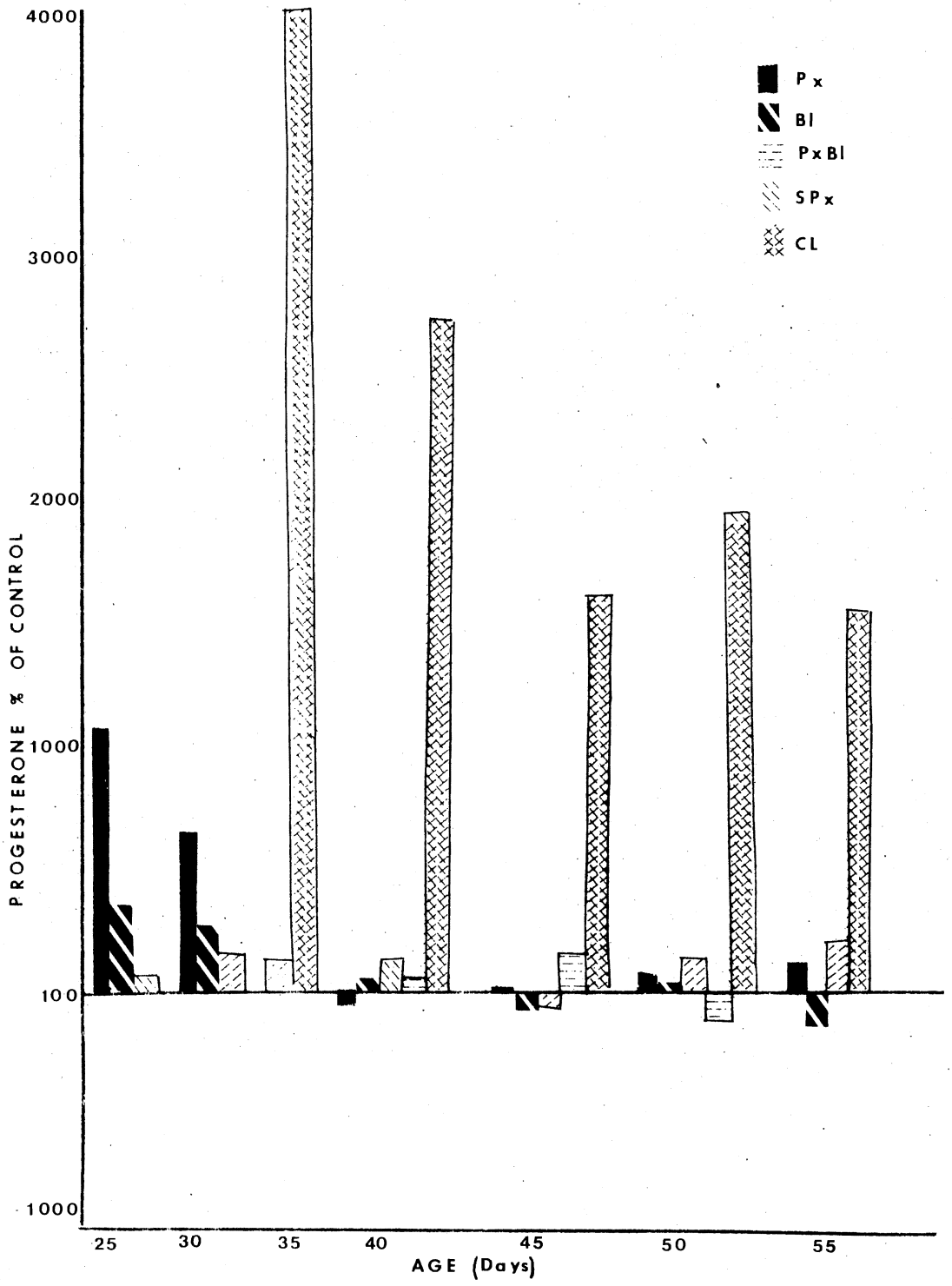

Figure 18. Progesterone as a Percentage of Control Versus Age In Immature Female Rats (refer to text for details).

CHAPTER V

DISCUSSION

There have been few, if any attempts in the past to correlate the onset of puberty with the function of the pineal gland. The data in the present study indicate an effect by the pineal gland on pubertal onset. However, the exact mechanism as to how the pineal influences this phenomenon is far from resolved. Perhaps, the scope of this work will contribute small, but significant information in the formulation of such a theory.

Effects of the Pineal on Body Weight

The mean body weight in each treatment group for the entire experiment showed no significant differences (Table 1). However, by comparing the weights of each group at each observation day, it was discovered that at day 35 there were significant differences between all of the groups except Px, Bl, and PxBl animals (Table II). Pinealectomy should increase GH (Sorrentino and Reiter, 1971), therefore, making these animals gain more weight and hasten their VO. CL animals should be similar to Px because of the lack of pineal function. Added to this is a decrease in prolactin in mothers, which may have caused a loss of milk to neonates. This would limit the amount of weight gained until after weaning. Frisch (1974) presented a theory which states that puberty is more closely re-

lated to the attainment of a critical weight than to the chronological age of the animal. However, at the time of VO, the lightest treatment group, Px, had the earliest VO and CL animals weighed approximately the same at VO time which occurred on day 39 and 41, respectively. The C animals were the heaviest group but the second to last to have VO.

Effect of the Pineal on Vaginal Opening Time

The vaginal opening times of the Px animals compared with Reiter's findings showed that this treatment to his animals caused VO to occur at 34 days of age. The C animals exhibited VO at 40 days of age, whereas Reiter recorded 41 days of age. CL animals showed an advanced sexual maturation as compared to the C animals which concurs with previous findings (Ramaley and Bartosik, 1975). Compared with the other groups, the Bl animals had a delayed vaginal opening time which is supported by previous reports. In this study, the delay was not significant when analyzed by Duncan's New Multiple Range test (Table III). However, this could be due to the procedure in which the VO was checked. As the experiment progressed, the animals were sacrificed, terminating all animals at 55 days which would eliminate any later VO, thereby influencing the mean VO time. Although this delay was not significantly different from the C animals, the pineal is thought to elaborate a substance which would modify the hormonal requirement for VO. Indeed, when blinded animals were pinealectomized, VO time was significantly earlier than the Bl and C animals, indicating an involvement of the pineal in this slight delay. However, the VO time of PxBl group of animals was more delayed then that of the Px group, indicating that the effects of blinding on VO may be mediated through another route in addition to the pineal.

Effects of the Pineal on Serum LH

The LH values reported herein are lower than those reported in previous studies (Ojeda and Ramirez, 1972; Dohler and Wuttke, 1974; Ojeda et al., 1975). This is probably due to the difference in techniques of the assays and a difference in reference preparations. However, it is not the absolute values which are critical in this study, but the fluctuations in the hormonal concentrations. Ojeda et al., (1975) reported that there is a rise in LH concentrations at the time of puberty. The data from this study compared with this statement, but only in the C animals. The other treatment groups showed no relationship between VO time and LH peak concentration levels. Comparison of the timing of the peak LH concentrations between the CL and Bl groups, indicated that the pineal may not influence the prepubertal surges of this hormone. Constant light should impose a restriction on the pineal function while blinding would activate secretion of an antireproductive substance. In these two groups, the LH surge occurs simultaneously and is statistically equivalent in concentration. The PxBl animals, normally should react the same as Px animals since the loss of light would not be modulating the pineal. From the results, it appears there is an alternate influence which decreased the effect of pinealectomy. Therefore, blinding, as a result of optic nerve severance, must affect something other than just the pineal.

Effects of the Pineal on Serum Prolactin

The absolute prolactin values were higher than previously reported values (Ojeda and McCann, 1974; Voogt, et al., 1970). Ojeda reported

a preovulatory discharge of prolactin which compared to the C group of animals, but none of the other treatment groups. Additionally, this prolactin surge should coincide with the LH surge and the timing of VO. Px animals exhibited VO before the prolactin peak concentration whereas, the other treated animals exhibited optimal values prior to vaginal opening. On days 45 and 50, after all groups had experienced puberty, there were significant differences between prolactin concentrations (Table IX). Generally, all of the groups except SPx, displayed parallel values or at least increases and decreases in prolactin and LH concentrations at the same ages.

Effects of the Pineal on Serum Progesterone

In their studies with prepubertal rats, Meijs-Roelofs, et al., (1975) reported progesterone concentrations of 1-3 ng/ml. The values in this study did not compare to the reported values. As was mentioned previously, this may be due to the discrepencies in assay techniques. Elevated concentrations of progesterone have been reported to occur at the time of onset of puberty (Dohler and Wuttke, 1974). These authors also reported that steadily increasing serum prolactin values between days 20 and 40 are accompanied by steadily increasing serum progesterone levels which remain elevated through diestrus. However, during elevated LH and FSH concentrations there were decreased progesterone levels which suggests an antagonistic relationship. The PxBl animals were the only group which exhibited elevated progesterone and prolactin concentrations at the same age. Control and SPx animals had overlapping times when prolactin levels were decreasing and progesterone levels were increasing. Px and Bl animals showed completely opposite peaks, that is when prog-

esterone was at an optimum, then LH and prolactin exhibited basal levels. It is known that there are two sources of progesterone, the ovaries and the adrenals. Since prepubertal animals have functionless ovaries, then the circulating progesterone must be secreted by the adrenal glands. Perhaps, then the pineal influences the activity of the adrenals in its secretion of progesterone.

CHAPTER VI

SUMMARY AND CONCLUSION

Sprague-Dawley female rats were divided into 6 groups. At day 0, the day of birth, one group of animals was placed in constant lighting. At 2 days of age, the animals to be pinealectomized and sham-operated were surgically treated. On day 10, optic enucleation was performed on another group of animals. With the other groups, a group of control animals were placed in an alternating photoperiod of 14 hours of light and 10 hours of dark (14L:10D), for a period of 55 days. Each animal was weighed every 5 days. The animals were sacrificed at 5 day intervals, starting at day 25. The serum samples from these rats were analyzed by radioimmunoassay (RIA) for LH, prolactin and progesterone. Vaginal opening (VO) time was checked during the experimental duration. After completion of all experiments, the data was analyzed first by an analysis of variance between each group for the entire 30 day interval and also between groups at each sampling day. When there was significance in the former case, then a student's t-test was calculated for within group significance over the 30 day interval. When there was significance in the latter instance, Duncan's New Multiple Range test was performed.

Significant differences were found between treatments for each hormone tested, body weights and VO time. However, none of these three

factors corresponded in any case to each other or published results.

In conclusion, there appears to be no relationship between body weight and vaginal opening time. LH and prolactin reach peak concentrations simultaneously but these peaks are out of phase with the vaginal opening times. The results from the progesterone assays are inconclusive at this time. Progesterone concentrations are low at the time of vaginal opening which is completely opposite to expected results. At this time, there is no apparent explanation for these results.

This study does not solve the problem of how the pineal is involved in puberty, but rather raises more questions concerning the mechanism of this reproductive phenomenon. However, this data leads to a couple of hypotheses. It may be possible that the pineal controls puberty by inhibiting PIF which causes increased prolactin levels until pubertal onset at which time the pineal loses its ability to inhibit PIF, thus allowing the surge of gonadotropins. In the final hypothesis, the pineal could indirectly, modulate the sensitivity of the vaginal epithelium; thus in pinealectomized animals VO is not inhibited and allowed to preceed the gonadotropin peak concentrations. Whereas, in the blinded animals, the pineal is hyperactive causing the vaginal epithelium to be less sensitive, delaying vaginal opening until after the gonadotropin surge.

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APPENDIXES

APPENDIX A

RADIOIMMUNOASSAY BUFFERS

0.05M EDTA-PBS

18.61 g Disodium EDTA
1000 ml PBS (Working Solution)
Stir until dissolved and adjust pH with 5N NaOH

0.25% NRS-EDTA-PBS

Reconstitute NRS with glass distilled water.
Add 2.5 ml NRS to 1000 ml of 0.05M EDTA-PBS

Phosphate Buffered Saline (PBS) (Stock Solution)

34.3 ml of 0.5M Sodium Phosphate, $NaH_2PO_4 \cdot H_2O$ (monobasic)
68.6 ml of 0.5M Sodium Phosphate, NaH_2PO_4 (dibasic)
40.86 g NaCl
0.5 g Thimerosol (Merthiolate)
Bring to volume of 1000 ml with glass distilled water

Phosphate Buffered Saline (PBS) (Working Solution)

1 part stock solution
4 parts glass distilled water

0.5M Sodium Phosphate Buffer pH 7.5

69.0 g $NaH_2PO_4 \cdot H_2O$ (monobasic) to 1000 ml glass distilled water (Solution A). Store at 5°C.
71.0 g NaH_2PO_4 (Dibasic) to 1000 ml with glass distilled water (Solution B). Store at 5°C.
Mix 1 part solution A with 4 parts solution B and adjust pH.

0.05M Sodium Phosphate Buffer pH 7.5

27.8 g $NaH_2PO_4 \cdot H_2O$ (monobasic) to 1000 ml with glass distilled water. Add 0.1 g thimerosol (Merthiolate). Store at 5°C. (Solution A)
28.38 g NaH_2PO_4 (dibasic) to 1000 ml with glass distilled water. Add 0.1 g thimerosol (Merthiolate). Store at 5°C. (Solution B)
Mix 16 ml of solution A and 84 ml solution B and bring to 500 ml with glass distilled water. Adjust pH. Store at 5°C.

APPENDIX B

RADIOIMMUNOASSAY FOR PROGESTERONE

A. Preparation of Tritiated Solution Recoveries.

1. Dilute tritiated solutions to approximately 20,000 cpm by:

   a. Take 10 µl of respective tritiated solution and evaporated.
   b. Add 10ml buffer of respective buffer, vortex.
   c. Add 100 µl to scintillation vial, add 10 ml cocktail solution and count.
   d. Repeat if it needs to be adjusted to 20,000 cpm by diluting or concentrating the original 10 ml buffered solution.

B. Extraction Procedures

1. Prepare 2 ml serum samples (or as much as there is available) in screw cap tubes. Several samples may be pooled together but the ratio of each sample must be noted.
2. Add tritiated recoveries to the 2 ml serum samples, 100 µl in each tube.
3. Add 2 ml fresh ether to each tube for extraction and then vortex.
4. Centrifuge 2 min at 2500 rpm.
5. Pipet off ether layer into clean screw cap tube and evaporate.
6. Repeat by adding another 2 ml fresh ether to serum, vortex, centrifuge and pipet into first evaporated ether tube.

C. Chromatography

1. Preparation of Columns

   a. Prepare eluting solvent (62:20:18) (See solutions)
   b. Weigh 0.9 g Sephadex LH-20 per column into a 15 ml disposible beaker
   c. Set up 10 ml syringes with valves in a supporting rack, rinse with methanol and plug with a filter paper disc, rinse column with (90:10) column wash to settle paper disc.
   d. Using a pasteur pipet, slurry the sephadex with column wash and pour into column.
   e. Rinse beaker with column wash until all sephadex is in the column.
   f. Fill the column with eluting solvent and allow it to saturate the column by running through. Repeat. Columns must never run dry.

g. When most of the air bubbles are removed, rinse the column walls with the solvent.
h. Flush columns with 22 ml solvent. Leave the meniscus of solvent slightly above sephadex column.
i. Columns may be stored in the above condition.

2. Elution of Samples

a. Set up and label one 10 X 75 mm test tube for each original sample.
b. Evaporate samples as in extraction procedures.
c. Lower column level, add 0.2 ml eluting solvent to sample tube mix thoroughly and apply mixture only to sephadex and not syringe walls.
d. Open valve and allow sample to enter oclumn, close valve and repeat with 0.1 ml solvent. Allow the sample to enter the column.
e. Add 4 ml of the eluting solvent and collect this entire sample. Run approximately another 30 ml of solvent through the column. Do not collect this fraction.

D. Progesterone Recovery and Assay

1. Recovery of Progesterone

a. Evaporate samples and reconstitute with 2 ml methanol.
b. Place 0.5 ml in a scintillation vial and evaporate for recovery. Add 10 ml cocktail and set aside.
c. Remove 100 µl and 200 µl in duplicate, into culture tubes, evaporate.

2. To the 0.5 ml in the 10 X 75 tubes, add:

a. 100 µl diluted progesterone antisera
b. 100 µl tritiated progesterone (20,000 cpm)
c. 100 µl progesterone buffer to the standards and 600 µl to the serum samples.

3. Incubate in cold room for 18 hours or longer.

4. Add charcoal solution by:

a. Keeping cold progesterone charcoal solution stirring
b. With 10 X 75 tubes on ice, add 200 µl charcoal solution per tube, vortex.
c. Incubate 20 min in centrifuge, spin 10 min at 2000 rpm.
d. Decant into scintillation vial and add 10 ml cocktail and count.

APPENDIX C

SOLUTIONS

Standards

Stock solution is prepared by adding 1 µg/ml absolute ETOH. Prepare standards in triplicate.

Solution A	20 µl of stock in 10 ml buffer	.5 ml = 1000 pg
Solution B	5 ml solution A + 5 ml buffer	.5 ml = 500 pg
Solution C	5 ml solution B + 5 ml buffer	.5 ml = 250 pg
Solution D	4 ml solution C + 6 ml buffer	.5 ml = 100 pg
Solution E	5 ml solution D + 5 ml buffer	.5 ml = 50 pg
Solution F	5 ml solution E + 5 ml buffer	.5 ml = 25 pg
Solution G	4 ml solution F + 6 ml buffer	.5 ml = 10 pg
Solution H	5 ml solution G + 5 ml buffer	.5 ml = 5 pg
Solution I	5 ml solution H + 5 ml buffer	.5 ml = 2.5 pg
Solution J	0 ml solution I + 5 ml buffer	.5 ml = 0 pg

Progesterone Buffer pH 7.4 - 7.5

8.175 g sodium phosphate dibasic heptahydrate
2.70 g sodium phosphate monohydrate
0.50 g sodium azide
4.50 g sodium chloride
500 ml glass distilled water
0.50 g gelatin

Solvent for columns - Iso-octane : Benzene : Methanol (62:20:18)

434 ml Isodone (2,2,4 trimethylpentane)
140 ml Benzene
126 ml Methanol

Charcoal Solution for Progesterone

0.625% Norite A
0.0625% Dextran T-80 or T-70
100 ml Progesterone Buffer

Cocktail

28 g PPO / gallon Toluene

Dilution of Progesterone Antisera

1 vial antisera
49.9 ml of progesterone buffer

Column Wash

Benzene : Methanol (90:10)

VITA[2]

KAREN J. BACHLE

Candidate for the Degree of

Master of Science

Thesis: THE INFLUENCE OF THE PINEAL GLAND ON PUBERTY IN THE NEONATAL FEMALE RAT

Major Field: Physiological Sciences

Biographical:

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