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**Studies on mechanisms of delayed puberty in female rats  
effected by dietary eicosapentaenoic acid**

**Zhang, Zhao, M.S.**

**The University of Arizona, 1992**

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STUDIES ON MECHANISMS OF  
DELAYED PUBERTY IN FEMALE RATS EFFECTED  
BY DIETARY EICOSAPENTAENOIC ACID

by

Zhao Zhang

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A Thesis Submitted to the Faculty of the  
DEPARTMENT OF ANATOMY  
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For the Degree of  
MASTER OF SCIENCE  
WITH A MAJOR IN ANATOMY  
In the Graduate College  
THE UNIVERSITY OF ARIZONA

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SIGNED: Zhao Zhang

## APPROVAL BY THESIS DIRECTOR

This thesis has been approved on the date shown below:

Bryant Benson

Bryant Benson  
Professor of Anatomy

12/9/92

Date

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## TABLE OF CONTENTS

	Page
LIST OF ILLUSTRATION.....	6
LIST OF TABLES.....	7
ABSTRACT.....	8
INTRODUCTION.....	9
Arachidonic Acid Metabolism.....	9
Effects of Eicosapentaenoic Acid on Prostaglandin Synthesis.....	10
Puberty in the Rat.....	12
Prostaglandin Involvement in the Development of the Reproductive System of the Female Rat.....	17
PGE <sub>2</sub> involvement in gonadotrophin release.....	17
PG involvement in ovulation.....	19
STATEMENT OF PROBLEM AND SPECIFIC AIMS.....	21
MATERIALS AND METHODS.....	22
Animals.....	22
Diets.....	23
Composition of diets.....	23
Compositions of fatty acids in dietary oils.....	24
Pair-feeding.....	25
Radioimmunoassays.....	26
FSH, LH and PRL.....	26
GnRH.....	26
Prostaglandin E <sub>2</sub> .....	27
E <sub>2</sub> Replacement.....	28
Prostaglandin E <sub>2</sub> .....	29

## TABLE OF CONTENTS CONTINUED

	Page
Classification of Phases of Puberty.....	29
Anestrus.....	29
Early proestrus I.....	30
Early proestrus II.....	30
Late proestrus.....	30
Estrus.....	30
First diestrus.....	30
Preparation of Ovaries for Histology and Morphometric Examination.....	31
Statistical Analysis.....	31
EXPERIMENTAL DESIGN AND RESULTS.....	32
Effects of Dietary FO on Puberty in Female Rats.....	32
Effects of Dietary FO on Neuroendocrine and Ovarian Events Culminating in First Ovulation.....	42
Effects of E <sub>2</sub> Treatment on Neuroendocrine and Ovarian Ovulatory Mechanisms in Rats Fed FO.....	53
Effects of Acute PGE <sub>2</sub> Injection on Neuroendocrine and Ovarian Ovulatory Mechanisms in Rats Fed FO.....	59
DISCUSSION.....	65
SUMMARY.....	81
REFERENCES.....	83

## LIST OF ILLUSTRATIONS

Figure	Page
1. Body weights gain in FO- and SO pair-fed female rats.....	35
2. Age at first estrus in FO- and SO-fed rats.....	37
3. POA/HYPO GnRH and PGE <sub>2</sub> in FO-fed and control female rats on diestrus.....	40
4. POA/HYPO PGE <sub>2</sub> levels at different days of age in FO- or SO-fed rats.....	47
5. Ovarian PGE <sub>2</sub> concentrations at different days of age after FO feeding.....	49
6. Serum LH levels at different days of age in FO- and SO-fed rats.....	50
7. POA/HYPO PGE <sub>2</sub> levels after E <sub>2</sub> treatment in FO- and SO-fed rats.....	56
8. Serum LH levels after E <sub>2</sub> treatment in FO- and SO-fed rats.....	58
9. Serum LH levels in FO-fed rats after acute PGE <sub>2</sub> injection.....	61
10. Pituitary LH in FO-fed rats after acute PGE <sub>2</sub> injection.....	62

## LIST OF TABLES

Table	Page
1. Composition of experimental diets.....	24
2. Percentage of fatty acid composition of dietary oils.....	25
3. Body and organ weights and femur lengths in rats fed FO and SO.....	34
4. Ovarian corpora lutea in rats fed FO or SO.....	38
5. Gonadotropins and prolactin in rats fed FO or SO.....	39
6. Puberty phases in female rats fed FO.....	45
7. POA/HYPO and ovarian PGE <sub>2</sub> and serum LH levels in female rats fed FO from 30 to 39 Days of Age.....	46
8. Organ weights, PGE <sub>2</sub> and serum LH.....	52
9. Ovulation in FO-fed rats after 24 hours of PGE <sub>2</sub> injection.....	63

## ABSTRACT

Marine oils contain eicosapentaenoic acid, a fatty acid that competes for cyclooxygenase and reduces the synthesis of dienoic prostanoids including prostaglandin E<sub>2</sub> (PGE<sub>2</sub>). Since PGE<sub>2</sub> plays an important role in the release of hypothalamic GnRH and the maturation of ovarian follicles and ova release, it was postulated that a diet containing fish oil (FO) would delay first ovulation through inhibitory effects on GnRH release, follicle development and ovulation.

Immature female Sprague-Dawley rats were fed a FO diet ad libitum. Controls were pair-fed an identical diet with the substitution of safflower oil. The age of the FO-fed rats was significantly increased at first estrus, and first ovulation was either delayed or inhibited. Preoptic area/hypothalamic and ovarian PGE<sub>2</sub> levels were reduced by FO feeding whereas hypothalamic GnRH was significantly increased. A FO-containing diet may delay the onset of puberty through attenuation of preovulatory GnRH release and local impairment of the ovulatory process.

## INTRODUCTION

## 1. Arachidonic Acid Metabolism

Arachidonic acid (all *cis*-5,8,11,14-eicosatetraenoic acid) is a carbon-20 polyunsaturated fatty acid which is essential for humans since its precursor, linoleic acid, must be provided from dietary sources. The oxygenated derivatives of this polyunsaturated fatty acid are defined as eicosanoids, which exert widely diverse biological activities in cells. Eicosanoids can be classified as autocoids, which are evanescent and exert their effects locally in the microenvironment of the tissue where they were generated, are not stored and must be synthesized de novo in response to stimulation.

Arachidonic acid in cells is present predominantly in an esterified form, usually in the sn-2 position of a glycerophospholipid, which is the source of arachidonic acid utilized for prostanoid formation (Lands and Samuelsson, 1968; Vonkeman and Van Dorp, 1968). Formation of these eicosanoids requires that arachidonic acid be in the nonesterified form. Therefore, the release of arachidonic acid is a key event in the biosynthesis of eicosanoids and is thought to be the rate-limiting step in this process. The release of arachidonic acid from glycerophospholipids is accomplished either directly, by the action of phospholipase A<sub>2</sub>, or indirectly, by the action

of phospholipase C; the latter mechanism requires the subsequent actions of diacylglycerol lipase and monoacylglycerol lipase.

Arachidonic acid released by these enzymes can be metabolized by at least three major pathways: fatty acid cyclooxygenase, arachidonic acid lipoxygenases and epoxygenases (Marcus AJ. 1978). The formation of prostaglandins (PGs) is carried out by way of the cyclooxygenase pathway, which begins with the action of prostaglandin endoperoxide synthase (synonymous with fatty acid cyclooxygenase) on arachidonic acid. There is an inherent peroxidase activity in the holoenzyme so that the  $\text{PGG}_2$  (15-hydroperoxy derivative) can be rapidly converted to  $\text{PGH}_2$  (15-hydroxy derivative).  $\text{PGH}_2$  is metabolized further to dienoic prostaglandins such as  $\text{PGE}_2$ ,  $\text{PGF}_2$  and  $\text{PGI}_2$ , by the actions of various isomerases. The catabolism of PGs is catalyzed by 15-hydroxyprostaglandin dehydrogenase (PGDH) and forms 15-keto-derivatives which are substantially biologically inactive and are rapidly converted to 13,14-dihydro-15-keto-derivatives that are the major circulating forms.

## 2. Effects of Eicosapentaenoic Acid on Prostaglandin Synthesis

Effects of dietary fatty acid modification on plasma and tissue fatty acid composition and prostaglandin (PG) synthesis have received considerable attention in both experimental

animals and humans (Mathias and Dupont, 1979; Galli et al., 1981; Willis, 1981). Generally, arachidonic acid (20:4, n-6) levels can be reduced by dietary enrichment with linolenic acid (18:3, n-3) (Mohrhauer and Holman, 1963; Hwang and Carroll, 1980) or marine oils containing eicosapentaenoic acid (EPA, 20:5, n-3) (Bang and Dyerberg, 1972; Siess et al., 1980), both of which result in a concomitant decrease in PG production (Ten Hoor et al., 1980; Schoene et al., 1981).

Kevin D. Croft and his coworkers (1984) studied dietary modification of fatty acid and PG synthesis in the rat. They found that PG synthesizing capacity was reduced by feeding fish oil (FO), and a substantial reduction in serum thromboxane A<sub>2</sub> formation resulted; this was consistent with diminished levels of 20:4 and increased amounts of 20:5 fatty acids in platelets. Their results suggested that dietary suppression of prostanoid synthesis was in part due to changes in available arachidonic acid and competitive inhibition of cyclooxygenase by (n-3) fatty acids. The observations from another study (Kelley et al., 1985) showed that a diet containing FO caused a generalized reduction in synthesis of the dienoic cyclooxygenase metabolites prostaglandin E<sub>2</sub> (PGE<sub>2</sub>), thromboxane B (TXB) and 6-keto prostaglandin F<sub>1 $\alpha$</sub>  (6-keto PGF<sub>1 $\alpha$</sub> ), and promoted a shift in synthesis to PGE<sub>3</sub>, a member of the trienoic series. The control mice in this study received dietary supplements of safflower oil (SO), a rich source of

linoleic acid (18:2, n-6) which is metabolized to the intermediate arachidonic acid and further converted to the dienoic prostaglandins (PG<sub>2</sub>). The FO-fed mice received menhaden oil, a rich source of EPA which is the precursor of trienoic prostaglandins (PG<sub>3</sub>). Indeed, certain investigators have found in vivo production of trienoic prostacyclin (PGI<sub>3</sub>) in primates (Abeywardena et al., 1989) and man (Fischer and Weber, 1984) after dietary fatty fish, FO supplements or ethyl esters of purified long-chain polyunsaturated n-3 fatty acids like EPA.

### 3. Puberty in the Rat

The rat is a convenient animal to use for the study of sexual development. It grows rapidly and external signs of sexual maturity are readily apparent. In working with this animal, we can also assume that most of the mechanisms in the process of sexual maturation are similar in other mammalian species including man.

The phases of sexual development in female rats have been defined in relation to the maturational stages of the ovary (Critchlow and Elwers Bar-Sela, 1967; Rennels, 1951). Ramirez (1974) established criteria of classification based on certain physiological changes including changes in circulating gonadotropin levels and alterations in steroid feedback mechanisms occurring at different postnatal stages. More

recently, another classification has been established which considers both morphological and physiological changes in female rat (Ojeda et al., 1980). According to these criteria, sexual development can be divided into four phase: 1) a neonatal period extending from birth to postnatal day 7; 2) an infantile period from days 8 to 21; 3) a juvenile period around days 30 to 32; and 4) a prepubertal period which has a variable duration, but culminates with the occurrence of first ovulation.

In the last decade studies have shown that the appearance of hypothalamic gonadotropin releasing hormone (GnRH) precedes that of gonadotropins in the anterior pituitary by several days. Immunoreactive GnRH can be detected in the brains of rat fetuses at day 12 (Aubert et al., 1985). Hypothalamic GnRH levels remain low until around days 17 to 18, after which they begin to increase (Nemeskeri et al., 1983). Pituitary luteinizing hormone (LH) can be detected around day 17, and a response to GnRH can be observed by days 17 to 18 (Salisbury et al., 1982). However, pituitary follicle stimulating hormone (FSH) is detected much later, between days 19 to 21. On the other hand, the significance that plasma gonadotropins may have for the development of the fetal ovary remains unclear.

The gonadotropin releasing system undergoes functional development changes. Beginning shortly after birth, plasma FSH levels start to increase, reaching maximum levels by day 12,

after which the values gradually decline (Döhler and Wuttke, 1974). Plasma LH is also more elevated in the neonatal-infantile than in the juvenile period (Döhler and Wuttke, 1974). The hypothalamic content of GnRH increases markedly between the day of birth and the end of juvenile development (Aubert et al., 1985). It is well known that the responsiveness of the pituitary to GnRH is much greater in infantile rats than in juvenile rats. This increased responsiveness may be due to increased number of gonadotrophs in the pituitary of younger rats (Denef et al., 1978).

Another factor that contributes to elevated gonadotropin levels in infantile rats is the relative ineffectiveness of estradiol ( $E_2$ ) negative feedback. Administration of  $E_2$  can more effectively depress gonadotropin levels in ovariectomized infantile rats than in juvenile rats (Ojeda and Ramirez, 1974). On the other hand, the  $E_2$  positive feedback system may also be an important factor in control of gonadotropin release since a stimulatory effect of  $E_2$  on LH release cannot be demonstrated before the third week of postnatal life. (Andrews et al., 1981; Puig-Duran and MacKinnon, 1976). In the study by Andrews and his colleagues (1981), implanted Silastic capsules containing  $E_2$  dissolved in oil (400  $\mu\text{g}/\text{ml}$ ) produced first proestrous levels of  $E_2$ ; serum LH levels were determined by RIA. Before day 15 of age  $E_2$  was unable to stimulate LH release. Between 16 and 20 days of age,  $E_2$  was found to be

effective in inducing an LH surge, but the dose required was twice as high as those observed on proestrus (400  $\mu\text{g}$  /ml). After day 20, proestrous  $\text{E}_2$  levels were sufficient to induce a LH surge. In these rats the LH surge occurred 54 hours after implantation of  $\text{E}_2$  capsules, but when  $\text{E}_2$  was administered to rats older than 28 days of age a LH surge occurred within 30 hours. The results indicate that as rats mature the hypothalamic-pituitary unit becomes more sensitive to the stimulatory effect of  $\text{E}_2$ , and suggest that the stimulatory effect of  $\text{E}_2$  on LH is due to the activation of GnRH release from the hypothalamus (Sarkar and Fink, 1979; Sarkar et al., 1976).

In the developing animal the capacity of endogenous  $\text{E}_2$  to stimulate LH release depends on the ability of the ovary to produce  $\text{E}_2$  in sufficient amounts and for a sufficient period of time (Ferin et al., 1969). The first unambiguous hormonal manifestation disclosing that puberty is eminent occurs only after the fourth postnatal week of development. It is expressed as a diurnal change in LH release that becomes apparent eight to nine days before the day of first proestrus, and appeared to be greater in the afternoon than in the morning (Meijs-Roelofs et al., 1983) This pattern was characterized by afternoon increases in LH pulse amplitude. Also, only when circulating  $\text{E}_2$  levels were slightly increased over juvenile values (via subcutaneous  $\text{E}_2$ -containing Silastic

capsules) did a minisurge of LH secretion occur, indicating that they are  $E_2$  dependent (Urbanski and Ojeda, 1986). At the end of the juvenile period the hypothalamic GnRH-secreting system becomes activated by multifactors. The involvement of norepinephrine (NE) and  $PGE_2$  in stimulating GnRH release was confirmed directly by Sarkar et al. (1981) and Ojeda and Campbell (1982). Other studies indicate also that  $E_2$  exerts its action by activating an NE- $PGE_2$ -dependent pathway (Ramirez et al., 1985; Ojeda et al., 1985).

Increased GnRH may be responsible for the pattern of LH release characterized by afternoon LH pulses of large amplitude and an overall increase in basal LH level. These changes may represent a major determining factor in the timing of the onset of puberty because, under the influence of LH, the ovary is stimulated to produce more  $E_2$ . In turn, increased  $E_2$  levels may evoke minisurges of LH secretion which can further induce ovarian activation.

Once the diurnal pattern of LH release becomes established, a new cascade of events develops that culminates with the first preovulatory surge of gonadotropin and the first ovulation. To examine this cascade it is important to divide the process of puberty into different phases. According to one classification (Ojeda et al., 1976), which is based mainly on morphological criteria, puberty in female rat can be divided into the following phases: anestrus, proestrus, estrus

and diestrus. The specific morphological criteria are listed in the materials and methods section.

#### 4. Prostaglandin Involvement in the Development of the Reproductive System of the Female Rat

It is becoming increasingly evident that eicosanoid derivatives from arachidonic acid play an important role in the regulation of hypothalamo-pituitary-gonadotrophic activity, and are widely involved in various components of the reproductive system. Early studies, employing the systemic administration of prostanoids or drugs which inhibit their biosynthesis, advocated a stimulatory role for certain prostaglandins.

##### A. PGE<sub>2</sub> involvement in gonadotrophin release

Numerous studies indicate that prostaglandins (PGs), and in particular PGE<sub>2</sub>, play a physiological role in the regulation of GnRH release. The capacity of the hypothalamus to synthesize PGE<sub>2</sub> increases remarkably at puberty, just prior to the first preovulatory LH surge (Ojeda and Campbell, 1982). Intracerebroventricular (i.c.v.) administration of PGs, which allows immediate access to the hypothalamus, induced ovulation in "pharmacologically-blocked" rats (Spies and Norman, 1973) and elevated serum LH and FSH concentrations in control rats (Harms et al., 1974; Ojeda et al., 1977; Ojeda et al., 1977). However, corresponding injections into the pituitary gland

produced no such effects in the rat (Harms et al., 1974; Eskay et al., 1975). All studies demonstrated that the most potent of the PGs given i.c.v. was PGE<sub>2</sub>, which has been shown repeatedly to elicit the release of LH and FSH (Ojeda et al., 1976). The increase in serum LH and FSH induced by i.c.v. PGE<sub>2</sub> was accompanied by increased GnRH in the hypophysial portal blood (Eskay et al., 1975) and was attenuated in rats in which GnRH activity was neutralized with an appropriated antiserum (Chobsieng et al., 1975; Drouin et al., 1976). Indomethacin, an inhibitor of cyclooxygenase, attenuated gonadotropin secretion when implanted in the median eminence (Sato et al., 1975) or medial basal hypothalamus (Ojeda et al., 1975). Complementary data have also been obtained from in vitro studies which confirm the ability of PGs, notably PGE<sub>2</sub>, to stimulate the release of GnRH from hypothalamic fragments and median eminence tissue maintained in static or dynamic conditions (Gallardo and Ramirez, 1977; Bigdeli and Snyder, 1978; Heaulme and Dray, 1984).

It can be concluded from the studies completed to date that an increase in PGE<sub>2</sub>-synthesizing capacity of the media eminence occurs at the time of the preovulatory LH surge in the rat, and that the administration of an inhibitor of PG synthesis interferes with the time of LH surge (Brown and Poyser, 1984 ); the latter action appears to be exerted at hypothalamic levels via inhibition of GnRH release.

### B. PG involvement in ovulation

In addition to the regulatory role of PGs as mediators of hormone action in the hypothalamus, the function of PGs at the ovarian level has also attracted more and more attention in recent years (LeMaire et al., 1987; Linder et al., 1980; Niswender and Nett, 1988). During the preovulatory period, the LH surge induces a three- to five-fold increase in both the quantity and activity of PG endoperoxide (PGH) synthase, the enzyme which regulates the conversion of arachidonic acid to PGH in whole rat ovaries (Huslin et al., 1987). LH stimulation increases ovarian PG production, which reaches a maximum concentration at the time of ovulation. This preovulatory increase in ovarian PGs appears to be essential for follicular rupture since inhibition of PG synthesis blocks ovulation (Linder et al., 1980; LeMaire et al., 1988; Lipner, 1988).

The increase of ovarian PGs under the influence of exogenous hCG or endogenous LH takes place only in the follicles that will mature and ovulate, and not in those that become atretic. Several studies indicate that PGE<sub>2</sub> appears to be involved in the dissolution of cell-to-cell contacts in preovulatory follicles, as well as in maturation of the oocyte (Murdoch, 1988; LeMaire and Marsh, 1975; Tsafiriri et al., 1972 ).

Furthermore, PGE<sub>2</sub> has been directly implicated in the process of ovulation. Administration of PGE<sub>2</sub> in the early

afternoon to proestrous rats elicited a rise in serum LH levels and induced ovulation, while indomethacin effectively blocked the same. This study also confirms that indomethacin does not block LH release but interferes with a later phase of the ovulatory process. PGE<sub>2</sub> reverses this action of indomethacin on the ovary (Tsafriri et al., 1973). It was suggested by Beers et al. (1975) that plasminogen activator may play a role in the process of follicular rupture that leads to ovulation. The protease plasmin, a product of the reaction catalyzed by plasminogen activator, was able to weaken follicle wall strips in vitro (Beers, 1975). In addition, rat granulosa cells released plasminogen-dependent fibrinolytic activity and such activity was greatest in granulosa cells obtained from preovulatory follicles. PGE<sub>2</sub> was shown to stimulate granulosa cells to produce the enzyme activator (Strickland and Beers, 1976). It was assumed that ovulation is due to LH stimulated cAMP production followed by increased follicular PG synthesis and resultant granulosa cell secretion of plasminogen activator.

## STATEMENT OF PROBLEM AND SPECIFIC AIMS

Marine oils contain eicosapentaenoic acid, a fatty acid that may alter eicosanoid metabolism and result in the synthesis of trienoic, in preference to dienoic, prostanoids. In order to develop and function properly, the female reproductive system in the rat requires the synthesis of dienoic prostanoids at both hypothalamic and ovarian levels. This is readily apparent during the period from the late juvenile stage to the onset of puberty.

Because of the important roles played by dienoic prostanoids in the reproductive system, these experiments were designed to examine the impact of dietary fish oil on the development of puberty in the female rat.

The specific questions addressed in the study are:

1. Is first ovulation delayed by feeding a diet containing FO?
2. Does dietary FO alter the neuroendocrine events leading to the first ovulation?
3. Does dietary FO affect estradiol-positive feedback system?
4. Can acute PGE<sub>2</sub> injection in FO-fed rats restore the neuroendocrine and ovarian events leading to ovulation?

## MATERIALS AND METHODS

### 1. Animals

Immature albino HSD rats were purchased from Harlan Sprague Dawley (Indianapolis, IN). This species was derived in 1925 through a series of crosses begun with a single hooded male and six albino females of unknown origin at Sprague-Dawley Company in Madison, Wisconsin. Current HSD colonies are direct descendants of this original colony.

Albino females were shipped overnight from San Diego, CA and received in Tucson at 22 days-of-age. All rats were maintained in a room where conditions of temperature (23° C) and photoperiod (L14:D10, lights on at 6:00 h) were controlled. They were housed two per hanging metal cage, each containing a removable stainless steel food cup. Water was provided ad libitum.

The animals were examined daily for vaginal opening and weighed at least every other day. Vaginal smears were obtained on the day of vaginal opening and everyday thereafter when required. This was accomplished by vaginal lavage with deionized water and a fine, fire-polished eye dropper. Small drops of each lavage were placed on a microscopic slides and examined immediately without cytological staining. The smears were allowed to dry and were subsequently stained with toluidine blue; in this manner a permanent serial record was

maintained of the vaginal smear cytology for each rat. To avoid adverse effects of anesthetics on hormonal and prostaglandin titers, all rats were killed by rapid decapitation at the termination of each experiment.

## 2. Diets

### A. Composition of diets

Two custom diets were prepared by ICN Biochemicals (Cleveland, OH). Immediately after preparation the diets were sealed in aluminum foil containers and shipped on dry ice to Tucson by air freight. The fish oil (FO) contained eicosapentaenoic acid (EPA) as the primary fat whereas the control safflower oil (SO) diet contained linoleic acid. With the exception of the fats all components in the diets were identical as indicated in the following table:

Table 1. Composition of experimental diets

	<u>Linoleic Acid Diet</u>	<u>Fish Oil Diet</u>
Safflower oil	*212	0
Menhaden oil	0	212
Casein	190	190
DL-Methionine	3	3
Sucrose	300	300
Cornstarch	200	200
Cellulose	50	50
Mineral mix	35	35
AIN Vitamin mix**	10	10

\* All components are g/Kg. \*\* contains 200mg/Kg of vitamin E  
 Vitamin E was added to the diets to prevent peroxidation.  
 The diets were maintained at -20° C before use.

#### B. Compositions of fatty acids in dietary oils

Menhaden oil is made from Menhaden, a member of the Herring family. These fish are found in abundance along the Atlantic coasts of Europe and the United States. Safflower oil is made from the safflower plant and consists mostly of linoleic acid (76.0-80.0%) and oleic acid (11.0-13.5%). The percentage fatty acid composition of the two experimental diets is shown in the following table:

Table 2. Percentage of fatty acid composition of dietary oils

<u>Fatty acid</u>	<u>Menhaden oil</u>	<u>Safflower oil</u>
C14	8.4	
C15	0.9	
C16	15.2	6.0-7.5
C16:1	11.6	Tr-0.1
C16:2	2.4	
C16:3	2.0	
C16:4	1.7	
C17	0.8	
C18	2.7	2.0-2.5
C18:1	2.7	11.0-13.5
C18:2	1.8	76.0-80.0
C18:3	1.8	Tr-0.1
C18:4	3.5	
C19	0.1	
C20	0.2	
C20:1	1.3	
C20:2	0.3	
C20:3	0.4	
C20:4	2.3	
*C20:5	16.1	
C21:5	0.8	
C22	0.1	
C22:1	0.3	
C22:4	0.3	
C22:5	3.9	
C22:6	10.8	
C24:1	0.3	

Data provided by ICN Biochemicals, Cleveland, Ohio. Tr = trace  
 \* EPA represents approximately 16.1% of the fatty acid composition of menhaden oil.

### C. Pair-feeding

The amount of FO diet consumed by individual rats in a 24-hour period was recorded and that quantity of SO diet weighed out and given to the paired control rats. In this

manner the caloric intake was identical for the rats in both groups.

### 3. Radioimmunoassays

#### A. FSH, LH and PRL

Pituitary and serum FSH, LH and PRL were measured by standard double-antibody radioimmunoassays with NIADDK kits for the rat provided by the National Hormone and Pituitary Program of the National Institute of Health. The assays employed LH-RP-2, FSH-RP-2 and rPRL-RP-3 as reference standards. Aliquots of 100-200  $\mu$ l of sera were used in the LH and FSH assays. Smaller aliquots of 50-100  $\mu$ l were tested in the assay for PRL. The lower limits of detection were: for LH = 0.21 ng/ml; for FSH = 0.42 ng/ml; and for PRL = 0.66 ng/ml. Intraassay coefficients of variations for LH, FSH and PRL were 7.4%, 8.1% and 10.1%, respectively; interassay variations were 7.5%, 8.5% and 7.0%, respectively.

#### B. GnRH

GnRH was determined in the preoptic area/hypothalamus (POA/HYPO) dissected as follows: brains were rapidly removed and a transverse section made caudally in the hypothalamus, immediately rostral to the mammillary nuclei. The optic nerves were removed and parasagittal cuts placed in the hypothalamic sulci and continued rostrally along either side of the optic chiasma and the septum/preoptic area. The entire middle

hypothalamus with the attached median eminence, as well as the preoptic area ventral to the anterior commissure, was taken, rapidly frozen in liquid N<sub>2</sub>, and stored at -80° C. For the GnRH radioimmunoassay the POA/HYPO segments were sonicated in iced 0.1 N HCL and stored overnight at -20° C. On the next day the acid extracts were centrifuged, the pellets discarded and the supernatant portions neutralized with 1.0 N NaOH. Recovery of GnRH during the extraction procedure, estimated by the recovery of radiolabelled GnRH, was greater than 90%. Adjustments for variations in the recovery were not performed.

Antiserum for the radioimmunoassays was provided by Dr. V.C. Ramirez and the synthetic GnRH used for reference standard as well as for iodination was purchased from Peninsula Laboratories (Belmont, CA). The radioimmunoassay incorporated routine competitive binding procedures with the exception that alcohol precipitation was used instead of a second antibody. The lower limit of detection was 0.2 pg. Both intraassay and interassay coefficients of variations for the GnRH RIA were less than 5%.

### C. Prostaglandin E<sub>2</sub> radioimmunoassays

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) concentrations were determined in extracts of dissected POA/HYPO segments and ovaries with kits purchased from NEN Research Products (Wilmington, DE). Tissues were placed in polypropylene tubes containing 10 µg/ml of indomethacin in phospho-buffered saline at pH 7.4. Each sample

was subsequently homogenized for 60 seconds, after which 100  $\mu$ l of formic acid was added and the homogenate mixed by vortex. The homogenate was centrifuged at 10,000 x g for 20 minutes at 4° C and the supernatant fraction decanted to a separate polypropylene tube held in an ice bath.

In preparation for prostaglandin extraction disposable C18 octadecyl columns (J.T. Baker Inc., Phillipsburg, NJ) were washed with 2.0 ml methanol and equilibrated with deionized water. Placement of the isotope-containing extracts onto the column was followed by sequential washing with: 1) 20 ml of deionized water; 2) 20 ml of a mixture of ETOH/H<sub>2</sub>O (15:85, v/v), and 3) 20 ml of petroleum ether. The eluates were discarded in turn. Prostaglandins were eluted with 10 ml of cold methyl formate which was collected in polypropylene tubes, evaporated under a nitrogen jet and stored at -80° C until the radioimmunoassays were performed. Extraction efficiency was consistently greater than 85%. In order to determine the efficiency of extraction, 0.05  $\mu$ C of <sup>3</sup>H-PGE<sub>2</sub> (200  $\mu$ C/mmol, New England Nuclear) was added to each supernatant fraction. Intraassay and interassay variations for PGE<sub>2</sub> RIA were remarkable trivial, in both cases consistently less than 2.0%.

#### 4. E<sub>2</sub> Replacement

Silastic tubing (ID: 0.040 in, OD: 0.085 in) was

purchased from Dow-Corning Corporation (Midland, MI). Silastic capsules were cut to a length of 20 mm/100 body weight (BW) and filled with a solution of E<sub>2</sub> in peanut oil (400 µg/ml). The capsules were sealed at both ends and allowed to incubate overnight at 37° C in 0.1% gelatin-normal saline to stabilize release of the steroid. E<sub>2</sub> (β-estradiol) was purchased from Sigma Chemical Company (St. Louis, MO).

#### 5. Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>)

PGE<sub>2</sub> was purchased from Cayman Chemical Company (Ann Arbor, MI) and stored at -20° C. Stock solutions of PGE<sub>2</sub> were prepared in 100% ethanol. Just prior to use, the stock solution was diluted with saline to bring the PGE<sub>2</sub> to the concentration of 1.0 mg/ml.

#### 6. Classification of Phases of Puberty

The various phases of puberty have been identified in Sprague-Dawley rats and a classification established based primarily on morphological criteria (Ojeda et al., 1976). The classification includes the following six phases:

##### A. Anestrus

This phase begins around 30 days-of-age and is characterized by the initiation of diurnal rhythms of LH secretion and a change in the mode of release to pulsatile patterns (Urbanski and Ojeda, 1985). The uterus is small (less

than 100 mg) and no intrauterine fluid can be detected. The vagina is closed.

B. Early proestrus I

This phase includes animals with vaginae closed but with large uteri; up to 140 mg containing intraluminal fluid.

C. Early proestrus II

This subphase has been proposed based on the accumulation of intraluminal uterine fluid. Rats in this phase would have uterine weights between 140 and 190 mg.

D. Late proestrus

This phase corresponds to the day of first proestrus when the vagina is still closed and the uterus is "ballooned" full of fluid. Uterine weight exceeds 200 mg. Most animals show closed vaginae but the vagina may be open. The first preovulatory surge of LH occurs in the afternoon on this day.

E. Estrus

First ovulation occurs on this day; uterine fluid is no longer present, the vagina is open and vaginal cytology shows mostly cornified cells. Fresh corpora lutea are readily discerned histologically in the ovary. This day usually coincides with vaginal opening.

F. First diestrus

There are many leukocytes in the vaginal cytology, as well as mature corpora lutea within the ovaries.

## 7. Preparation of ovaries for histology and morphometric examination

After weighing, one ovary from each animal was fixed in Bouin's solution. After 24 hours the ovaries were transferred to 70% ethanol before dehydration and embedding in paraffin.

Ovaries were sectioned in two different ways: in certain experiments only one section was taken through the center of each ovary, while others were serially sectioned at 8.0 micrometer. All sections were stained with hematoxylin and eosin. Corpora lutea were counted on serial section and the numbers of recruited follicles were determined from counts on one section from each ovary.

## 8. Statistical analysis

Significant differences between control and treatment groups were determined by analysis of variance with ANOVA and Student's t test. In addition, analysis of the distribution of puberty phases and the number of ovulation between control and treatment groups was carried out using Chi-square ( $\chi^2$ ) test.

## Experiment One. Effects of Dietary FO on Puberty in Female Rats

### 1. Rationale and Hypothesis

Menhaden oil contains EPA, a fatty acid which may alter the eicosanoid metabolism and result in the synthesis of trienoic, in preference to dienoic, prostanoids (Dulp et al., 1979; Needleman et al., 1979; Fischer and Weber 1984). In order to develop and function properly the reproductive systems of female rats require the synthesis of dienoic prostanoids at both hypothalamic and ovarian levels (Armstrong and Grinwich, 1972; Tsafiriri et al., 1973; Ojeda et al., 1975). This is readily apparent during the period of transition from the late juvenile stage to the onset of puberty (Ojeda and Urbanski, 1988; Bauminger and Lindner, 1975).

This experiment was undertaken to examine the impact of dietary FO on the maturation of the female reproductive system. We postulated that FO would reduce hypothalamic PGE<sub>2</sub> levels, prevent the preovulatory release of hypothalamic GnRH and in this manner impair and/or delay first ovulation. Furthermore, if ovarian PGE<sub>2</sub> levels were decreased by dietary FO, the mechanical processes involved in ova extrusion might be disabled.

## 2. Experimental Design

Immature female rats were received at 22 days of age and maintained in a room where conditions of temperature (23° C) and lighting (14L:10D; lights on at 6:00 AM) were controlled. Thirty rats were fed a diet containing FO ad libitum; control rats were pair-fed an identical diet containing safflower oil as the fatty component. Water was provided ad libitum for both groups.

Body weights were recorded every other day and on the day of sacrifice. Beginning at 30 days of age the rats were inspected daily for vaginal opening, after which smears were prepared by vaginal lavage and evaluated by microscopic examination. All rats were decapitated on the morning of metestrus (diestrus one) after the first estrous smears were observed. Some of the FO-fed rats were killed on the first day of metestrus after demonstration of predominately cornified cells in vaginal smears over two or more consecutive days. Trunk blood, ovaries, pituitaries and uteri were collected, and a portion of brain was rapidly dissected containing the combined preoptic and hypothalamic areas. Tissues saved for prostaglandin radioimmunoassay were immediately frozen in liquid N<sub>2</sub> and stored at -80° C. Pituitary, ovarian and uterine weights were recorded; additionally, after removal of soft tissues the length of each right femur was measured with fine calipers to the nearest 0.1 mm.

### 3. Results

#### A. Body and organ weights

Body weights of FO-fed and control rats are shown in Figure 1. No differences were apparent before 38 days of age when the body weights of the controls increased sharply above that for the rats fed FO. The differences in body weights between the FO-fed and pair-fed controls groups were highly significantly at 38 ( $P < 0.001$ ) and 40 ( $P < 0.001$ ) days of age. Rats fed FO achieved the same final body weights five to six days later, at which time estrous vaginal smears were observed and the animals sacrificed on the following metestrus.

Table 3. Body and organ weights and femur lengths in rats fed FO and SO.

<u>Diet</u>	<u>Body Weights(g)</u>	<u>Organ Weights(mg/100 g BW)</u>			<u>Femur lengths (mm)</u>
		<u>Pituitary</u>	<u>Ovaries</u>	<u>Uteri</u>	
<u>SO</u>	111.8±2.0	3.8±0.1	33.0±1.4	133.1±8.1	25.5±0.2
<u>FO</u>	124.4±3.9 <sup>a</sup>	3.4±0.1*	21.2±1.3*	94.9±5.9*	25.7±0.2

<sup>a</sup>  $P < 0.05$  vs. SO; \*  $P < 0.001$  vs. SO; Values = mean±S.E.

As shown in Table 3, mean body weights of all FO-fed rats were significantly increased at the time of sacrifice on first metestrus ( $124.4 \pm 3.9$  for FO-fed rats vs.  $111.8 \pm 2.9$  for controls;  $P < 0.01$ ). This difference probably reflected the

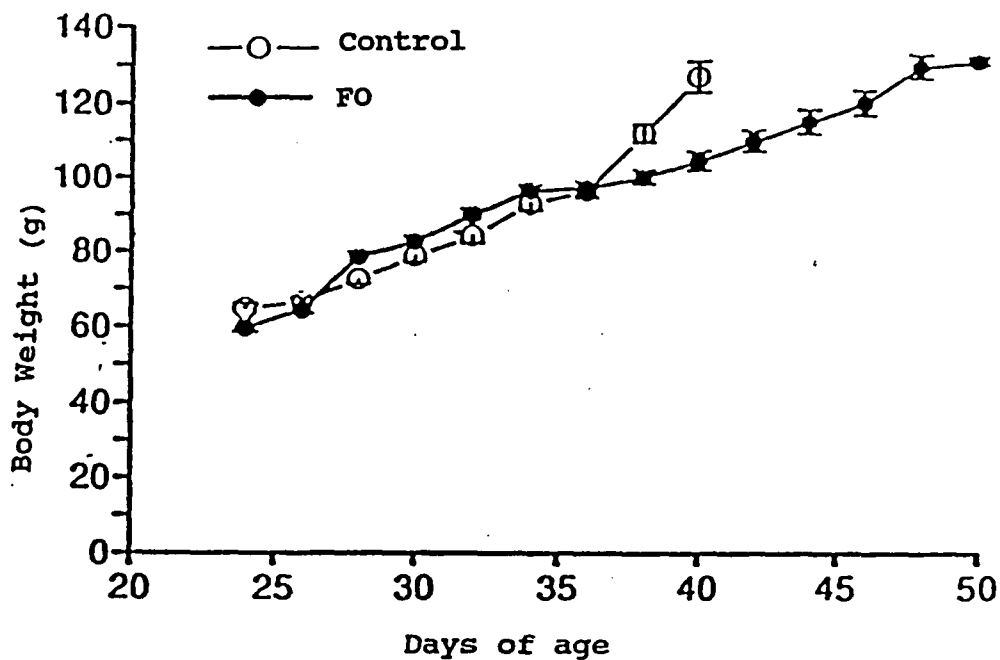


Figure 1. Body weights gain in FO- and SO pair-fed female rats. Before 34 days-of-age each point represents the mean $\pm$ S.E. for 30 rats; the number in each group declined after that time as individual animals were killed on the day of first diestrus. The differences between the FO- and pair-fed control groups were highly significant at 38 ( $P < 0.001$ ) and 40 days of age.

significant increase in age of the FO-fed rats at the time they reached first estrus.

When corrected for body weights pituitary, ovarian and uterine weights were significantly lower in the rats fed FO compared with the controls ( $P < 0.001$ ). The lengths of femurs were not different however, indicating that skeletal growth was not significantly affected.

#### B. Age at first estrus

Although the mean age of vaginal opening was not different between the two groups of thirty rats (in both cases between 34 and 36 days of age), the time at which an estrous vaginal smear was first recorded was significantly delayed in the FO-fed rats compared with that for the controls (see Figure 2). The control rats demonstrated their first estrous smears between 35 and 39 days of age (mean  $\pm$  S.E. =  $36.1 \pm 0.3$  days of age), whereas mean age at first estrus in the FO-fed rats was clearly delayed ( $42.9 \pm 1.0$ ). This difference was highly significant ( $P < 0.001$ ).

#### C. Ovarian morphological changes

As shown in Table 3, ovarian weights corrected for body weights were significantly reduced in the rats fed FO. The reduced ovarian weights probably reflects either absence or reduced numbers of corpora lutea. Most of the FO-fed rats which showed a delay in the demonstration of an estrous vaginal smear eventually exhibited estrous smears extending

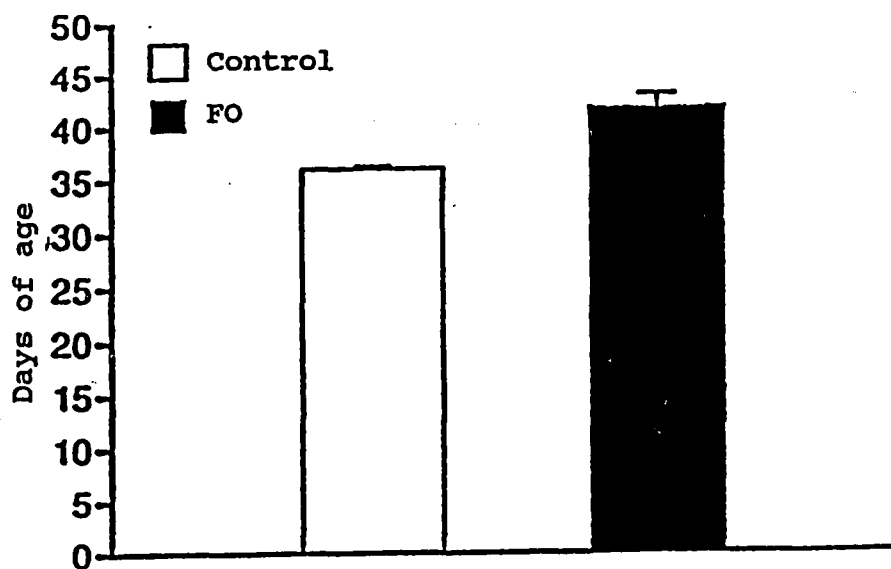


Figure 2. Age at first estrus in FO- and SO-fed rats. Each bar represents the mean  $\pm$  S.E. for 30 rats. Mean age at first estrus for FO-fed rats was  $42.9 \pm 1.0$  compared with  $36.1 \pm 0.3$  days for controls ( $P < 0.001$ ).

over several days.

Table 4. Ovarian corpora lutea in rats fed FO or SO.

<u>Diet</u>	<u>CL</u>	<u>No. rats with CL</u>
<u>SO</u>	4.8±0.6 <sup>*</sup>	28/30
<u>FO</u>	2.3±0.4 <sup>a</sup>	18/30

\* Corpora lutea (CL) in one ovarian section; Values = Mean±S.E.

<sup>a</sup> P<0.001 vs. SO.

It was notable that only 18 of the 30 rats fed FO ovulated as indicated by the presence of corpora lutea on first metestrus after the demonstration of an estrous vaginal smear on one or more days (see Table 4). The remaining 12 rats in this group did not ovulate despite the clear demonstration of estrous vaginal smears; microscopically all of their ovaries showed large antral follicles. Additionally (Table 4), the numbers of corpora lutea were significantly reduced in the ovaries of FO-fed animals that did ovulate ( $2.3 \pm 0.4$  vs.  $4.8 \pm 0.6$  for controls;  $P < 0.01$ ). These morphological differences probably account for the reduction in ovarian weights observed in FO-fed rats.

#### D. Gonadotropins and Prolactin

Circulating and pituitary gonadotropin levels were

similar at sacrifice on first metestrus ( see Table 5). Serum PRL also was not significantly altered by dietary FO at this time, whereas pituitary PRL was significantly increased ( $P < 0.01$ ). Since the rats were killed on first metestrus, when blood levels of these gonadotropins are baseline, this result was not unexpected.

Table 5. Gonadotropins and prolactin in rats fed FO or SO

	<u>FSH</u>	<u>LH</u>	<u>PRL</u>
<u>Serum</u> (ng/ml)			
<u>SO</u>	5.9±0.2	0.27±0.02	3.4±1.4
<u>FO</u>	5.5±0.1	0.30±0.03	2.7±0.7
<u>Pituitary</u> (ng/mg)			
<u>SO</u>	96.4±9.8	115.9±14.5	368.9±36.0
<u>FO</u>	86.8±9.8	127.4±13.5	553.1±51.8*

\*  $P < 0.01$  vs. SO. Values = mean±S.E.

#### E. POA/HYPO PGE<sub>2</sub> and GnRH

The weights of the dissected POA/HYPO segments of the FO-fed rats ( $27.8 \pm 0.7$  mg) were not significantly different from that of the pair-fed controls ( $26.3 \pm 0.7$  mg). Since no significant correlation could be demonstrated between these weights and their contents of PGE<sub>2</sub> ( $r = 0.3$ ,  $P > 0.05$ ), the data were expressed as pg of PGE<sub>2</sub> per POA/HYPO segment. As shown in Figure 3, POA/HYPO PGE<sub>2</sub> concentrations were reduced in FO-fed

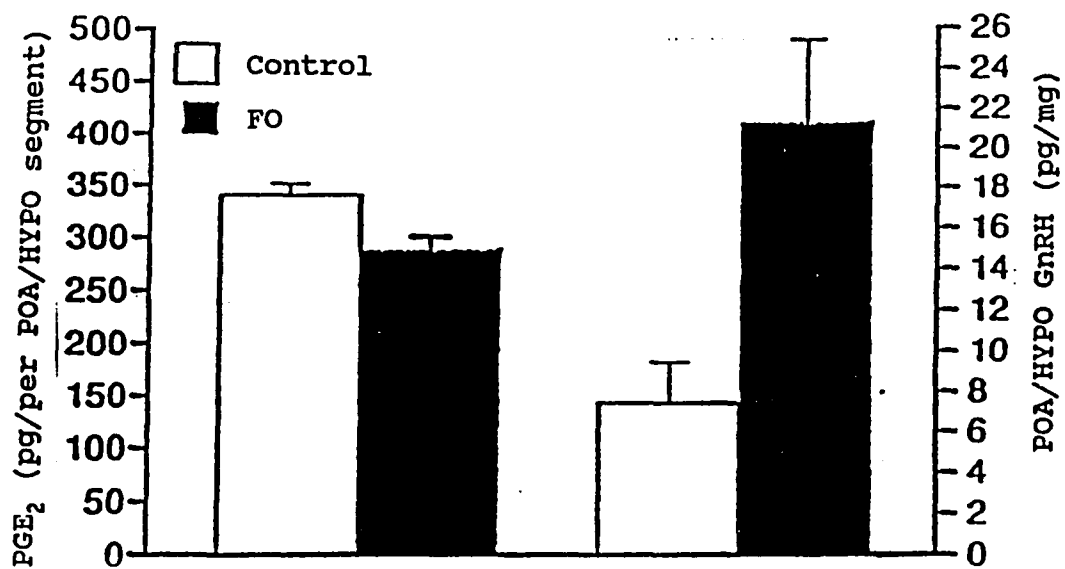


Figure 3. POA/HYPO GnRH and PGE<sub>2</sub> in FO-fed and control female rats on diestrus. Each bar represents the mean  $\pm$  S.E. for 30 rats. Both the suppression of PGE<sub>2</sub> content ( $P < 0.02$ ) and increase in GnRH concentration ( $P < 0.001$ ) in the FO-fed rats were significantly different from the pair-fed controls.

rats compared with the controls ( $P < 0.01$ ). This significant difference was not altered when the data were expressed as pg/mg of POA/HYPO.

On the other hand, POA/HYPO GnRH concentrations were significantly increased in the rats fed FO ( $21.4 \pm 4.0$  vs.  $7.6 \pm 2.2$  pg/mg for controls;  $P < 0.001$ ), suggesting perhaps either increased synthesis of this decapeptide or reduced release.

F. Comparison of POA/HYPO GnRH and PGE<sub>2</sub> levels in ovulatory and anovulatory FO-fed rats

When the FO-fed rats which ovulated were compared with those that did not ovulate, it became apparent that there were notable differences. The FO-fed rats which did ovulate had higher hypothalamic PGE<sub>2</sub> ( $314.2 \pm 10.8$  pg/POA/HYPO segment) and lower GnRH levels ( $13.5 \pm 2.4$  pg/mg) compared with the FO-fed rats which did not ovulate ( $258.6 \pm 10.0$  pg/POA/HYPO segment and  $35.5 \pm 6.8$  pg/mg), further indicating reduced PGE<sub>2</sub> in the failure of ovulation (refer to Table 4).

#### 4. Summary

The mean age at first estrus of the rats fed FO was significantly increased, indicating that dietary FO delayed the onset of puberty as was postulated. This effect of FO was obviously independent of skeletal growth since femur lengths were not affected. On the other hand, pituitary, uterine and

ovarian weights were significantly lower in the rats fed FO, suggesting that the development of these organs was deterred, numbers of corpora lutea reduced and circulating estrogen levels lowered by FO feeding. Compared with the controls the number of rats with corpora lutea, as well as the number of corpora lutea per ovary, were significantly reduced in FO-fed rats, indicating that first ovulation was impaired or prevented altogether. Since hypothalamic GnRH was significantly increased in FO-fed rats while hypothalamic PGE<sub>2</sub> levels were concomitantly decreased, the results suggest that dietary FO impaired or prevented first ovulation, through neuroendocrine mechanisms. Effects mediated via changes in prostanoid synthesis in the ovary were not ruled out however.

## Experiment Two. Effects of Dietary FO on Neuroendocrine and Ovarian Events Culminating in First Ovulation

### 1. Rationale and Hypothesis

The results of Experiment One suggested that dietary FO delayed the development of the reproductive system in female rats and impaired or prevented first ovulation. However, the animals in Experiment One were killed on metestrus, immediately following the first demonstration of an estrous vaginal smear, rather than on proestrus when dynamic

neuroendocrine events occur. Therefore, this experiment was conducted in order that we might examine the spectrum of effects of dietary FO throughout the proestrous-estrous period, when rising  $E_2$  levels stimulate hypothalamic  $PGE_2$  synthesis (Ojeda et al., 1979; Ojeda and Campbell, 1982; and Ojeda et al., 1986) and effectively modulate the NE-stimulated release of GnRH (Ojeda et al., 1979; Ojeda et al., 1982 and Ojeda et al., 1986).

Certain investigators have examined ovarian  $PGE_2$  synthesis (LeMaire and Marsh, 1975; Bauminger et al., 1975; Bauminger and Linder, 1975; Advis et al., 1979) and its relation to follicle development (LeMaire and Marsh, 1975; Tsaafiriri et al., 1972) and ovulation (Tsaafiriri et al., 1973; Strickland and Beers, 1976). These studies have pointed out a significant role for dienoic prostanoids in these processes. Accordingly, the effects of dietary FO on ovarian  $PGE_2$  synthesis were examined in this experiment in an attempt to assess its role in ovarian follicular development and ovulation.

We postulated that FO feeding would reduce the synthesis of hypothalamic  $PGE_2$ , result in failure of  $PGE_2$ -mediated GnRH release during the dynamic proestrous phase, and culminate in inadequate pituitary release of LH and ovarian stimulation. It was also expected that concomitantly reduced ovarian  $PGE_2$  levels would retard the maturation of ovarian follicles and/or

disrupt the mechanical processes of ova release.

## 2. Experimental Design

Immature Sprague-Dawley rats were maintained in a room where conditions were identical to those described in Experiment One. They were fed dietary FO ad libitum beginning at 22 days of age; control rats were pair-fed the SO diet. Vaginae were inspected daily for opening and body weights were recorded every other day.

Starting at 30 days of age, and on each day through 39 days of age, subgroups of five FO-fed and five SO-fed rats were killed by rapid decapitation between 9:30 - 10:00 h. Trunk blood was collected, pituitary, uterine and ovarian weights were recorded and the status of uterine fluid (ballooning) noted. POA/HYPO segments were rapidly dissected from each rat. POA/HYPO PGE<sub>2</sub> as well as serum LH were measured by RIA. One ovary from each rat was stored at -80° C for PGE<sub>2</sub> analysis while the contralateral ovary was placed in Bouin's fixative and prepared for histological examination.

All animals between 30 and 39 days of age were classified in different phases of puberty according to previously established criteria (Ojeda et al., 1976; Advis et al., 1979; and Ojeda and Campbell, 1982).

### 3. Results

#### A. Effects of FO diet on different phases of puberty

Rats were classified into different phases of puberty from days 30 to 39 according to established criteria (Ojeda et al., 1976; Advis et al., 1979). Significantly different distributions in these phases were seen between FO- and SO-fed rats (see Table 6,  $P < 0.01$ ). It was notable that 86.0% of control rats ovulated while only 50% of the FO-fed rats ovulated, confirming the result of the first experiment.

Table 6. Puberty Phases in Female Rats Fed FO  
(From 30 to 39 Days of Age)

	<u>Anestrus</u>	<u>Proestrus</u>	<u>Estrus</u>	<u>Diestrus</u>
<u>SO</u>	4.0% (2/50)	10.0% (5/50)	42.0% (19/35)	44.0% (22/50)
<u>FO</u>	29.0%* (14/48)	20.8%* (10/48)	16.7%* (8/48)	33.3%* (16/48)

\*  $P < 0.01$  vs. SO.

#### B. POA/HYPO PGE<sub>2</sub> levels during the onset of puberty

As shown in Figure 4, compared with controls POA/HYPO PGE<sub>2</sub> levels were consistently and significantly lower in FO-fed rats throughout the period we examined ( $P < 0.01$ ), except at 30 days-of-age. POA/HYPO PGE<sub>2</sub> levels did not change in FO-fed rats throughout the period of study, whereas POA/HYPO PGE<sub>2</sub> appeared to increase gradually in controls compared with

baseline levels at 30 days-of-age; this was especially the case between 34 to 36 days-of-age at which time a large increase was observed.

As shown in Table 7, POA/HYPO PGE<sub>2</sub> concentrations were significantly reduced in the FO-fed rats at all phases of puberty compared with those in the controls (P<0.001). POA/HYPO PGE<sub>2</sub> levels changed with different phases of puberty in SO-fed rats, increasing from anestrus to maximal levels at proestrus, then declining at estrus and reaching lowest levels at diestrus. Rats fed FO showed a similar pattern of changes in POA/HYPO PGE<sub>2</sub> levels, however, these levels in the FO-

Table 7. POA/HYPO and Ovarian PGE<sub>2</sub> and Serum LH Levels in Female Rats Fed FO from 30 to 39 Days of Age

	<u>Anestrus</u>	<u>Proestrus</u>	<u>Estrus</u>	<u>Diestrus</u>
<u>SO</u>				
POA/HYPO	9.8±0.8	13.1±1.4	9.8±0.4	8.9±0.5
PGE <sub>2</sub> (pg/mg)				
Ovarian PGE <sub>2</sub> (pg/mg)	21.1±1.1	25.5±3.4	13.0±0.9	11.7±0.8
Serum LH (ng/ml)	0.24±0.01	0.26±0.02	0.16±0.01	0.15±0.01
<u>FO</u>				
POA/HYPO	6.4±0.8*	7.6±0.6*	7.0±0.5*	6.2±0.4*
PGE <sub>2</sub> (pg/mg)				
Ovarian PGE <sub>2</sub> (pg/mg)	9.3±0.8*	14.1±1.6*	7.1±0.7*	6.7±0.8*
Serum LH (ng/ml)	0.14±0.01*	0.15±0.02*	0.12±0.01*	0.11±0.01*

\* P<0.01 vs. SO. Values = mean±S.E.

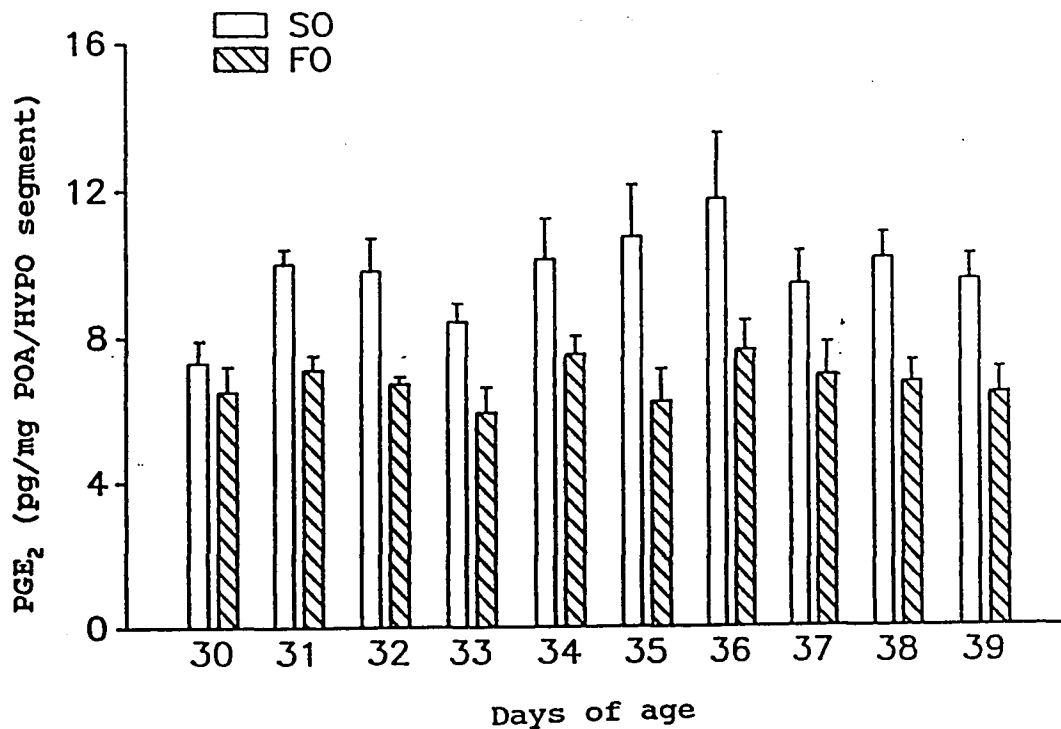


Figure 4. POA/HYPO PGE<sub>2</sub> levels at different days of age in FO- or SO-fed rats. Each bar represents mean $\pm$ S.E. for five rats. Except at 30 days-of-age, POA/HYPO PGE<sub>2</sub> levels were consistently and significantly lower in FO-fed rats throughout the period examined compared with pair-fed controls ( $P < 0.01$ ).

fed group failed to increase PGE<sub>2</sub> to the degree as that in the SO controls did at the critical period of proestrus.

C. Ovarian PGE<sub>2</sub> levels during the onset of puberty

Compared with those in SO controls ovarian PGE<sub>2</sub> levels in FO-fed rats (see Figure 5) remained low throughout the entire period examined, (P<0.01) except on days 30, 32 and 36. Ovarian PGE<sub>2</sub> levels in certain groups of SO-fed rats were two to three times higher than those in FO fed rats on days 34, 37, 38 and 39. It is clear that after 36 days-of-age, at which time the SO controls were approaching first ovulation, their ovarian PGE<sub>2</sub> levels were higher than those for the FO group.

Significant differences in ovarian PGE<sub>2</sub> levels were apparent when the animals were grouped according to different phases of puberty (see Table 7). Ovarian PGE<sub>2</sub> in SO-fed rats increased from anestrus to highest levels at proestrus, and declined thereafter on estrus and diestrus. Also, consistently lower PGE<sub>2</sub> levels were observed in FO-fed animals compared with the controls in every phase of puberty, It was clear that ovarian PGE<sub>2</sub> did not increase in FO-fed rats similar to SO-fed rats did at proestrus, an increase which may be essential for ovulation.

D. Serum LH levels during the onset of puberty

As shown in Figure 6, LH levels in the control group appeared to decrease from day 30 to day 37 before they rose

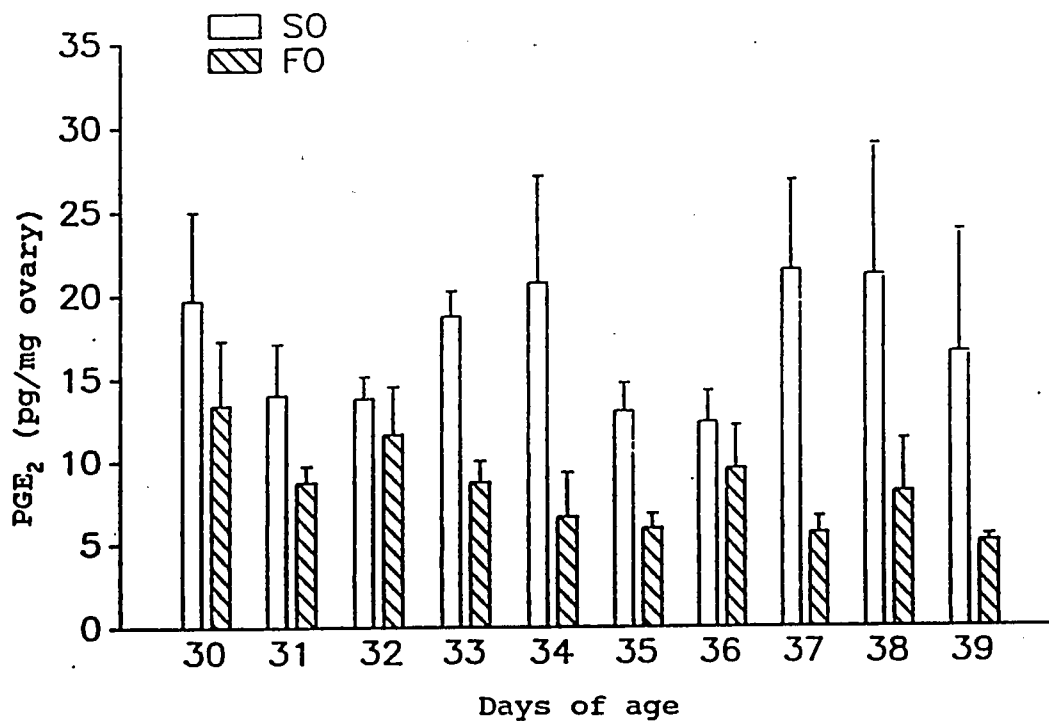


Figure 5. Ovarian PGE<sub>2</sub> concentrations at different days of age after FO feeding. Each bar represents the mean  $\pm$  S.E. for five rats. The differences in ovarian PGE<sub>2</sub> between FO-fed and SO-fed rats were significant on all days ( $P < 0.01$ ) except days 30, 32 and 36.

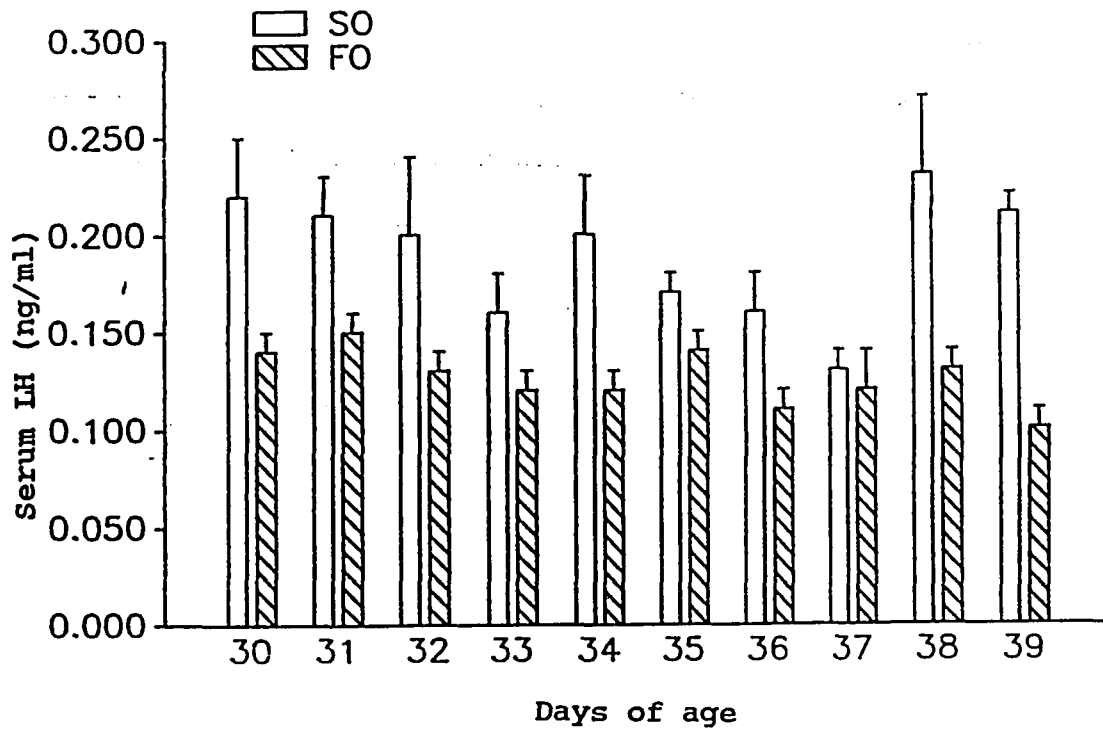


Figure 6. Serum LH levels at different days of age in FO- and SO-fed rats. Each bar represents mean  $\pm$  S.E. for five animals. Serum LH levels were significantly reduced in FO-fed rats compared with those in SO-fed controls ( $P < 0.01$ ) during all the times examined except at 37 days of age.

and reached almost two fold higher than those in the FO group after 37 days of age. Serum LH levels were significantly reduced in FO-fed rats compared with the SO-fed controls ( $P < 0.01$ ) during all the times examined except at 37 days of age, indicating reduced release or synthesis of this gonadotropin.

When grouped into different phases of puberty, serum LH levels in SO-fed rats were higher at anestrus and proestrus and declined at estrus and diestrus (see Table 7). Serum LH levels in FO-fed rats were significantly reduced at every phase of puberty compared with those in the controls, especially at proestrus when LH levels in the FO-fed rats remained markedly lower.

#### E. Organ weights, prostaglandin $E_2$ and serum LH

Since the events of puberty proceed with a degree of variability between the age 30 and 39 days in rats, the data were examined previously either on a daily basis or after grouping into various phases of puberty. Here (see Table 8) we have compared organ weights,  $PGE_2$  and LH levels in all FO- and SO-fed rats in this experiment without regard to age. There were no difference in mean body weights between all SO-fed and FO-fed rats ( $110.5 \pm 2.4$  vs.  $110.6 \pm 2.6$  g). Pituitary, uterine and ovarian weights corrected for body weights were significantly reduced in FO-fed rats compared with those in SO-fed controls ( $P < 0.01$ ). Ovarian and POA/HYPO  $PGE_2$  as well as

serum LH were also significantly lower in the rats fed FO compared with those in SO-fed controls ( $P < 0.01$ ).

Table 8. Organ Weights,  $PGE_2$  and Serum LH

	<u>Uterine Weights</u> (mg/100 g B.W.)	<u>Pituitary Weights</u> (mg/100 g B.W.)	<u>Ovarian Weights</u> (mg/100 g B.W.)
<u>SO</u>	173.0±7.0	4.4±0.1	34.7±0.8
<u>FO</u>	138.0±5.9*	3.8±0.1*	29.9±0.8*
	<u>Ovarian <math>PGE_2</math></u> (pg/mg ovary)	<u>Serum LH</u> (ng/ml)	<u>POA/HYPO <math>PGE_2</math></u> (pg/mg POA/HYPO)
<u>SO</u>	17.2±1.5	0.19±0.018	9.7±0.4
<u>FO</u>	8.3±0.7*	0.12±0.00*	7.1±0.3*

\*  $P < 0.01$  vs. SO. Values = mean±S.E.

#### 4. Summary

The results from this experiment indicate that dietary FO delays puberty in female rats, confirming the primary observation in Experiment One. Ovulation was clearly impaired by FO feeding in both experiments. Uterine weights in the FO-fed rats were reduced throughout the period examined compared with the SO controls, suggesting lower  $E_2$  levels. Overall, lower hypothalamic and ovarian  $PGE_2$  levels in FO-fed rats suggested a failure of  $PGE_2$ -mediated GnRH release during the proestrous-estrous period, resulting in inadequate pituitary

release of LH and the delay and/or failure to generate a preovulatory LH surge. The latter conclusion was supported by the consistently lower serum LH levels in the FO-fed rats.

In addition to neuroendocrine effects, FO feeding lowered ovarian PGE<sub>2</sub> levels and may have affected ovarian follicular development and the mechanical process of ovulation. Although the results of this experiment confirmed the hypothesis that FO feeding delays puberty, questions were raised about specific mechanisms. For example, impaired ovarian follicular development might have impaired estrogen synthesis resulting in a delay, or in some cases failure, to reach and maintain critical levels required for positive effects on GnRH and LH release. This possibility will be explored in the following experiment.

### Experiment Three. Effects of E<sub>2</sub> Treatment on Neuroendocrine and Ovarian Ovulatory Mechanisms in Rats Fed FO

#### 1. Rationale and Hypothesis

An E<sub>2</sub>-induced GnRH surge is an obligatory component of the first preovulatory release of gonadotropin (Sarkar and Fink, 1979; Andrews et al., 1982). It is well known that puberty can occur only when the ovaries develop the capacity to produce E<sub>2</sub> over a period of time and in sufficient amounts

to trigger an LH surge of preovulatory magnitude (Ojeda et al., 1986). This capacity is acquired concomitantly with the development of large antral follicles in the ovaries (Richards, 1980). It has been suggested that in female rats the development of E<sub>2</sub>-positive feedback mechanisms consists of several phases corresponding to age (Andrews et al., 1982). By the fourth phase, around 26-28 days of age, the rats have developed the ability to elicit an LH surge in response to blood E<sub>2</sub> levels of proestrous magnitude. During this period rising estrogen levels enhance both the sensitivity and the responsiveness of GnRH neurons to PGE<sub>2</sub> and NE (Ojeda et al., 1986).

Our previous results showed that rats fed FO had significantly depressed uterine weights, indicating that ovarian E<sub>2</sub> production may have been compromised by FO feeding. This could result from the observed lower ovarian PGE<sub>2</sub> levels since this prostaglandin induces follicular maturation. In this experiment a preovulatory level of E<sub>2</sub> was created in immature FO-fed rats in order to assess the hypothalamic responses. It was postulated that the ability of the POA/HYPO to synthesize PGE<sub>2</sub> in response to E<sub>2</sub> would be reduced in the rats fed FO and that this would result in the inability to release GnRH and evoke a timely LH surge.

## 2. Experimental Design

Rats were fed FO beginning at 22 days of age and controls were pair-fed SO. At 32 days of age FO-fed and control rats received subcutaneously Silastic capsule implants containing  $E_2$  in peanut oil (400  $\mu\text{g}/\text{ml}$ ; capsule length 20 mm/100 g BW) between 9:00 and 10:00 h. Groups of eight FO-fed and control rats were decapitated at two hours intervals beginning at 10:00 h on the following day (33 days of age), and continuing until 18:00 h. POA/HYPO segments were rapidly dissected and saved for  $\text{PGE}_2$  measurements. As in previous experiments serum LH levels were determined by RIA.

## 3. Results

### A. POA/HYPO $\text{PGE}_2$ after $E_2$ treatment

As shown in Figure 7, POA/HYPO  $\text{PGE}_2$  levels were significantly lower in the FO-fed rats than those in the SO-fed controls at all time points examined after  $E_2$  treatment ( $P < 0.01$ ). It can be seen that the greatest increase in these levels occurred in the SO-fed rats after 16:00 h, while a comparably slight increase was effected in the FO-fed rats. The results indicate that the central component of  $E_2$  positive feedback was impaired in the FO-fed rats.

### B. Serum LH after $E_2$ treatment

Similar to the POA/HYPO  $\text{PGE}_2$  response to  $E_2$ , significant differences were seen in that of serum LH between FO- and SO-

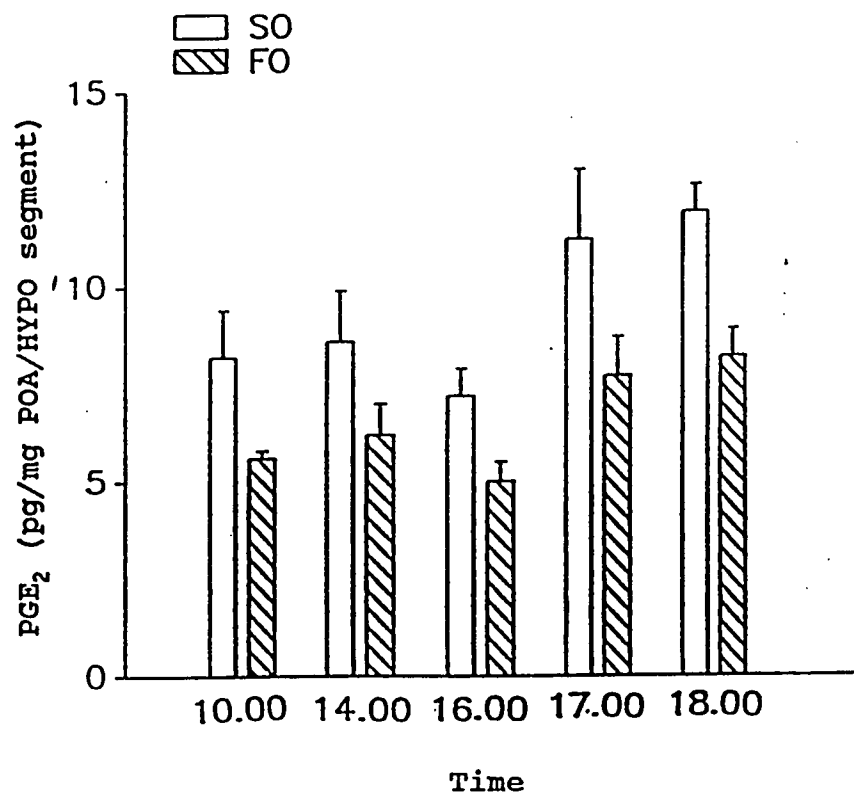


Figure 7. POA/HYPO PGE<sub>2</sub> levels after E<sub>2</sub> treatment in FO- and SO-fed rats. FO-fed rat and control rats received subcutaneously Silastic capsule implants containing E<sub>2</sub> in peanut oil at 32 days of age. POA/HYPO PGE<sub>2</sub> levels in FO-fed rats were significantly reduced at all time points compared with the controls (P<0.01).

fed rats (see Figure 8). Beginning at 14:00 h serum LH showed a gradual increase with time in the control rats. On the other hand, FO-fed rats remained at basal levels, increasing only slightly at 17:00 and 18:00 h. At 18:00 h LH was increased in SO-E<sub>2</sub> treated rats more than four fold over that for FO-E<sub>2</sub> treated rats.

#### 4. Summary

The results of this experiment indicate that FO-fed rats fail to generate surges in serum LH levels after E<sub>2</sub> treatment. Since the response of hypothalamic PGE<sub>2</sub> levels to E<sub>2</sub> was minimal, FO feeding appeared to result in the impaired ability to release GnRH, as was suggested by previous experiments. It is suggested that the positive feedback effects of estrogen are altered and FO-fed rats fail to elicit preovulatory levels of LH due to reduced release of hypothalamic GnRH. It is known that PGE<sub>2</sub> acts as a second messenger in GnRH release, and is increased by E<sub>2</sub> stimulation. If, as has been suggested, POA/HYPO PGE<sub>2</sub> synthesis is reduced in FO-fed rats, replacement of PGE<sub>2</sub> should reverse this impairment. Accordingly, the next experiment will test the effects of acute PGE<sub>2</sub> injection on neuroendocrine and ovarian ovulatory mechanisms in FO-fed rats.

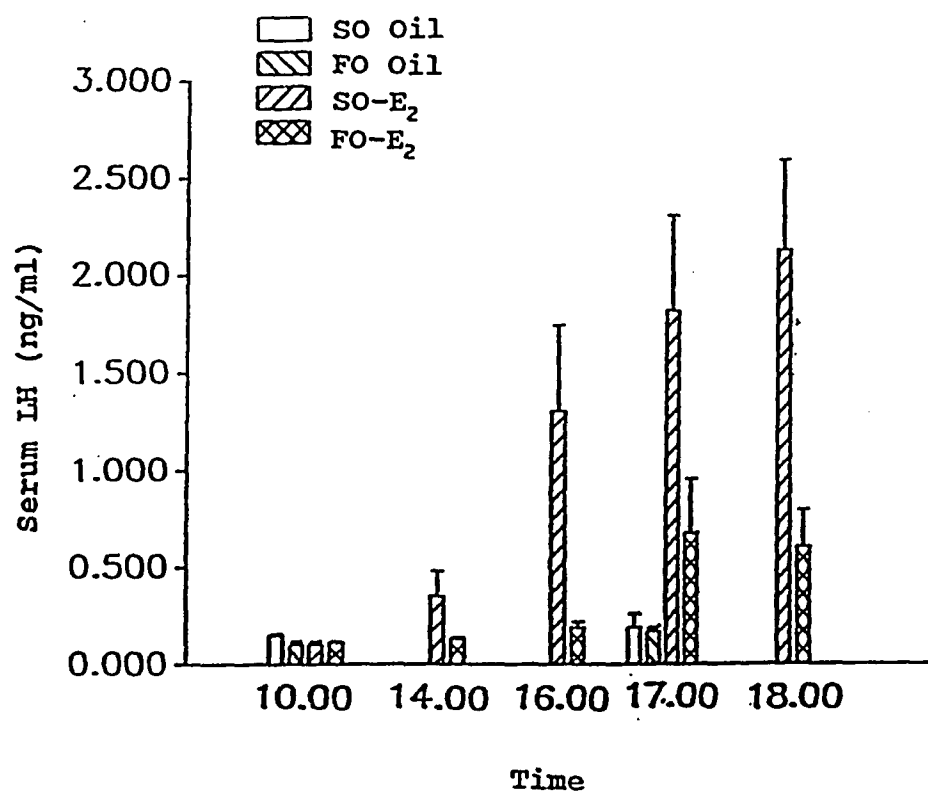


Figure 8. Serum LH levels after E<sub>2</sub> treatment in FO- and SO-fed rats. The mean±S.E. values for eight rats are shown at each time point. LH levels in FO-fed E<sub>2</sub> treated rats were significant lower compared with the controls at all times after 14:00 h (P<0.01).

## Experiment Four. Effects of Acute PGE<sub>2</sub> Injection on Neuroendocrine and Ovarian Ovulatory Mechanisms in Rats Fed FO

### 1. Rationale and Hypothesis

As pointed out in the introduction, PGE<sub>2</sub> is involved in the mechanism of GnRH release from the hypothalamus (Harms et al., 1973; Eskay et al., 1975; and Ojeda et al., 1975) and inhibition of prostaglandin synthesis with indomethacin in vivo decreases plasma LH levels (Carlson et al., 1974; Ojeda et al., 1975). Also GnRH levels in either portal or jugular vein blood increases after intraventricular infusion of PGE<sub>2</sub> (Ojeda et al., 1975; Eskay et al., 1975). Since the results of the first three experiments showed that dietary FO may have reduced release of hypothalamic GnRH by decreasing the synthesis of PGE<sub>2</sub>, it was postulated that PGE<sub>2</sub> replacement will result in GnRH release and effect preovulatory levels of LH in FO-fed rats.

### 2. Experimental Design

In order to avoid the surgical stress associated with indwelling cannula placement, the rats in this experiment were given PGE<sub>2</sub> intravenously. They were fed a diet containing FO beginning at 22 days of age. At 36 days of age subgroups were given either an alcohol-saline diluent or the diluent containing PGE<sub>2</sub>. PGE<sub>2</sub> (1.0 µg/g BW) was injected slowly into

a tail vein in an approximate volume of 0.2 ml. Groups of eight-nine PGE<sub>2</sub>- or saline diluent-treated rats were killed at 30 and 60 minutes postinjection. Others were killed at 24 hours postinjection and their ovaries were taken for assessment of ovulation by histology. Serum and pituitary LH levels were measured by RIA.

### 3. Results

#### A. Serum and pituitary LH in FO-fed rats after acute PGE<sub>2</sub> injection

As shown in figure 9, serum LH levels were significantly increased at both time points after iv PGE<sub>2</sub> injection compared with the response of the rats receiving saline (P<0.001). LH levels in PGE<sub>2</sub>-treated rats reached 0.45±0.03 ng/ml which was two times higher than those in controls at 30 min postinjection. The results indicate that the GnRH release system in FO-fed rats is capable of responding to PGE<sub>2</sub>.

Pituitary LH levels (see Figure 10) were significantly reduced in FO-fed rats receiving PGE<sub>2</sub> compared with those in controls (P<0.01). This was expected considering the marked increase in serum LH after PGE<sub>2</sub> treatment. LH levels in the control groups remained stable at baseline at both time points. The results suggest that the pituitaries of FO-fed rats responded to GnRH, however, further study will be needed to assess the full response of pituitary in FO-fed rats.

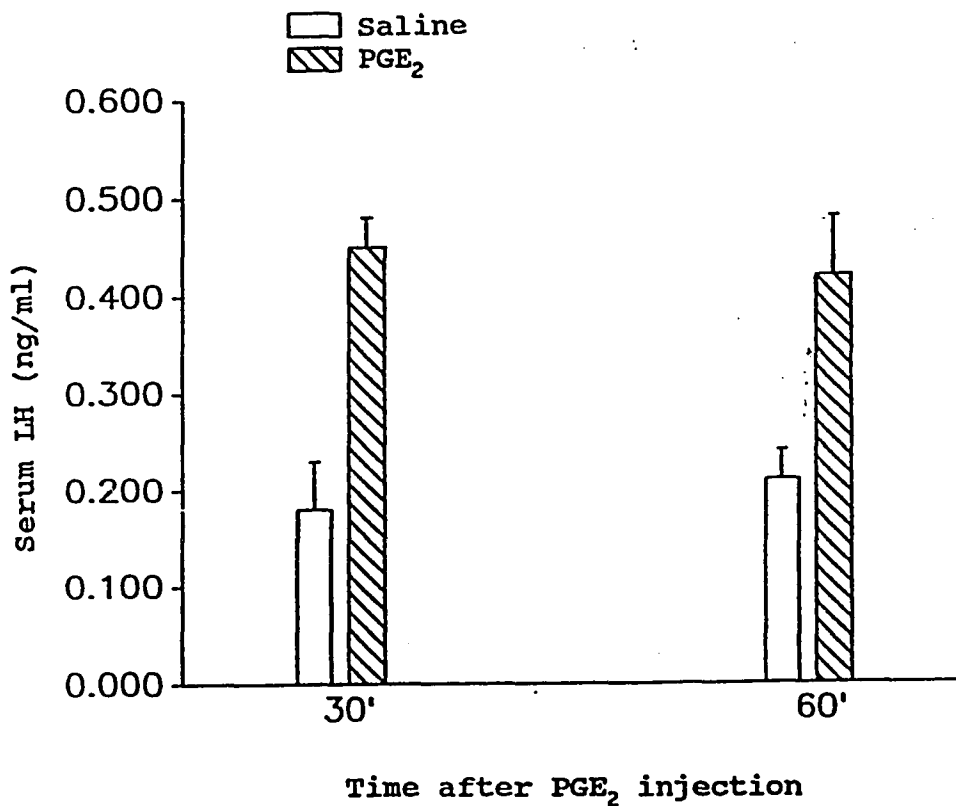


Figure 9. Serum LH levels in FO-fed rats after acute PGE<sub>2</sub> injection. FO-fed rats were given PGE<sub>2</sub> intravenously at 36 days of age. Each time point represents the mean  $\pm$  S.E. of eight rats. Serum LH levels in PGE<sub>2</sub> treated rats were significantly increased compared the rats receiving saline ( $P < 0.01$ ).

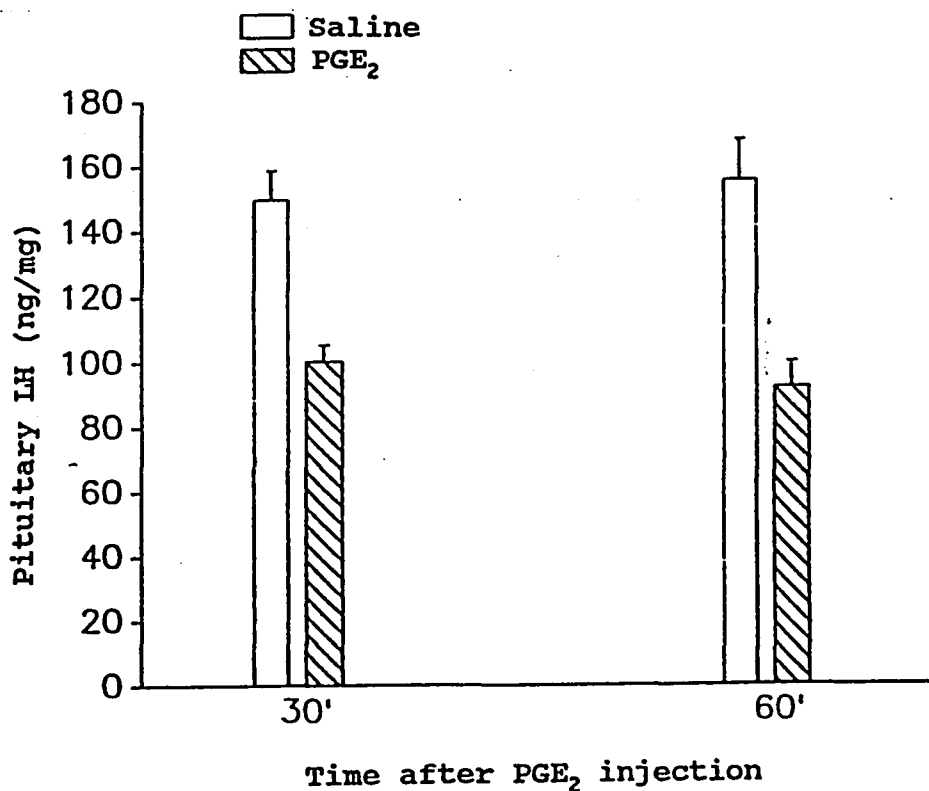


Figure 10. Pituitary LH in FO-fed rats after acute PGE<sub>2</sub> injection. FO-fed rats were given PGE<sub>2</sub> intravenously at 36 days age. Eight rats were killed at each time point postinjection. Pituitary LH levels were decreased in PGE<sub>2</sub> treated rats at both time points ( $P < 0.01$ ) compared with the controls.

Table 9. Ovulation in FO-fed Rats after 24 Hours  
of PGE<sub>2</sub> Injection

	<u>Number of ovulation</u>	<u>Percentage of ovulation</u>
<u>Control group</u>	1(1/8)	12.5%
<u>PGE<sub>2</sub> group</u>	6(6/9)	67.8%*

\* P<0.01 vs. SO.

B. Ovulation in FO-fed rats 24 hours after PGE<sub>2</sub>  
injection

As shown in Table 9, eight rats in both groups were killed 24 hours after PGE<sub>2</sub> injection. The ovaries were examined microscopically for the presence of corpora lutea. The results revealed that six out of nine rats that received PGE<sub>2</sub> ovulated, whereas only one out of eight rats ovulated after saline injection.

4. Summary

The results of this experiment showed that iv PGE<sub>2</sub> injection significantly increased the release of LH compared with the rats receiving saline. The numbers of ovulation in PGE<sub>2</sub>-treated rats were also higher than those in the controls. These results indicate that the GnRH release system of FO-fed

rats can respond to PGE<sub>2</sub> and stimulate the pituitary to release LH.

This study confirms that PGE<sub>2</sub> replacement can reverse the impairment of GnRH release, increase serum LH levels in FO-fed rats and induce ovulation. The latter effect is probably through the interactions of LH and increased ovarian PGE<sub>2</sub> after replacement.

## DISCUSSION

## 1. Morphological Effects of Dietary FO on Puberty

The physiological progression towards puberty is a dynamic process. During early proestrus, the ovaries increase in weight as follicles are recruited to produce estradiol. POA/HYPO GnRH stores increase and are released in late proestrus, prompting the release of pituitary gonadotropins and the surge in blood levels required to complete first ovulation.

The results of the present study clearly indicate that dietary FO impaired gonadal development in female rats, and either retarded or prevented the first ovulation. FO diet-delayed puberty was probably not due to impaired nutrition since the two groups of animals were pair-fed isocaloric diets and grew at the same rates up to the point of first estrus. However, the increase in body weights just prior to the first metestrus (at 38 and 40 days of age) in SO-fed rats in the Experiment One may relate to acute renal retention of water from elevated circulating estrogen levels (Christy and Shaver, 1974). That the growth of rats was not affected by dietary FO is also indicated by the observation that the lengths of right femurs were not different in the FO- and SO-fed rats in this experiment. The results suggest that dietary FO produced no sign of toxicity.

The mean age of vaginal opening was not different between SO- and FO-fed rats, whereas age at first estrus was significantly delayed at FO-fed rats. This suggests that the time of vaginal open and first ovulation were not concomitant in FO-fed rats, and the former may be more sensitive to lower levels of estrogen. An examination of ovarian morphology revealed that many of the FO-fed rats had not ovulated, despite open vaginae and the demonstration of estrous smears. It is interesting that many of the FO-fed animals without corpora lutea in their ovaries had numerous mature antral follicles, suggesting no impairment in folliculogenesis itself but a defect in the process of extrusion of ova from the ovary. The latter process has been linked to diene PG synthesis, which is locally augmented in the follicle just prior to rupture (Bauminger and Lindner, 1975; LeMaire and Marsh, 1975; Tsafriri et al., 1972; Strickland and Beers, 1976).

Pituitary, ovarian and uterine weights were significantly reduced in FO-fed rats at metestrus in Experiment One and at different days of age in Experiment Two. These reductions indicate that the development of these organs in FO-fed animals was deterred or impaired. The reduction in uterine weights suggests especially that rats fed FO had lower circulating estrogen levels regardless of the presence of healthy antral follicles. Although estrogen production was

sufficient to induce a timely vaginal opening, it is possible that the levels of estradiol were insufficient in many of the FO-fed rats to stimulate the required preovulatory surge of LH.

To our knowledge the effects of FO feeding on the onset of puberty have not been reported previously, although other reproductive effects have been observed. As examples, Olsen et al. (1986) found that the ingestion of marine oil prolongs gestation in women, probably by inhibiting the production of dienoic prostaglandins which are mediators of uterine contractions and cervical ripening; and sows fed fish oil were noted to have fertility problems (Opstvedt et al., 1988).

The present results demonstrate that the delayed reproductive development observed in rats fed FO diet appears to stem from alterations in both hypothalamic and ovarian function. These possibilities will be discussed separately in the following paragraphs.

## 2. Neuroendocrine Effects of Dietary FO.

### A. PGE<sub>2</sub>

The delay or prevention of first ovulation in rats fed FO may have been due to depressed POA/HYPO PGE<sub>2</sub> synthesis. This autocoid appears to serve as a signal transduction mediator of GnRH release (Ojeda et al., 1989; Ojeda and Campbell, 1982). Estradiol stimulates hypothalamic PGE<sub>2</sub> synthesis (Ojeda et

al., 1986), and GnRH release requires activation of norepinephrine-PGE<sub>2</sub> (DePaolo et al., 1982; Ojeda et al., 1979), and cAMP-PGE<sub>2</sub> pathways (Kim and Ramirez, 1986). Indomethacin, a potent cyclooxygenase inhibitor, has been shown to block GnRH release (Ojeda et al., 1975). The present study reveals significant depressions of POA/HYPO PGE<sub>2</sub> levels in rats fed FO, with marked retention of GnRH, suggesting failure of activation of these PGE<sub>2</sub>-dependent pathways for its release.

Previous studies (Ojeda and Campbell, 1982; Brown and Poyser, 1984) confirmed that there is an increase in PGE<sub>2</sub>-synthesizing capacity of the hypothalamus at the time of the preovulatory LH surge in the rat, and that the administration of an inhibitor of PG synthesis interferes with the timing of the LH surge (Brown and Poyser, 1984;). In the present study, the POA/HYPO from rats fed FO diet exhibited a marked reduction in PGE<sub>2</sub> concentration, suggesting a reduced ability to synthesize PGE<sub>2</sub> at different days of age during the prepubertal period. It was apparent that animals fed the SO diet displayed increased hypothalamic PGE<sub>2</sub> levels after 34 days of age in which the rats were approaching the onset of puberty (first ovulation), however, rats fed the FO diet failed to do so during this critical time.

Although FO effects have not been assessed previously, the importance of dietary essential fatty acids and dienoic

prostanoids to the onset of puberty was demonstrated in a recent study by Smith and his coworkers (1989). Immature female rats were fed a diet deficient in the essential fatty acids linoleic and linolenic acids. This dietary treatment reduced arachidonic acid availability and hence the capacity for PGE<sub>2</sub> synthesis. In this study the times of first ovulation were significantly delayed in the animals with essential fatty acid deficiency. In the present study, we provided EPA in menhaden oil as an alternative to the essential fatty acid linoleic acid, found in safflower oil. The FO-fed rats were not deprived of essential fats since menhaden oil contains a variety of fatty acids. Yet, as in the animals with essential fatty acid deficiency (Smith et al., 1989), first ovulation was delayed or prevented altogether by FO feeding. This dietary maneuver appeared to interfere with POA/HYPO production of the arachidonic acid metabolite PGE<sub>2</sub>.

The results from acute injection of PGE<sub>2</sub> clearly demonstrated that PGE<sub>2</sub> can stimulate release of LH in FO-fed rats. This confirms our hypothesis that PGE<sub>2</sub> replacement would increase GnRH release and effect the release of LH. The plasma LH levels in PGE<sub>2</sub>-injection rats fed FO were significantly increased compared with those in saline-injection rats fed FO at both 30 and 60 minutes postinjection. In contrast to serum LH, pituitary LH in PGE<sub>2</sub>-treated group were significantly reduced while saline-treated controls remained at higher

levels at both time points after injection, suggesting that PGE<sub>2</sub>-injection induced hypothalamic GnRH and pituitary LH release consequently increased. Saline, however, had no apparent effect on GnRH release, resulting in higher stores of LH in the pituitary and no significant release.

According to previous studies (Harms et al., 1973; Eskay et al., 1975; Harms et al., 1974), the site of action for intravenous PGE<sub>2</sub> is primarily at the level of the central nervous system since a marked increase in gonadotropin release followed the intraventricular injection of PGE<sub>2</sub> whereas injection into the anterior pituitary was without response in ovariectomized rats. Additionally, evidence for the effect of PGE<sub>2</sub> injection on the preovulatory LH surge, which is essential for ovulation, was obtained in the present study from histological examination of the ovaries at 24 hours postinjection. 67.8% of the FO-fed rats ovulated after PGE<sub>2</sub> treatment whereas only 12.5% ovulated in the saline-treated control animals. The difference between these two groups was significant, and the result indicates that PGE<sub>2</sub> injection induced a preovulatory LH surge, and resulted in ovulation in most FO-fed animals. From our present data no apparent evidence implies that the pituitary becomes unresponsiveness to GnRH after FO feeding.

#### B. GnRH

The observed decrease in PGE<sub>2</sub> production could also

affect the hypothalamic response to NE, an important step involved in GnRH release. Since the preovulatory LH surge is associated with an increased capacity of NE to induce hypothalamic PGE<sub>2</sub> synthesis and an increased GnRH response to PGE<sub>2</sub>, the LH surge may have been delayed or altogether nonexistent in FO-fed rats. Thus, lower POA/HYPO PGE<sub>2</sub> levels observed at different days of age in FO-fed rats may have indirectly resulted in the consistently lower LH levels observed during the prepubertal period.

In the first experiment POA/HYPO concentrations of GnRH were significantly elevated by FO feeding. Apparently, as a consequence of reduced GnRH release from the POA/HYPO during the prepubertal period, the rats fed FO in Experiment Two showed significant reduction in circulating LH levels compared with those in SO controls. It was also noted that SO-fed animals had significantly increased LH after 37 days of age, while LH in the FO-fed group remained at basal levels. This suggests that FO feeding resulted in a failure to manifest an LH surge, probably due to the reduction of GnRH release into the hypophysial portal blood vessels. From these results, the conclusion can be made that FO feeding probably depressed POA/HYPO PGE<sub>2</sub> production and impaired the release of GnRH in female rats during the prepubertal period. This dysfunction affected sexual development and delayed the onset of puberty.

### C. E<sub>2</sub> feedback

It is known that the capability of the hypothalamic-pituitary unit to respond to an increase in circulating estrogen levels develops gradually as rats matures. The development of the positive feedback of estrogen is a process characterized by a gradual, quantitative increase in LH and FSH sensitivity and responsiveness to steroid. Thus, estrogen can elicit an adult type of LH release of "surge" magnitude as the rats approached puberty. Under physiological conditions, the first preovulatory surge of gonadotropins is preceded by a four-fold increase in circulating estrogen levels and a three-fold increase in uterine weight.

$E_2$  can influence certain of the intraneuronal mechanisms involved in GnRH release during the onset of puberty in female rats. The capacity of median eminence (ME) nerve terminals to secrete GnRH increases significantly during the juvenile-early prepubertal periods of development which occurs in the rat between postnatal days 22-34 (Ojeda et al., 1986). The first preovulatory surge of gonadotropin secretion appears to be primarily the consequence of an  $E_2$ -induced increase in GnRH release. Simulation of first proestrous serum  $E_2$  levels in late juvenile female rats (28 days-of-age) enhances the sensitivity and the responsiveness of GnRH-containing terminals to  $PGE_2$  (Ojeda et al., 1986). Furthermore,  $E_2$  also enhances the sensitivity and the responsiveness of GnRH terminals to norepinephrine (NE). Since  $E_2$  can modify specific

components of the GnRH-secreting system by increasing the capacity of the juvenile medial basal hypothalamus to synthesize PGE<sub>2</sub> from arachidonic acid (Ojeda and Campbell, 1982), this effect appeared to be related to both the increased GnRH response to PGE<sub>2</sub> and an enhanced sensitivity of the PGE<sub>2</sub>-synthesizing pathway to NE. It is suggested that one of the mechanisms by which E<sub>2</sub> activates positive feedback at puberty on the preovulatory GnRH surge in female rats is by facilitating the occurrence of two different but sequentially related events: 1) the stimulation of PGE<sub>2</sub> formation by NE and 2) the enhancement of GnRH release by PGE<sub>2</sub>. In addition, it appears that maintenance of GnRH responsiveness to PGE<sub>2</sub>, which has been implicated as an obligatory component of NE-induced GnRH release, is also E<sub>2</sub>-dependent.

The present study provides insights into the mechanisms by which FO-feeding delays or prevents first ovulation. After E<sub>2</sub> treatment, hypothalamic PGE<sub>2</sub> levels in FO-fed rats were lower at all time points examined compared with SO-fed controls which showed, especially, a gradual increase after 16:00 h. This result implies that the hypothalamus of rats fed FO could not respond to juvenile serum E<sub>2</sub> levels by synthesizing PGE<sub>2</sub> from arachidonic acid. Concomitant with the effect of E<sub>2</sub> on hypothalamic PGE<sub>2</sub>, serum LH levels in rats fed FO were also reduced at all time points compared with SO controls (Experiment Two). It was noted that E<sub>2</sub> elicited a

sharp increment in LH levels in rats fed SO, which showed almost a four fold surge in LH.

The results suggest that FO-fed rats developed impairment of their  $E_2$  positive feedback systems, which deterred the maturation of the GnRH release mechanism in the hypothalamus and resulted in the failure to generate the preovulatory LH surge. The current data also give more support for the hypothesis that FO feeding affects the synthesis of  $PGE_2$  at the hypothalamic level, and also indicates that FO feeding resulted in low hypothalamic  $PGE_2$ , not only at diestrus but at all stages in the prepubertal period. Because of this change, the GnRH release process was impaired and GnRH accumulated in the hypothalamus. This culminated in a significantly different distribution of puberty phases between FO- and SO-fed animals.

### 3. Ovarian Effects of Dietary FO

An additional factor that probably contributed significantly to the delayed puberty in rats fed FO diet is altered ovarian synthesis of  $PGE_2$ . A preovulatory rise in the  $PGE_2$  content of the rat ovary has been reported, and the time of its occurrence could be advanced by the injection of LH before the onset of the endogenous LH surge (Bauminger and Linder, 1975). The preovulatory rise in ovarian PG's as well as in ovarian PG-synthetase activity could be prevented by treatment by nembutal, and restored by exogenous LH or FSH.

This finding clearly established a causal relationship between the proestrous gonadotropin surge and ovarian PG formation. The present results showed that FO-fed rats had consistently lower ovarian PGE<sub>2</sub> levels between 30 to 39 days of age, while SO-fed rats showed significantly higher ovarian PGE<sub>2</sub> levels throughout this period. It was notable that after 36 days of age, generally after vaginal opening and first ovulation has occurred, SO-fed animals showed gradually increasing PGE<sub>2</sub> levels whereas FO-fed rats remained at lower levels during this critical time.

The effect of lower ovarian PGE<sub>2</sub> after FO feeding should be considered a very important factor contributing to the observed delayed first ovulation. The reduced ovarian PGE<sub>2</sub> in FO-treated rats may be the consequence of two factors: 1) one is the possibility that EPA directly altered ovarian PGE<sub>2</sub> synthesis; 2) the other is that lower LH levels failed to increase PG synthetase activity and to stimulate ovarian PGE<sub>2</sub> synthesis. The latter factor was demonstrated in the study by Bauminger and Lindner who showed LH stimulated PG production in isolated follicles in rats (1975).

On the other hand, PGE<sub>2</sub> has been suggested to mediate some of the effects of LH on the ovary. For instance, PGs have been implicated in the process of ovulation (LeMaire and Marsh, 1975; LeMaire et al., 1975); it was shown that rat granulosa cells released plasminogen-dependent fibrinolytic

activity with increased PGE<sub>2</sub> concentration in ovarian follicles (Beers et al., 1975). Such activity was greatest in granulosa cells obtained from preovulatory follicles, and was inducible by LH treatment in vitro. As previously reported (Weiner and Kaley, 1972; Osvaldo-Decima, 1970), ovarian PGE<sub>2</sub> is also involved in the process of follicle rupture in which PGE<sub>2</sub> acts on lysosome and causes the release of lytic enzymes and affects smooth muscle fibers in the follicular wall. In addition, PGE<sub>2</sub> stimulates granulosa cells to produce plasminogen activator. The results of the present study indicate that FO feeding may affect the ovary directly in its delay of first ovulation via effects on PG synthesis.

Previous studies demonstrated that the pubertal changes in uterine weights are well correlated with both the ability of the ovary to release E<sub>2</sub> in response to gonadotropin stimulation and with the actual pubertal changes in circulating E<sub>2</sub> levels (Ojeda et al., 1976; Advis et al., 1979). Although we did not measure estrogen levels, the fact that the uterine weights of rats fed FO were significantly lower than those of SO-fed rats suggests that basal circulating estrogen titers were reduced by FO feeding throughout the prepubertal period. These observations strongly indicate that the inability of the ovary to secrete estrogen in quantities large enough to trigger a gonadotropin surge is also the mechanism by which FO feeding delays the onset of

puberty in female rats. Low ovarian estrogen production probably reflected both effects of lower ovarian  $\text{PGE}_2$  and lower gonadotropin stimulation directly on follicle development. The development of large preantral follicles into mature, large antral follicles may have been delayed since both  $\text{PGE}_2$  and LH are involved in the process of follicle maturation. Because of lower estrogen levels in blood the FO-fed rats failed to achieve a timely LH surge. Additionally, lower LH in FO-fed rats may have failed to stimulate plasminogen activation, an important step in the ovulatory process. All these factors could contribute to the delay in and/or failure of first ovulation in FO-fed rats.

It is known that the ovarian content of both LH and FSH receptors increases strikingly, and in a sequential manner, during prepubertal development of the rat (White and Ojeda, 1981). Marked increases in LH receptors, particularly in granulosa cells, occur during the onset of puberty, attaining maximal binding during the hours preceding the first preovulatory LH surge. Earlier studies have demonstrated that FSH is capable of inducing not only the formation of its own receptors but also, in the presence of  $\text{E}_2$ , the formation of LH receptors in granulosa cell (Richard et al., 1976). The probable sequence of changes in GnRH release and depressed gonadotropin and estrogen levels observed in FO-fed rats during the prepubertal period in the present study raises the

possibility that the aforementioned hormonal interactions would result in the alteration of the formation of LH receptors in granulosa cells in FO-fed rats which is undoubtedly an important requirement for the ovary to respond to gonadotropin stimulation.

Maintenance of elevated plasma  $E_2$  levels, for a defined period of time, results in an abrupt enhancement of GnRH release from the hypothalamus and an increased pituitary responsiveness to GnRH (Castro-Vazquez and Ojeda, 1977; Sarkar and Fink, 1979). The timely expression of these two events induces the first surge of gonadotropin secretion. The observation from the present experiments that FO feeding reduced both ovarian and uterine weights in the prepubertal period suggests that rats fed FO had lower plasma  $E_2$  levels in that defined period of time and the two events mentioned above were not expressed in the usual time. Furthermore, consistently lower circulating  $E_2$  levels throughout the prepubertal period may have affected the maturation of the GnRH-releasing system since the secondary component of this system is  $E_2$ -dependent (Ojeda et al., 1986).

#### 4. Summary

It can be concluded that FO feeding reduced  $PGE_2$  in both the POA/HYPO and ovaries of immature female rats. The release of GnRH and LH was probably altered in the prepubertal period

because of reduced PGE<sub>2</sub> synthesis. The development of follicles in the ovaries may also have been impaired, resulting in consistently lower E<sub>2</sub> levels and impairment of the development of the E<sub>2</sub> positive feedback system during the prepubertal period. Alterations in these interdependent mechanisms probably resulted in the delay or prevention of first ovulation in FO-fed rats. The hypothesis that delayed puberty resulted from reduced synthesis of POA/HYPO PGE<sub>2</sub> was strengthened by the observation that replacement of PGE<sub>2</sub> partly restored GnRH release and ovulation.

If EPA serves as the alternative to arachidonic acid for cyclooxygenase action, then it may be assumed that a certain amount of PGE<sub>3</sub> is produced in the FO-fed rats instead of PGE<sub>2</sub>. Although the present study demonstrated that POA/HYPO PGE<sub>2</sub> content was diminished in rats fed FO, PGE<sub>3</sub> was not measured. Furthermore, although trienoic prostanoid synthesis has been demonstrated in man and primates (Needleman et al., 1979; Abeywardena et al., 1989), the capacity of rodents to produce these eicosapentaenoic metabolites remains controversial (Hornstra et al., 1981). A complete understanding of the influence of dietary FO on reproduction awaits further clarification of this point, as well as delineation of the biological activities of the trienoic analogues of arachidonic acid metabolites.

In summary, the present study provides evidence for the

importance of dietary fatty acids in the development of the reproductive system in the rat. This initial investigation into the mechanism of the effect of FO on the maturation of the reproductive system suggests that the PGE<sub>2</sub>-dependent mechanisms that trigger GnRH release from the POA/HYPO, and ova extrusion from the ovary, may be primarily impaired . However, clarification of the role of trienoic prostanoids will ultimately be required for a complete understanding of the impact of a fish-rich diet on the reproductive system.

## SUMMARY

This study was undertaken to define the effects of dietary FO on the development of the reproductive system in the female rat. From the results presented, it is evident that dietary FO not only altered POA/HYPO PGE<sub>2</sub> synthesis, but also depressed ovarian PGE<sub>2</sub> levels, consequently resulting in the attenuation of GnRH release and the probable impairment of the mechanical processes involved in ova extrusion. Specifically, the answers to the questions addressed in this study are:

1. Is first ovulation delayed by FO feeding?

Yes. The age at first of estrus of the rats fed fish oil was significantly increased compared with the controls. Also, a different distribution of puberty phases between two groups indicate that puberty was delayed and first ovulation was prevented in FO-fed rats. In certain cases first ovulation was impaired or prevented altogether, as indicated by the histological observation that the number of CL, as well as the number of CL per ovary, were significantly reduced in FO-fed rats.

2. Does dietary FO alter the neuroendocrine events leading to the first ovulation?

Yes. POA/HYPO and ovarian PGE<sub>2</sub> levels were consistently lower during the prepubertal period in

the FO-fed rats while GnRH levels were increased.

3. Does dietary FO affect estradiol-positive feedback system?

Yes. The significantly lower uterine weights in rats fed FO indicate lower circulating  $E_2$  levels. No LH surge was produced in FO-fed rats by  $E_2$  treatment, and  $E_2$ -stimulated POA/HYPO  $PGE_2$  levels were significantly lower compared with oil-treated controls. The results suggest that FO feeding delayed or impaired the maturation of the central component of the  $E_2$ -positive feedback system.

4. Can acute  $PGE_2$  injection restore the neuroendocrine and ovarian events leading to ovulation?

Yes.  $PGE_2$  replacement induced pituitary LH release and increased serum LH levels in FO-fed rats. 67.8% of the FO-fed rats ovulated 24 hours postinjection.

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