PSYCHOLOGICAL AND NUTRITIONAL FACTORS INFLUENCING THE DYNAMICS OF GONADOTROPHIN SECRETION IN THE FEMALE RAT

by

John Manning de Wet B.A.(Hons), M.A.

A Thesis Submitted to the University of Cape Town
in Fulfilment of the Requirements
for the Degree of
Doctor of Philosophy

1981

The University of Cape Town has been given the right to reproduce this thesis in whole or in part. Copyright is held by the author.

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

ACKNOWLEDGMENTS

I wish to express my appreciation to the following people for their invaluable assistance:

To my supervisors Dr. C.J. Beardwood and Professor G.S. Saayman for their guidance and support.

An additional expression of gratitude to Dr. C.J. Beardwood for providing the laboratory facilities and materials for the radioimmunoassay determinations.

To Professor P.J.V. Beumont for arousing my interest in the field of anorexia nervosa.

To Associate Professor L. Viney for her helpful suggestions and comments during the preparation of the manuscript.

To Mr. Alex Reynolds and Mr. Francois Eggar for their assistance in constructing much of the apparatus used in this research.

To Mr. Laurie Kellaway whose dedication and perseverance served as an inspiration to me.

To my friends Lynne and Garrielle for their painstaking typing.

Finally, to Joan whose unswerving support made this work possible.

TABLE OF CONTENTS

List of Tables

List of Figures

ABSTRACT

This research has been concerned with the separate and combined effects of caloric deprivation and stress induction on the reproductive physiology of the female rat of the Long-Evans strain. Its objective was to determine whether the findings of controlled experiments provided support for existing hypotheses about the aetiology of amenorrhoea in anorexia nervosa.

The relationship between alterations in body weight and reproductive function was investigated at regular intervals during caloric deprivation and nutritional rehabilitation. The aim was to determine the extent and duration of caloric deprivation required to induce anoestrus and whether the anoestrus was reversible. The latency of resumed oestrous cycling and associated body weight changes was investigated with rats on different rehabilitation regimens. A related objective was to determine whether a relationship existed between duration of malnutrition and recovery of the oestrous cycle. Pharmocological tests were used to investigate which components of the hypothalamic-pituitary-ovarian axis were dysfunctional during anoestrus.

Groups of sexually-mature female rats were fed diets in which the caloric contents were systematically reduced. After 21 days feeding on a dextrin and fat free (DFF) diet, groups of rats were nutritionally rehabilitated at different rates. At regular intervals blood samples were collected for serum LH determination by double antibody radioimmunoassay. Pituitary LH levels were determined, organ weights were measured, the surface appearance of the ovaries were examined and the oviducts were inspected for the presence of ova. Anoestrous rats with indwelling jugular cannuli were injected with 50ng LHRH after slow infusion of either 50ng LHRH or saline and blood samples for serum LH assay were collected at regular intervals. In separate studies emaciated, anoestrous rats were injected with 2µg oestradiol

benzoate (OB) and 20IU pregnant mares serum (PMS) to determine whether ovulation could be induced. The PMS test was also used at intervals during different rates of nutritional rehabilitation.

It was found that the mechanisms regulating the oestrous cycle were resistant to relatively severe caloric deprivation. Only after complete removal of carbohydrate and fat components was anoestrus observed within a 21 day period. It took more than 7 days feeding on a DFF diet before serum LH levels and ovarian and uterine weights were significantly reduced. It was established that a relationship existed between weight loss and the onset of anoestrus. The average body weight of rats at the onset of anoestrus was 18-19% below initial body weight. Nutritionally induced anoestrus was readily reversible with no evidence of permanent damage to the reproductive system. The resumption of oestrous cycles was dependent upon weight gain of a critical magnitude. Oestrous cycles were first observed when rats were 1-3% below initial body weight. No relationship between duration of emaciation and speed of recovery was demonstrated. The results of the pharmocological studies suggested that the hypothalamus was the primary site of dysfunction in anoestrous rats. Injections of OB and PMS failed to induce ovulation in anoestrous rats. A normal LH response to LHRH was observed only in rats whose pituitaries were previously primed with LHRH. This provided support for the hypothesis that the major effects of inanition are exerted on the hypothalamus rather than on the pituitary.

The second research programme involved stress induction with fully fed rats. Conditioned emotional response procedures were employed to investigate the effects of signalled shocks and conditioned fear on serum LH levels and reproductive function. The effects of chronic administration of unpredictable shocks on the ovarian cycle were

examined. The presentation of predictable shocks during conditioning trials, and of the CS during extinction trials, failed to produce significant changes in serum LH concentration or reproductive functioning. Daily administration of three unpredictable shocks succeeded in blocking oestrous cycles in 20% of rats.

In the third research programme three unpredictable shocks were administered daily to groups of rats during the caloric deprivation and rehabilitation phases. The aim was to determine whether the inferior nutritional status resulted in an increase in the susceptibility of the reproductive system to the effects of a stressor which alone was not capable of causing widespread anoestrus. In the final study 20IU PMS was administered to groups of rats receiving unpredictable shocks daily during different forms of nutritional rehabilitation in order to determine whether imposition of stress inhibited induction of ovulation by PMS.

Unpredictable shock administration hastened the onset of anoestrus during the weight loss phase and inhibited the resumption of oestrous cycles during the rehabilitation phase, even after normal body weight had been restored. Anoestrus persisted for as long as daily stress was imposed. The stress treatment also inhibited the induction of ovulation by PMS. Inhibition of ovulation in PMS-injected rats occurred in rats above normal weight as well as in rats still 30% below initial body weight.

It is proposed that the inhibition of spontaneous resumption of oestrous cycles following rehabilitation and the failure to ovulate in response to PMS provocation was due to a hypothalamic dysfunction that persisted as a consequence of stress. Since the stress treatment alone was incapable of producing these effects it is proposed that the functional state of the hypothalamus was initially disturbed by

caloric deprivation and prolonged by stress. The findings of the research are discussed with particular reference to hypotheses about the aetiology of amenorrhoea in anorexia nervosa.

INTRODUCTION

The stimulus to carry out the present research can be traced back to polemical clinical discussions concerning the aetiology of secondary amenorrhoea in anorexia nervosa. It appeared that a number of variables were implicated in the onset and maintenance of amenorrhoea; the most prominent variables being nutritional and psychological ones. Since the effects of these variables on reproductive function could not be investigated singly in anorexic subjects recourse to experimentation with laboratory animals was required. The aim was to study the separate and combined effects of caloric deprivation and experimentally induced stress on serum LH levels, rhythmicity of the oestrous cycle and other aspects of reproductive physiology.

In the first chapter a brief review of the current state of knowledge about the regulation of the mammalian reproductive cycle is presented. This provides the framework within which the findings of the present research are interpreted.

In Chapter 2 the clinical picture of anorexia nervosa is described and the controversy surrounding the aetiology of the secondary amenorrhoea is discussed. A number of issues which stimulated this research are identified. The literature dealing with endocrine changes in anorexia nervosa and with pharmocological tests carried out in order to determine the nature and location of the endocrine dysfunction is reviewed.

In Chapter 3 the reader is introduced to methodological details common to many of the experiments. They are presented here in order to avoid repetition in later chapters or repeated reference to appendices.

Chapter 4 is prefaced by a review of studies on the effects of nutritional factors on reproduction and endocrine function. Thereafter a series of experiments aimed at investigating the effects of caloric restriction and nutritional rehabilitation on reproductive function are described. Studies involving the administration of pharmocological agents are described in the latter part of this chapter.

In Chapter 5 the focus is turned to the literature dealing with the effects of stress on reproductive function. This is followed by a description of a series of experiments carried out in order to establish the nature and duration of stress treatment required to alter serum LH levels and to induce anoestrus.

Chapter 6 is devoted to a description of experiments performed in order to investigate the combined effects of altered nutritional status and stress induction on certain aspects of reproductive function.

In the final chapter the research is discussed with particular reference to the relevance of the findings to our understanding of secondary amenorrhoea in anorexia nervosa.

Please note: In the preparation of this thesis many of the guidelines recommended in the Publication Manual of the American Psychological Association (1974) were followed.

Chapter 1

REGULATION OF THE MAMMALIAN REPRODUCTIVE CYCLE

The cyclical variations which characterize the mammalian reproductive cycle are the result of complex interrelationships between the hormones of the hypothalamus, pituitary gland and the ovaries. What follows in this chapter cannot be considered to be an exhaustive review of the contemporary literature pertinent to its title. The focus of this review will be on hormonal patterns and the regulation of gonadotrophin secretion in post-pubertal, non-pregnant mammals. Where relevant, attention will be drawn to the differences that exist between the reproductive physiology of humans and rats. The purpose of this review is to provide the framework for subsequent discussions of the endocrine disturbance in anorexia nervosa and the findings of the present research.

The female reproductive cycle is characterized by the release of a hypothalamic releasing factor or factors which stimulate the synthesis of, and regulate the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by the anterior lobe of the pituitary gland. In the normal cycle FSH stimulates the growth of the ovarian follicle and together with LH stimulates follicular development and oestrogen secretion. There is general agreement that rising titres of oestrogen are the endocrinological signal for the surge in release of LH, which in turn, induces ovulation (Banks & Freeman, 1978; Goodman, 1978). The positive feedback effect of oestrogen is believed to occur at a site in the hypothalamus where the oestrogen acts by stimulating the release of luteinizing hormone releasing hormone (LHRH) into the pituitary portal blood (Sakar et al., 1976). There is evidence that oestrogen, in addition, exerts a stimulatory effect at the level of the anterior pituitary (Arimura & Schally, 1971; Reeves

et al., 1971). The presumed anatomical sites where oestrogen exerts its positive feedback effect will be discussed later in this chapter together with evidence for tonic control of gonadotrophin secretion by negative feedback action of the sex steroids.

Normal patterns of gonadotrophin and gonadal steroid secretion

Although different in many respects, the human menstrual cycle and the oestrous cycle of the rat and other spontaneously ovulating mammals share some characteristics. In both humans and rats the most dramatic hormonal events, involving the gonadotrophins and the sex steroids, occur just prior to, and following ovulation.

The human menstrual cycle

The levels of gonadotrophins and gonadal hormones during different phases of the menstrual cycle have been described by Midgley & Jaffe (1971), Ross et al. (1970), Speroff & VandeWiele (1971) and VandeWiele (1970).

Menstruation represents the termination of the previous ovarian cycle and the life of the corpus luteum. During menses there is a small but constant increase in plasma FSH concentration. The rising FSH concentration stimulates several follicles to develop. After a few days one of these matures rapidly while the others begin to involute. During the follicular phase the maturing follicle produces increasing amounts of oestrogen. Since corpora lutea are absent during the preovulatory period progesterone secretion is relatively low. The steadily increasing oestrogen levels control the release of the gonadotrophins by negative feedback inhibition. However, as in other mammals, a critical level of oestrogen is reached, stimulating a surge in the release of LH and FSH; ovulation follows about 30 hours after

this surge (Newton, 1972). There is some question whether the peri-ovulatory increase in progesterone occurs simultaneously with the gonadotrophin surge or as a consequence of the latter.

There is a transitory drop in circulating steroids in the immediate post-ovulatory period. The ruptured follicle starts to luteinize and the luteal cells start producing increasing amounts of progesterone and oestrogen. The high levels of circulating gonadal steroids inhibit, by negative feedback action, the release of gonadotrophins. In the absence of implantation of a fertilized ovum the corpus luteum eventually degenerates, presumably as the result of the luteolytic action of prostaglandins, particularly PGF2α (Henderson & McNatty, 1977). With degeneration of the corpus luteum there is a sharp decline in circulating levels of both oestrogen and progesterone. The menstrual flow that follows is the consequence of the withdrawal of the steroid stimulants. The gonadotrophin levels which have declined during the luteal phase increase significantly at the onset of the menses. Thereafter there is a constant increase in FSH concentration initiating the start of the new ovarian cycle (Bentley, 1976; Tepperman, 1973).

In addition to the cyclical changes described so far, it has also been demonstrated that gonadotrophins fluctuate episodically during the course of a day (Midgley & Jaffe, 1971; Santen & Bardin, 1973). The rhythmic secretion of LH at 1-3 hour intervals has been described in sub-human primates as well as in other species (Bhattacharya et al., 1972; Knobil et al., 1972). The term "circhoral" was applied to this rhythm since there is a mean frequency of one LH burst per hour (Dierschke et al., 1970).

Santen & Bardin (1973) found secretory LH spikes occurring approximately 3 times every 6 hours in normal women during the follicular phase. In contrast women in the luteal phase of the menstrual cycle

had LH pulses of higher amplitude and lower frequency (approximately 1.6 pulses every 6 hours) suggesting that gonadal steroids may modulate LH pulses. Furthermore Santen & Bardin (1973) reported a significant concordance between LH and FSH secretory pulses.

Other studies of the 24 hour pattern of gonadotrophin secretion have demonstrated that, in normal women, the LH pulses occur with equal frequency and magnitude during the waking and sleeping periods (Kapen et al., 1973). In contrast, prepubertal children show low levels of LH and FSH that oscillate around a constant mean. The onset of puberty in both boys and girls is heralded by the augmentation of LH secretion synchronous with sleep (Boyar et al., 1972, 1973).

The oestrous cycle of the rat

The rat oestrous cycle lasts 4-5 days. In the present work the days of the 4 day cycle are designated oestrus, metoestrus, dioestrus and pro-oestrus.

As observed earlier the most dramatic hormonal events occur just prior to, and following ovulation. Of these events the surge of LH, and its role in inducing ovulation, has received the greatest attention. Everett (1956) showed that in rats, subject to controlled illumination (Light: 0500-1900h), the ovulatory surge of LH is released between 1400h and 1600h on the afternoon of pro-oestrus. Ovulation is dependent on the LH surge and occurs between 0100h and 0230h on the day of oestrus (Everett, 1948, 1969).

The onset of the pro-oestrous LH surge and the timing of other mid cycle events, reported by Everett, are not necessarily applicable to all rats. Other investigators have reported the events occurring at earlier or later times. This variation is, most likely, the result of a combination of factors including: (a) random variation between

individual rats of the same species (b) inter-species variation (c) differences in light-dark illumination schedules (d) blood sampling procedures.

Oestrogen: In the 4 day cycling rat, oestrogen levels increase throughout dioestrus to reach peak levels by 0900-1200h on pro-oestrus (Brown-Grant et al., 1970; Butcher et al., 1974; Hori et al., 1968; Smith et al., 1975; Yoshinaga et al., 1969). Butcher et al. (1974), sampling at 3 hour intervals, found a rise in oestradiol levels late on dioestrus, with a peak at noon on pro-oestrus. The plasma levels of oestradiol started falling before the peak LH concentrations were reached on the afternoon of pro-oestrus and had declined to near base levels at midnight of pro-oestrus. These results support those of Exley & Dutton (1970) who found that the oestrogen peak precedes, by several hours, the ovulatory release of LH. Throughout the remainder of the cycle oestrogen remains at base levels, except for small peaks on the afternoon of oestrus and metoestrus (Butcher et al., 1974; Yoshinaga et al., 1969).

Progesterone: Progesterone levels increase above baseline on two occasions during the oestrous cycle. The first is a small rise on metoestrus, representing the short functional life of the corpora lutea (Butcher et al., 1974; Piacsek et al., 1971). Following this small elevation progesterone levels drop by the morning of dioestrus. Piacsek et al. (1971) found no change in progesterone secretion between the afternoon of dioestrus and the early afternoon of pro-oestrus. The same investigators found a similar pattern of 20αOH-P secretion, except that peak levels on the afternoon of pro-oestrus were observed approximately 2 hours before progesterone levels started increasing. Similarly, Barraclough et al. (1971) reported an earlier elevation of

20αOH-P levels. Piacsek et al. (1971) suggested that the 20αOH-P may be secreted by corpora lutea of previous ovulations in response to an initial elevation in LH levels.

Although Barraclough et al. (1971) observed an increase in peripheral progesterone levels prior to the LH surge, this increase was small and is believed to be of extra-ovarian origin. Butcher et al. (1974) reported that progesterone appeared to rise simultaneously with the LH surge on the afternoon of pro-oestrus. Other investigators, however, using more frequent sampling, have found that the rise in plasma LH precedes that of progesterone on the afternoon of pro-oestrus (Barraclough et al., 1971; Piacsek et al., 1971; Schneider et al., 1970). These observations have been interpreted as secretion of progesterone in response to LH stimulation. Indeed Banks & Freeman (1978) observed that "in the presence of ovaries, the LH "surge" during proestrus initiates a simultaneous "surge" of progesterone" (p.426) which occurs before ovulation. Since the marked elevation in plasma progesterone concentration precedes the formation of the corpora lutea it presumably arises as a result of a luteotrophic effect of the LH on ovarian interstitial tissue (Bentley, 1976).

Barraclough et al. (1971) found that progesterone levels in ovarian vein plasma continued to rise after plasma LH levels started dropping on the evening of pro-oestrus. Thereafter progesterone levels start declining towards base line concentrations, reaching them by early morning of oestrus (Butcher et al., 1974) or the afternoon of oestrus (Piacsek et al., 1971).

The role of progesterone in the regulation of the oestrous cycle will be discussed later in this chapter.

Peripheral LH: With some exceptions investigators report that serum/

plasma concentrations of LH are low during all stages of the oestrous cycle except during the afternoon and evening of pro-oestrus (Banks & Freeman, 1978; Brown-Grant & Greig, 1975; Butcher et al., 1974; Daane & Parlow, 1971; Gay et al., 1970; Greig & Weisz, 1973; Mann & Barraclough, 1973; Monroe et al., 1969; Piacsek et al., 1971). In addition to the pre-ovulatory surge some investigators have observed a small increase in serum LH on the afternoon of metoestrus (Naftolin et al., 1972; MacKinnon & terHaar cited in Naftolin et al., 1972). The physiological significance of this slight rise is not known. Gay et al. (1970) have observed diurnal variation in LH secretion during the 3 days following pro-oestrus with low levels during darkness and higher levels during periods of light.

Examination of the literature reveals differences in the recorded times of the onset of the pro-oestrous LH rise, the magnitude of the rise as well as in other features of the preovulatory LH surge (Barraclough et al., 1971; Blake, 1976a; Gay et al., 1970; Monroe et al., 1969; Naftolin et al., 1972). It is generally found, however, that the LH surge occurs on the afternoon and evening of pro-oestrus and that LH levels have declined to basal concentrations later the same evening. Some investigators report finding elevated LH levels during the early morning of oestrus (Naftolin et al., 1972). The majority of studies, however, have found reduced LH levels before 2400h on pro-oestrus.

Blake (1976a) has presented a detailed characterization of the pro-oestrous LH surge based on a composite of data obtained from different groups of rats. He identifies (i) an "initial rising phase" of LH (ii) a "rapid rising phase" during which plasma LH rises quickly, starting between 1445 and 1650h, and lasting for 20-50 minutes (iii) a "plateau phase" (iv) a "declining phase" characterized by a rapid

drop in LH levels, but commonly interrupted by one or more rapid increases in plasma LH. Blake (1976a) observed that the pro-oestrous LH rise does not appear to be made up of intermittent pulses of LH release.

Pituitary LH: Using the ovarian ascorbic acid depletion assay Schwartz & Bartosik (1962) found that the pituitary LH content is lowest at oestrus and metoestrus and maximal (2 times oestrus values) at dioestrus and on the morning of pro-oestrus. The serum LH surge is accompanied by a drop in pituitary content of LH on the day of pro-oestrus (see Schwartz, 1969 for review of studies employing the ovarian ascorbic acid depletion assay). These findings have been confirmed by investigators employing radio-immunoassay procedures (Naftolin et al., 1972).

Peripheral FSH: It is most frequently reported that the FSH rise on pro-oestrus appears to occur simultaneously with the LH surge. It is generally observed, however, that the decline in FSH levels is slower i.e. plasma FSH levels remain high while LH levels have started to decrease (Aiyer et al., 1974; Brown-Grant & Greig, 1975; Butcher et al., 1974; Daane & Parlow, 1971; Gay et al., 1970). The difference in half lives of the two gonadotrophins is not sufficient to account for this observation.

By noon of oestrus FSH levels have returned to base line (Butcher et al., 1974) and during the three days following pro-oestrus peripheral FSH levels decrease progressively (Gay et al., 1970).

Peripheral prolactin: Serum prolactin levels also increase on the afternoon of pro-oestrus (Gay et al., 1970; Neill & Reichert, 1971). The prolactin rise, however, does not necessarily occur simultaneously

with the gonadotrophin surge (Gay et al., 1970). Butcher et al. (1974), for example, observed that plasma prolactin levels started to rise a few hours before the rise of FSH and LH.

The regulation of gonadotrophin secretion

Hypothalamic control of gonadotrophin secretion

Moore & Price (1932) proposed that the cyclical variations of the oestrous cycle were the result of a reciprocal interrelationship between the gonads and the pituitary. More recently, however, it became apparent that the production and release of gonadotrophins from the anterior pituitary is under hypothalamic control. Largely due to the pioneering work of G. W. Harris and his colleagues (Harris, 1955) it was recognized that the hypothalamus exerts its control by means of chemical messenger molecules. These neurohormonal substances are transmitted along a pathway linking the hypothalamus and the anterior pituitary; the pathway being partly neural and partly vascular.

The neural component consists of a tract of nerve fibres which have their origin in nerve cells in the hypophysiotrophic area of the hypothalamus. The nerve fibres run down the tubero-infundibular tract, many terminating on or in the vicinity of the primary capillary bed in the median eminence that feeds the hypophysial portal vessels. Here they transmit, into the blood stream, their neurohormones which are then passed down the portal veins to control the output of hormones from the epithelial cells in the anterior pituitary.

In addition to the anatomical considerations there is biochemical evidence that the hypothalamus controls the secretion of anterior pituitary hormones by means of releasing hormones or factors (Campbell et al., 1961; McCann et al., 1960).

Initially it was believed that the secretion of LH and FSH was regulated by discrete releasing hormones; LHRH and FSH-RH respectively (McCann & Ramirez, 1964; Schally et al., 1968). Later studies, however, indicated that both natural and synthetic LHRH possess LH and FSH releasing activity (Arimura et al., 1972). Support for the notion that LHRH alone may control both LH and FSH release came from reports that plasma LH and FSH levels fluctuate in parallel during the menstrual cycle (Cargille et al., 1969; Swerdloff & Odell, 1969). Despite the overall similarity of LH and FSH secretory patterns, dissociations of the secretion of these hormones have nonetheless been reported (Barraclough et al., 1971; Gay et al., 1970; Rubin et al., 1971). Schally et al. (1971) proposed that the secretion of LH and FSH is regulated by one hypothalamic releasing hormone, LHRH decapeptide, and that the differential release of the gonadotrophins is regulated by modulators such as gonadal steroids. Studies by Franchimont et al. (1974) produced results suggesting that gonadotrophin secretory responses to LHRH stimulation are influenced by the sex steroids which may enhance pituitary sensitivity to LHRH.

The question of whether LHRH is the sole factor involved in the hypothalamic control of both LH and FSH secretion has yet to be resolved to the satisfaction of all investigators. A detailed discussion of this issue goes beyond the scope of the present review. In conclusion, however, the consensus of opinion favours the notion of a single decapeptide LHRH which stimulates the release of both LH and FSH.

The hypothalamic releasing factor has been named and abbreviated in different ways by different authors. The variations are: luteinizing hormone-releasing hormone (LHRH or LRH), luteinizing hormone-releasing factor (LRF), or gonadotrophic releasing hormone (GnRH). Legan & Karsh (1975) use GnRH to designate synthetic preparations

which elicit LH release, whereas they use LRF to refer to the natural releasing factor. In the present work the abbreviation LHRH will be used throughout to refer to both synthetic and natural releasing factors. Where applicable the distinction between endogenous and exogenous LHRH will be made.

In conclusion, hypothalamic LHRH regulates the secretion of gonadotrophins and thereby the secretion of the gonadal steroids. This relationship, however, is not unidirectional. A complete discussion of the hypothalamic control of gonadotrophin secretion requires consideration of the feedback action of gonadal steroids.

Gonadal steroid hormone control of gonadotrophic secretion

It is now generally recognised that gonadal steroids exert a regulatory effect on gonadotrophin release by means of complex positive and negative feedback mechanisms acting at both the hypothalamic and pituitary levels.

Steroid hormone control of tonic secretion of gonadotrophins

During the follicular phase of the cycle the low, basal or tonic release of gonadotrophins appears to be regulated by ovarian hormones acting in the region of the arcuate and ventromedial nuclei of the hypothalamus (Barraclough, 1973; Davidson, 1969; Donovan, 1972) as well as at the level of the pituitary (Greeley et al., 1975).

The tonic secretion of LH and FSH is regulated by a negative feedback action of oestrogen which allows the discharge of gonadotrophins in sufficient quantity to maintain normal follicular development and oestrogen secretion but not sufficient to independently initiate the ovulatory gonadotrophin surge. A variety of procedures have been employed to demonstrate the negative feedback action of the sex steroids. Interruption of the negative feedback loop by ovariectomy (OVX)

leads to elevated levels of plasma gonadotrophins (Greep, 1961). The enhanced release of LH which occurs in the OVX female can be suppressed by oestrogen administration (Kalra et al., 1971; McCann & Taleisnik, 1961). Implantation or infusion of oestrogen directly with the pituitary has been found to exert an inhibitory action on gonadotrophin release. This suggests that the pituitary is one of the sites at which oestrogen acts (Bogdanove, 1963; Ramirez et al., 1964; Rose & Nelson, 1957). On the other hand implants of oestrogen in the hypothalamus also inhibited LH release and resulted in a reduction in its content of stored LHRH (Chowers & McCann, 1965; Ramirez et al., 1964).

With regard to the pulsatile discharges of LH, the balance of evidence is against attributing these spikes of gonadotrophin secretion to fluctuations in negative feedback control by ovarian steroids. It has been suggested by Brown-Grant (1977) that the episodic secretion may be superimposed on a system that may be sensitive only to sustained changes in steroid levels.

So far mention has only been made of the inhibitory effects of oestrogen. Progesterone also exerts an inhibitory effect on gonadotrophin secretion at certain stages of the mammalian reproductive cycle. Everett (1948), for example, found that an injection of progesterone, early in the oestrous cycle, delayed ovulation. During the luteal phase progesterone acts together with oestrogen in inhibiting gonadotrophin secretion. During pregnancy progesterone inhibits further ovulation, possibly by inhibiting the positive feedback effect of oestrogen.

Steroid hormone control of the ovulatory surge of gonadotrophins

It is a formidable task to attempt a clear exposition of this topic because the role played by the individual gonadal steroids in the regulation of the ovulatory gonadotrophin surge is still not fully understood.

Although it is now apparent that the sex steroids interact in regulating gonadotrophin secretion the effects of oestrogen and progesterone will be examined separately.

Stimulatory effects of oestrogen: Holweg & Junkmann (1932) were the first to propose that oestrogen exerts a facilitating effect on LH release. There is now widespread, but not universal agreement that the spontaneous mid-cycle gonadotrophin surges are the result of the rising titre of oestrogen during the latter part of the follicular phase with a subsequent positive feedback action of oestrogen (i) at the level of the brain to release hypothalamic LHRH, and (ii) at the level of the pituitary to facilitate LHRH induced gonadotrophin release (Arimura & Schally, 1971; Aiyer et al., 1974; Davidson, 1969; Ferin et al., 1969; Gordon & Reichlin, 1974; Jaffe & Keye, 1974; Kalra & Kalra, 1974; Kastin, Gual & Schally, 1972; Knobil et al., 1972; Neill et al., 1971; Piacsek & Meites, 1966; Schneider & McCann, 1970; Wang & Yen, 1975; Yen, Vandenberg & Siler, 1974; Zeballos & McCann, 1975).

It has been demonstrated that the rise in urinary oestrogens and plasma oestradiol precedes the ovulatory peak of LH in women (Burger et al., 1968; Corker et al., 1969) and in the Rhesus monkey (Knobil, 1972). In rats the peak level of plasma oestradiol is found on the morning of pro-oestrus, before the onset of the LH surge (Brown-Grant et al., 1970; Butcher et al., 1974; Smith et al., 1975). Furthermore, the ability of antisera, or chemical antagonists of oestrogen action, to block the surge, provides convincing evidence for the prominent role of oestrogen in the control of ovulation.

Administration of exogenous oestrogen has provided further evidence of its positive feedback action. Everett (1948) observed that injection of oestrogen on the second day of dioestrus (5-day cycle) would advance

ovulation by one day.

The anatomical site where oestrogen exerts its positive feedback action remains a matter of active investigation. As Brown-Grant (1977) remarks: "The crucial question is where and how is the estrogen acting" (p.63). Present evidence suggests that oestrogen exerts its stimulatory effect at the level of the brain as well as at the level of the pituitary. To argue in favour of one or the other as the "main site" of action seems fruitless since it appears that the action of oestrogen at the two levels is complimentary and not competitive.

With regard to "where" in the brain oestrogen exerts its positive feedback action, it is now generally agreed that the stimulatory effects are mediated in the suprachiasmatic region and the preoptic area of the hypothalamus; and possibly also in parts of the limbic system which are known to contain oestrogen retaining neurons (Stumpf, 1970). This view is based on the fact that lesions in the suprachiasmatic region prevent ovulation (McCann et al., 1968), whereas implantation of oestrogen in this area can advance puberty in the rat (Smith & Davidson, 1968). Kalra & McCann (1975) and Goodman (1978) succeeded in advancing ovulation with oestradiol implants in the preoptic area. Moreover, knife cuts, which separate the preoptic area from the remainder of the hypothalamus block ovulation (Halasz & Gorski, 1967).

As to the question of "how" oestrogen acts, it is presumed that the facilitatory action of oestrogen is to increase the release of hypothalamic LHRH and subsequently the release of gonadotrophins by the pituitary. Present evidence suggests that the facilitatory effects of oestrogen are not confined to the hypothalamus. It would appear that oestrogen also exerts a direct effect on the pituitary, influencing gonadotrophin secretion.

One of the main arguments advanced by those workers favouring the

pituitary as the main site of oestrogen feedback action was the failure of researchers to demonstrate increased levels of LHRH in the hypophysial portal vessel blood at the appropriate time, i.e. before or during the spontaneous LH surge (Eskay et al., 1975; Fink & Jamieson, 1976). A breakthrough in this area was eventually made by Sakar et al. (1976). Using Althesin, an anaesthetic that does not block ovulation, Sakar and his colleagues demonstrated increased release of LHRH from nerve terminals in the median eminence with significantly increased levels of LHRH in portal vessel blood on the afternoon of pro-oestrus. It is significant that they also stressed the importance of increased pituitary responsiveness to LHRH on pro-oestrus. The increased pituitary sensitivity to LHRH could be due to either the sensitizing action of oestrogen or the priming effect of LHRH on the pituitary or both.

Another objection raised by those favouring the pituitary as the primary site of positive feedback action of oestrogen was the possibility that oestrogen implanted in the hypothalamus could be distributed to the pituitary by the portal circulation (Bogdanove, 1963; Palka et al., 1966). In order to resolve this methodological issue Goodman (1978) monitored the pituitary oestradiol concentrations produced by the hypothalamic implantation of oestradiol in the rat and compared these concentrations with the pituitary oestradiol levels normally associated with the LH surge. The results of his study suggest that the oestradiol levels found in the pituitary during pro-oestrus is not sufficient to induce the preovulatory LH surge. Implantation of oestradiol in the medial basal hypothalamus caused a significant increase of pituitary oestradiol but elicited an LH surge in only 6% of animals. In contrast, oestradiol implanted in the preoptic area elicited an LH surge in 56% of animals without significantly increasing pituitary oestradiol levels.

These findings do not preclude the possibility that oestrogen has a

direct influence on gonadotrophin release by the pituitary. Indeed it is now appreciated that the magnitude of the changes occurring at the pituitary level are far greater than was previously believed. Döcke & Dörner (1965) first proposed that increased sensitivity of the pituitary gland induced by direct effect of gonadal steroids is, at least, partly responsible for the preovulatory surge. This viewpoint was supported by Weick & Davidson (1970) who went further to conclude that the anterior pituitary is the main site of the positive feedback action of oestrogen.

Oestrogen has been demonstrated to have a direct effect on LH release at the level of the pituitary (Piacsek & Meites, 1966; Schneider & McCann, 1970). Evidence that oestrogens may enhance the sensitivity of the pituitary to LHRH comes from the work of Arimura & Schally (1971) who showed that the response to LHRH, on day dioestrus-2, of rats with 5 day cycles could be facilitated by the administration of oestradiol benzoate on day dioestrus-1. Arimura & Schally (1972) were also the first to present experimental evidence that the pituitary response to LHRH is greater at the time of the preovulatory surge of gonadotrophins in sheep and hamsters. Aiyer et al. (1973) observed that pituitary responsiveness to exogenous and endogenous LHRH increased markedly on the afternoon of pro-oestrus in the rat. Since then the results of a number of other studies have added support for the notion that the gonadal steroids exert a direct effect on the pituitary gonadotrophs by enhancing their sensitivity to the action of LHRH (Aiyer et al., 1974; Jaffe & Keye, 1974; Keye & Jaffe, 1974; Krey et al., 1973; Wang & Yen, 1975; Yen et al., 1974).

Implantation of anti-oestrogens into the pituitary has the effect of blocking ovulation (Bainbridge & Labhsetwar, 1971). This observation, together with the evidence reviewed so far, provides support for

the argument that the stimulatory effects of oestrogen at the level of the pituitary are essential for the release of an ovulatory quota of LH. Although oestrogen appears to play an important role in determining the magnitude of the preovulatory surge, it is likely that the timing and duration of the surge depends on other factors as well.

Stimulatory effects of progesterone: Everett (1948) showed that, although the administration of progesterone early in the oestrous cycle of the rat would delay ovulation, injection of the same dose later in the 5 day cycle (dioestrus-3) advanced ovulation by 24 hours. Later studies provided experimental support for the suggestion (Everett, 1948) that the facilitatory effect of progesterone on ovulation was seen only when the hypothalamo-pituitary system had recently been exposed to oestrogen (Brown-Grant, 1969; Grayburn & Brown-Grant, 1968). A number of studies have demonstrated that the administration of progesterone to oestrogen-primed ovariectomized rats results in a surge of LH within a few hours (Caligaris et al.,1971a; Jackson, 1972; Kalra et al., 1972).

Furthermore it has been observed that an injection of progesterone on the morning of pro-oestrus advances the time of LH release on that day (Brown-Grant & Naftolin, 1972). Further evidence that progesterone facilitates LH release on pro-oestrus comes from the studies performed by Fink et al. (1975). They found that oestradiol benzoate administered immediately after ovariectomy, on the morning of dioestrus-2 in 4 day cycle rats, augmented the LH response to LHRH at 1330 h on pro-oestrus. However, it failed to completely restore the response, at 1830h, to that level observed in sham operated rats. The administration of oestradiol benzoate immediately after the operation and of 2.5 mg progesterone at 1300 h on pro-oestrus completely restored the gonadotrophin response to LHRH.

It appears that progesterone requires the prior and not simultaneous stimulation of oestrogen. Progesterone administered simultaneously with oestradiol suppresses the pituitary sensitivity to LHRH (Arimura & Schally, 1972). While this finding does not exclude the possibility that progesterone acts directly on the gonadotrophs, it does suggest that progesterone, together with oestrogen, acts at the level of the brain by modulating LHRH secretion. That is, it is likely that the effect of the steroid hormones on pituitary responsiveness to LHRH is the sum of actions exerted at the level of the brain as well as on the gonadotrophs. Eshkol et al. (1975) came to a similar conclusion that the site of the "modulatory effects of ovarian steroids [can] not be localized since the end result, ie the elevation of plasma gonadotropins, represents the sum total of an effect on the whole hypothalamic-pituitary axis" (p.1065).

It appears that progesterone plays yet another important role in the regulation of the ovulatory gonadotrophin surge. Freeman and his colleagues (Banks & Freeman, 1978; Freeman et al., 1976) provide convincing experimental evidence that progesterone not only enhances the LH surge at the appropriate time but also plays the role of limiting the expression of the spontaneous LH surge to once every 4 to 5 days in the rat. They observed that, in the presence of ovaries, the LH surge apparatus retains the capability for daily expression even though it is only activated once every 4-5 days. In the absence of ovaries a single injection of oestrogen causes occurrence of a daily surge of LH for at least 3 days after oestrogen administration (Caligaris et al., 1971a; Legan & Karsh, 1975; Neill, 1972). However, in intact animals the progesterone surge on pro-oestrus acts to abolish the expression of a proposed "daily neural timing signal for LH release" (Banks & Freeman, 1978, p.426). Their results provide no indication whether the

progesterone acts directly on the pituitary gland or at the hypothalamic level.

The priming effects of LHRH

Evidence is gathering that LHRH itself acts to increase pituitary LH release in response to later LHRH secretion i.e. LHRH primes or sensitizes the pituitary to itself (Aiyer et al., 1973, 1974; Fink et al., 1975; Gordon & Reichlin, 1974).

Aiyer et al. (1974) showed that after injection of two successive doses of LHRH in phenobarbitone blocked pro-oestrous rats the rise in plasma LH was much greater after the second injection. Gordon & Reichlin (1974) found that LHRH was more effective in elevating LH if administered after the start of the LH surge than if administered before. Similarly Zeballos & McCann (1975) reported that LHRH injection at a time when plasma LH levels were already elevated on pro-oestrus, elevated plasma LH more, and more rapidly, than LHRH injection earlier in the afternoon.

Fink et al. (1975) examined the possible mediating role of gonadal steroids in the LHRH priming of the pituitary. They injected two successive doses of LHRH at various intervals into pro-oestrous rats. The LH response to the second injection was significantly greater than that of the first, and was greatest when the two injections of LHRH were separated by 60 minutes. However, they found that the increased LH response to a second injection of LHRH was even greater in rats ovariectomized immediately before the first injection. This finding would suggest that LHRH priming is not mediated by gonadal steroids. On the other hand it has been observed that ovariectomy on the morning of di-oestrus resulted in a considerable reduction in the response to LHRH on the afternoon of pro-oestrus. Furthermore oestradiol administration at 1000h on metoestrus caused a significant increase in the response

to the second of two injections of LHRH given on the afternoon of di-oestrus (Aiyer et al., 1974b). It is difficult to resolve the issue of where and how the gonadal steroids may be acting. On the one hand oestradiol may cause increased endogenous LHRH output which, in turn, primes the anterior pituitary. Alternatively the oestradiol may act on pituitary gonadotrophs increasing their sensitivity to LHRH; or act at both hypothalamic and pituitary levels.

The results of studies by Blake (1978) suggest that the LHRH priming mechanism is more a function of the length of time that LHRH stimulates the pituitary than of the amount of LHRH which stimulates the gland. This is in agreement with the observation by Fink et al. (1975) that the LH response to LHRH is greatest when the two injections of LHRH are separated by 1 hour.

"Short loop" feedback mechanisms in the regulation of gonadotrophin secretion

In addition to the feedback effects of the gonadal steroids and the priming effects of LHRH it is now generally accepted that another type of control exists, whereby LH and FSH are able to control their own secretion by influencing hypothalamic function. For this feedback the name "short", "internal" or "auto" feedback has been proposed (Martini et al., 1968). Evidence in favour of such feedback loops comes from studies like that of David et al. (1966) who found that implantation of LH into the median eminence results in a decrease in pituitary and plasma LH and that of Martini et al. (1968) who demonstrated that implants of FSH in the median eminence lower pituitary FSH levels.

At present the physiological significance of such short loop feedback mechanisms is not known.

The influence of environmental factors on reproductive function

It has been established that environmental factors exert an influence on the reproductive system via central nervous pathways. For example, it has been shown that temperature (Bohanan, 1939), light (Piacsek & Hautzinger, 1974) and sound (Zondek & Tamari, 1960) exert an effect on the reproductive cycle of lower animals. However, further discussion of this research is beyond the scope of the present review. In later chapters the effects of nutrition and emotional factors will be examined in greater depth.

A final comment

The tremendous advances in the field of reproductive physiology during the last decade have been made possible by two important developments.

The first is the development of the radio-immunoassay procedure; that:

> exquisitely sensitive analytic technique capable of detecting minute quantities of hormone in the blood stream
>
> (Chedd, 1977, p.144)

The second development:

> has been the virtual founding of a new science, that of neuroendocrinology - the isolation, identification, synthesis and modification of the chemical messengers by which the brain exercises control over several of the body's major hormone systems, including those involving thyroid function, growth and fertility
>
> (Chedd, 1977, p.144)

While progress has been rapid the complex and dynamic hormonal mechanisms regulating the mammalian reproductive system have not yet been fully elucidated. For the present, at least, the research described in later chapters will be interpreted in the light of available knowledge.

Chapter 2

ANOREXIA NERVOSA - THE ORIGINS OF THE INQUIRY

Anorexia nervosa is a complex psychological disturbance seen typically in adolescent girls, although its onset in the mid-30's is recognised and a similar syndrome occurs rarely in children (Blitzer et al., 1961) and in males (Beumont, 1970; Dally, 1969).

The descriptions of the psychological and physical features of anorexia nervosa are numerous. Nevertheless, most clinicians would agree that the following features, listed by Beumont et al. (1976), are characteristic of the disorder:

(a) evidence of a pattern of behaviour aimed at inducing weight loss. Such behaviours include food refusal, avoidance of carbohydrate and fat containing foods, strenuous exercise, self induced vomiting and purgation.

(b) a persistent pursuit of thinness; an admitted aversion to regaining a normal weight, and denial of the severity and extent of weight loss.

(c) emaciation to a body weight at least below 80% of standard (standard weight refers to the average weight of same sexed individuals of the same age and height and may be derived from the Tables of the Society of Actuaries (1959).

(d) cessation of menstruation for at least 3 months.

(e) absence of other physical of psychiatric ~~symptoms~~ illness.

Symptoms such as disorders of temperature regulation, lanugo hair, peripheral oedema are presumed to be secondary to the malnutrition. To this list of diagnostic features some clinicians would add further psychological symptoms such as a disorder of perception relating to body image, a morbid fear of becoming fat, and other disturbances of the patient's mental state.

The symptom of central interest in the present work is the secondary amenorrhoea and the other symptoms which may, in turn, be causing the amenorrhoea. The aetiology of the amenorrhoea in anorexia nervosa is still subject to active debate. It is to this controversy that attention will now be directed.

The controversy surrounding the aetiology of secondary amenorrhoea in anorexia nervosa

The controversy surrounding the aetiology of secondary amenorrhoea in anorexia nervosa continues to be generated by a number of clinical observations:

(a) Secondary amenorrhoea is frequently observed in anorexic patients before the onset of dieting or before significant weight loss has occurred (Kanis et al., 1974; Kay & Leigh, 1954; King, 1963). There is no unanimity on this issue since the reported frequency of amenorrhoea preceding weight loss or other characteristic symptoms, varies considerably (Crisp, 1967).

(b) The restoration of normal body weight of nutritionally rehabilitated anorexic patients does not invariably result in the resumption of normal menstrual function (Russell, 1969). Indeed some females remain amenorrhoeic for 3 years after regaining normal weight. The patients who fail to menstruate continue, for long periods, to show an absence of the cyclical changes in gonadotrophins and oestrogens which characterize the normal menstrual cycle (Crisp et al., 1973; Russell & Beardwood, 1968).

(c) It is firmly established that involuntary malnutrition and undernutrition can result in amenorrhoea. Studies of famine victims have revealed that women can become amenorrhoeic in the absence of psychological disturbance, apart from the stress directly related to food deprivation (Gomez-Mont, 1959; Zubiran & Gomez-Mont, 1953).

To add to the controversy about the aetiology of amenorrhoea in anorexia nervosa is the observation that the amenorrhoea of famine victims is readily reversible. Females previously suffering from chronic malnutrition show increased gonadotrophin secretion and resume normal menstrual function relatively rapidly following nutritional rehabilitation (Zubiran & Gomez-Mont, 1953).

It has already been observed that a cardinal feature of anorexia nervosa is behaviour aimed at inducing weight loss - food refusal, avoidance of carbohydrate and fat containing foods, self-induced vomiting and purgation. In spite of the fact that it is likely that a part of the protein intake is being utilized for gluconeogenesis, Russell (1967) found in anorexic patients an extreme emaciation resulting from calorie deficiency and yet a relatively preserved protein metabolism. That is, the malnutrition in anorexia nervosa is likely to be in the form of a carbohydrate and fat calorie deficiency.

Although it is generally agreed that malnutrition and weight loss play a dominant role in maintaining the amenorrhoea during the acute stages of anorexia nervosa there is contention about (i) the extent of the malnutrition or the degree of weight loss required to precipitate the cessation of menses. Some authors suggest that weight loss need not be marked. For example, Shearman (1969) reported that the amenorrhoea in teenagers on "crash diets" occurred abruptly and preceded significant weight loss. (ii) the extent to which the persistent amenorrhoea, occurring in some patients after restoration of normal weight, is a function of nutritional or body weight factors.

In the present study both of these issues are investigated by means of animal experimentation. The effects of malnutrition and

and undernutrition on human and animal reproductive physiology is discussed at greater length in Chapter 4.

(d) It is now firmly established that psychological factors alone can be responsible for the cessation of menses. These amenorrhoeas have been termed the "psychogenic" or "hypothalamic" amenorrhoeas (Klinefelter et al., 1943; Reifenstein, 1946). Clinicians have observed that traumatic experiences, emotional turmoil and even changes in environment may be followed by amenorrhoea (McCormick, 1975). The physiological mechanisms operating in these instances are still to be elucidated, although they are assumed to be exerting an effect at the hypothalamic level (Russell, 1972a) by inhibiting hypothalamic regulation of gonadotrophin secretion.

The psychological disturbance in anorexia nervosa is not as clearly understood as, for example, in the neuroses. There appears to be no common psychological pattern or developmental history that characterizes all anorexics. Admittedly, a relentless pursuit of thinness, possibly as the result of a morbid fear of fatness, appears to be a central theme in the psychopathology but this gives us little insight into more fundamental psychological processes; processes which are presumably operating in those cases in which the amenorrhoea has an early onset.

Because of the wide variety of different situations or events that appear to precipitate menstrual disturbances, the tendency is to refer to the patient as being in a state of "stress". The broad and vague nature of the term "stress", simply reveals our ignorance about the precise psychological processes involved. In the case of anorexia nervosa this is understandable since there appears to be no consistent description of the mental state of the anorexic patient nor supporting evidence of heightened physiological

arousal. Nevertheless, clinical observations seem to suggest that psychological factors are prominent, in some patients, in precipitating the amenorrhoea and in maintaining it, even after nutritional rehabilitation.

The effects of experimentally induced stress on gonadotrophin secretion and other aspects of the reproductive physiology of the female rat have been investigated in this study and are reported in Chapter 5.

Having examined the source of the controversy it is now appropriate to discuss the possible aetiological factors responsible for the amenorrhoea in anorexia nervosa.

It is probably true to say that at the physiological level the amenorrhoea is the result of an inhibition of the cyclical hormonal changes, particularly the mid-cycle LH surge (Nillius & Wide, 1972; Russell, 1972a). What causes the inhibition of the ovulatory surge and where these causal factors are exerting their effect is a matter of some speculation. A number of different hypotheses have been proposed.

(a) One of the earliest views was that the amenorrhoea is a result of a primary pituitary dysfunction. The bulk of the evidence does not support this view and it is no longer believed that anorexia nervosa resembles Simmond's disease or pan hypopituitarism. The general consensus of opinion favours the hypothalamus as the site of the dysfunction; but there is presently insufficient evidence to specify the cause of the hypothalamic dysfunction.

(b) One hypothesis is that the amenorrhoea is due to a primary hypothalamic dysfunction/lesion of unknown origin. It has been observed that patients with tumours in the hypothalamic region present with symptoms similar to that of anorexia nervosa (Kagan, 1958; Lewin et al., 1972). However, the finding that the menstrual disturbance

is reversible, even though prolonged in some cases, does not provide support for the notion of a primary hypothalamic defect. As Mecklenberg et al.(1974) observed the persistence of defects of water balance and thermo regulation, found in some patients, may be due to irreversible changes in hypothalamic function caused by the previous malnutrition. Fries (1974) raised the possibility that a primary hypothalamic disorder makes some women susceptible to acute emotional conflicts and/or weight change. According to Fries (1974), however, there is no present evidence of a hypothalamic "locus minoris resistentiae" (p.31).

(c) A further possibility is that the hypothalamic dysfunction is secondary to malnutrition. Few would disagree that malnutrition plays a prominent role in maintaining the amenorrhoea during the acute stages of anorexia nervosa. However, malnutrition can't be the cause of the amenorrhoea in all cases because many patients cease menstruating prior to weight loss and in some cases menses do not automatically follow correction of the malnutrition. It is still not known whether malnutrition can cause damage to the hypothalamus; a form of damage from which the patient takes some time to recover following restoration of normal weight.

(d) The hypothesis most frequently proposed to account for the early onset of amenorrhoea and its persistence after nutritional rehabilitation is that there is a functional disturbance of the hypothalamus that is induced in some, as yet, undefined way by psychological conflict or anxiety. Mecklenburg et al. (1974) claim that this is the traditional view to which most psychiatric investigators subscribe. Establishing that psychological factors are primary is a hazardous venture since anorexia nervosa is a disorder characterized by both psychological disturbance and malnutrition and most patients

present only when the malnutrition is relatively advanced.

(e) Since both psychological and nutritional factors are known to cause secondary amenorrhoea investigators generally refer to one or both factors in accounting for the menstrual disturbance. However, in view of our ignorance we must accept the possibility that an unknown factor may be causing the amenorrhoea as well as the feeding and psychological disturbance (Russell, 1972a).

(f) A viewpoint not frequently expressed, but one which could account for the variety of patterns of menstrual disturbance, is that the aetiology may be multifactorial. It seems feasible that several aetiological factors either acting on their own, or in combination, could account for the different clinical observations. Such a multifactorial interpretation is in agreement with the findings of an epidemiological study of aetiological factors in secondary amenorrhoea (Fries et al., 1974).

Endocrine changes in anorexia nervosa

It is now appropriate to review the studies reporting on the endocrine disorder in anorexia nervosa and to examine what support they provide for the various hypotheses proposed to account for the amenorrhoea.

Hormonal levels during the acute stage

During the acute phases of anorexia nervosa decreased excretion of gonadotrophins has been observed (Beardwood, 1974; Mecklenburg et al., 1974; Russell et al., 1965). With the development of more sophisticated radioimmunoassay procedures the levels of circulating gonadotrophins and gonadal steroids have been investigated more thoroughly and precisely.

During the acute phase of anorexia nervosa the levels of plasma LH are markedly reduced and there is an absence of the cyclical changes in

gonadotrophins that characterize the normal menstrual cycle (Beumont et al., 1973; Danowski et al., 1972; Lundberg et al., 1972; Marshall & Fraser, 1971; Warren & VandeWiele, 1973).

Santen & Bardin (1973) reported that the episodic LH secretion pattern in anorexia nervosa differs from that found in normal females in the follicular phase. The LH impulses, while still present, were of low amplitude and low frequency.

The 24 hour LH secretory pattern of patients during the acute phase of anorexia nervosa has been shown, in two separate studies, to resemble that found in normal prepubertal or pubertal premenarchal girls (Boyar et al., 1974; Kalucy, 1974). The LH secretion pattern is characterized by very low hormone levels during wakefulness and substantially higher levels in plasma during sleep. These findings are interpreted by Boyar et al. (1974) as suggesting a regression to more immature LH secretory patterns.

Brown et al. (1977) reported a significant correlation between plasma LH levels and weight loss. In contrast they found no relationship between plasma LH levels and caloric intake or duration of amenorrhoea. Similarly Beumont et al. (1976) found that reduced plasma LH levels are related to the weight loss and not to caloric intake.

Although the secretion of FSH is usually reduced, the plasma FSH levels are not as consistently low as the plasma LH levels. That is, the impairment of FSH secretion appears to be less pronounced (Danowski et al., 1972; Lundberg et al., 1972; Marshall & Fraser, 1971; Warren & Vande Wiele, 1973). The results of a study by Beumont et al. (1976) suggest that FSH secretion is less susceptible to body weight loss. They reported that FSH was abnormally low only in patients below 70% of standard weight. The mean plasma FSH levels for patients greater than 70% of standard weight did not differ significantly from those of normal

women during the follicular phase. In contrast, the mean plasma LH values were significantly lower than normal in patients below 80% of standard weight.

Associated with the low gonadotrophin levels is decreased oestrogen secretion. The low oestrogen levels found during the acute phase of anorexia nervosa is considered to be secondary to the reduced gonadotrophin secretion (Aono et al., 1975).

Abnormal prolactin levels during the acute phase of anorexia nervosa have been reported (Mecklenburg et al., 1974). In the majority of patients, however, the plasma prolactin levels have been found to fall in the normal range (Beumont et al., 1974; Mecklenburg et al., 1974).

Studies of pituitary hormones, other than the gonadotrophins, have yielded conflicting findings. As Brown et al. (1977) observe, plasma cortisol and plasma growth hormone are reported as normal or elevated; some patients have been described as euthyroid, while instances of reduced levels of thyroxine and triiodothyronine have been reported. Anorexia nervosa is not associated with a global failure of anterior pituitary function and the long held view that anorexia nervosa resembles Simmond's disease, or panhypopituitarism, is now recognized as a myth.

Hormonal levels during rehabilitation and after weight gain

As weight is gained during rehabilitation, the levels of circulating gonadotrophins and gonadal steroids tend to rise, but this is not an invariable finding (Crisp et al., 1973; Marshall & Fraser, 1971; Wakeling et al., 1976, 1977). In some cases the basal LH levels remain abnormally low even after the restoration of normal weight (Nillius & Wide, 1977; Wakeling et al., 1976).

In those instances in which gonadotrophins levels do rise to

normal levels there appears to be a relationship between weight gain and increased gonadotrophin levels. Furthermore it has been reported that FSH shows earlier recovery than LH during the course of nutritional rehabilitation (Beumont et al., 1976; Nillius & Wide, 1977). Beumont et al. (1976) found that basal levels of both LH and FSH rise with weight gain, with a linear correlation between gonadotrophin levels and body weight (expressed as % of standard). The return to normal basal FSH levels occurred earlier during rehabilitation, while the patients were still in the 70-79% standard weight category. Plasma LH and oestradiol values did not reach the normal range until the patients were weighing 80% or more of standard weight.

The observation that basal LH levels remain persistently low in some nutritionally rehabilitated patients has been the source of some speculation. Russell (1969) suggested that difference in the severity and duration of weight loss may account for the different secretion patterns found in some rehabilitated patients. However, Kanis et al. (1974) found that the rise in gonadotrophin levels in their patients after treatment did not appear to be related to the severity or duration of the weight loss. This is likely to remain a moot point until the severity and duration of inanition and its relationship with gonadotrophin levels after treatment is further investigated.

Wakeling et al. (1976) proposed some alternative explanations for the persistently low basal LH levels after restoration of normal weight. One proposal is that the centre responsible for the basal or tonic release of gonadotrophins remains unresponsive, possibly because of greater sensitivity to the negative feedback effects of the albeit low levels of oestrogen. Other explanations may be a persistent unresponsiveness of the anterior pituitary due to depletion of gonadotrophins consequent upon decreased LHRH secretion, or a refractoriness of the ovarian

follicle (Marshall & Fraser, 1971).

With the resumption of normal weight the amenorrhoea may persist for months or even years (Dally, 1969). Studies of the patterns of hormone excretion and secretion in such cases have revealed an absence of the cyclical changes in gonadotrophins and oestrogens which characterize the normal menstrual cycle (Bell et al., 1966; Crisp et al., 1973; Marshall & Fraser, 1971; Russel & Beardwood, 1968). That is, there are some patients who have basal LH levels restored to normal but who fail to show resumption of the cyclical events found in ovulating women. This suggests a persistent dysfunction of the centre(s) responsible for controlling the cycles of gonadotrophin release and ovulation.

The failure to resume normal rhythmical changes of gonadotrophins and gonadal steroid secretion after restoration of normal body weight is difficult to explain simply in terms of weight loss or malnutrition. With regard to the latter, Mecklenburg et al. (1974) raise the possibility that starvation damages the hypothalamus. This hypothesis, however, so far lacks experimental support.

Returning to the issue of body weight it may be said that restoration of normal body weight is a prerequisite for the resumption of menstruation but that other, as yet unidentified, factors, operate in perpetuating the amenorrhoea. Dally & Sargent (1966) suggested that normal menses return in anorexic patients only after the psychological disturbance has been resolved, in addition to their maintaining a normal body weight. Results of animal studies have suggested that basal gonadotrophin secretion is regulated separately from the mid cycle surge (Barraclough & Gorski, 1961; Halasz & Gorski, 1967). Evidence suggesting that basal secretion, but not phasic secretion, is affected by changes in body weight comes from studies of obese patients on reduction diets. Although gonadotrophin levels decreased the rhythmical

pattern of gonadotrophin output was maintained, in contrast to the acyclic secretion pattern found in anorexic patients (Beardwood, 1974; Russell, 1972a). Russell (1972a) proposed that deprivation of calories and carbohydrates in anorexia nervosa might interfere with basal levels but that it does not account for the interruption of gonadotrophin cycles. Beardwood (1974) observed that the phasic mid cycle surge in animals is, to a large extent, neurally mediated and suggested that the phasic secretion in anorexics may be more readily affected by psychological distress than by weight loss. Although conceding that alternative explanations may exist, Russell (1972a) also proposed that central nervous mechanisms, triggered off by psychological disturbance, may be implicated in the acyclic patterns found in anorexic patients.

Studies of the 24 hour LH secretory patterns of nutritionally rehabilitated anorexics (Boyar et al., 1974; Boyar & Katz, 1977) also provide support for the notion that weight loss alone is insufficient to account for the endocrine changes found in anorexia nervosa. The findings are more suggestive of a multifactorial aetiology. For example, Boyar et al. (1974) observed that one of their patients, who had regained normal weight, showed a "maturation" from an early pubertal LH pattern to one characteristic of a normal adult woman. This, however, is not an invariable finding in nutritionally rehabilitated patients. Studies of other patients which revealed abnormal 24 hr LH secretory patterns, even when they had reached near-normal body weight, led Boyar and Katz (1977) to conclude that

> a regression or arrest of the 24-hr LH secretory "program" is not solely dependent on weight loss in patients with anorexia nervosa. The finding that "immature" LH secretory patterns can occur in patients who fulfil the psychiatric criteria for anorexia nervosa but who are of near-normal ideal body weight suggests that the psychiatric abnormality and its neurochemical correlate can also be associated with abnormalities of gonadotrophin secretion.
>
> (p. 183)

This observation does not exclude the possibility that weight factors are implicated in the abnormal LH secretion patterns in the nutritionally rehabilitated patients of Boyar & Katz (1977). Although all their patients had reached the "critical weight" (47.5 ± 0.8kg) at which the menarche occurs in normal pubertal girls (Frisch, 1972), recent evidence suggests that an even greater weight needs to be achieved before normality is restored. Frisch & McArthur (1974) suggested that fatness may prove to be an even more critical determinant of the menarche and ovulation. It seems that adolescent girls who have had an episode of secondary amenorrhoea need to achieve a higher weight and a greater amount of fat before menstrual bleeding resumes (Editorial, BMJ 1975). A similar conclusion was drawn with regard to anorexia nervosa patients by Crisp & Stonehill (1971). They found that patients who had become amenorrhoeic at a time when their weight was greater than 47.5 kg, had to regain weight to a level equal to that at the onset of the amenorrhoea before menses reappeared.

In conclusion, the relationship between body weight and the onset, maintenance and persistence (after nutritional rehabilitation) of the amenorrhoea or abnormal gonadotrophin secretory patterns in anorexia nervosa is still to be fully elucidated. The balance of available evidence seems to favour a multifactorial aetiology with not all factors operating equally in all patients. Indeed, in some cases a single factor may be dominant; in other cases a separate factor may dominate, whereas in other cases the factors may interact. At present, all that can be concluded is that, in different patients, either body weight, residual psychological disturbance or other factors may account for the observed abnormalities, either separately or in interaction. This viewpoint is supported and most clearly illustrated by the observations that (a) some patients become amenorrhoeic prior to dieting and (b)

some nutritionally rehabilitated patients remain amenorrhoeic and/or continue to display abnormal gonadotrophin secretory patterns, and (c) other rehabilitated patients resume normal menstrual function shortly after normal body weight has been restored.

Pharmacological tests with anorexia nervosa patients during the acute phase and nutritional rehabilitation

In an attempt to investigate more fully the nature and location of the endocrine dysfunction in anorexia nervosa researchers have administered exogenous hormones and other provocative agents to anorexic patients. Before reviewing these studies it is appropriate to introduce a note of caution. Whereas these tests are likely to provide some valuable insights into understanding the mechanisms underlying the endocrine disturbance, it should be remembered that responses to these pharmacological tests may not provide a true depiction of the endogenous hormonal milieu (Boyar & Katz, 1977). Because of the complex interrelationships which exist between the hormones of the HPO system, and the intricacy of the various feedback systems interpretations of observed responses must be offered with caution; that is, interpretion of results are, at best, speculative. These tests may nevertheless provide some vital clues to understanding this baffling disorder.

The three main stimulatory or provocative tests employed in the investigation of anorexia nervosa patients are (a) the clomiphene citrate stimulation test (b) the oestrogen-provocation test (c) the LHRH test.

(a) The clomiphene citrate stimulation test:

Clomiphene citrate is thought to act by competing for and thus blocking oestradiol receptors in the tonic centre of the hypothalamus and in the anterior pituitary; thus preventing the negative

feedback effects of oestrogen (Franchimont, 1971; Maurer & Woolley, 1971; Newton & Dixon, 1971). It is presumed that the blocking of the negative feedback action at the hypothalamic and pituitary levels results in the increased synthesis and secretion of LHRH and gonadotrophins respectively (Ross et al., 1970).

The use of this test in endocrine investigation, as distinct from its therapeutic application, is to evaluate the integrity of the hypothalamic-pituitary axis and, potentially, the entire HPO axis (Katz, 1977).

The normal response to clomiphene is described as an initial rise of serum LH and oestradiol levels during administration of clomiphene. There is a fall in LH levels before a secondary LH peak which occurs 3-9 days (Marshall et al., 1973) or 5-8 days (Jacobson et al., 1968; Ross et al., 1970) after clomiphene is stopped. Ovulation may thereby be induced in women with anovulatory infertility whose pituitary and ovaries are responsive (Wakeling et al., 1976).

Acute phase: Marshall & Fraser (1971) and Beumont et al. (1973) found that clomiphene, administered during the acute stage of anorexia nervosa failed to cause an increase in blood LH levels or to induce menses. These findings have been generally confirmed by Travaglini et al. (1976) who found that clomiphene administration induced an increase in gonadotrophins in only 1 out of 7 patients and by Wakeling, et al.(1976) who report that, in the malnourished state, the response to clomiphene was usually either absent or incomplete. Aono et al. (1975), however, found an increase in serum LH in 3 out of 5 patients during the administration of clomiphene, with the secondary peak of LH occurring in one of these 3 patients. All three patients who showed a response

weighed less than 80% of normal weight. However, it is not clear whether these patients had entered the recovery stage or not. Aono et al. (1975) do, however, describe the patient showing the secondary LH peak as being "at the recovery stage". The finding of responses to clomiphene in patients below 80% of standard weight (Aono et al., 1975) is inconsistent with the findings of Marshall & Fraser (1971) and Beumont et al. (1973) who found responses only in those patients who had achieved 81% of the ideal body weight and 80% of standard weight respectively.

During and after nutritional rehabilitation: Marshall & Fraser (1971) and Beumont et al. (1973) found an increase in basal levels of LH as well as a LH response to clomiphene in patients weighing more than 80% of standard showed the secondary LH peak, followed by menstrual bleeding. A relationship between body weight and responsiveness to clomiphene has been demonstrated by Brown et al. (1977). They report that the LH response to clomiphene was significantly correlated with weight loss and that there was no relationship between the response to clomiphene and the duration of amenorrhoea.

Restoration of weight, however, does not invariably result in a normal response to clomiphene. Marshall et al. (1973) found that 6 out of 8 anorexic patients who had regained weight showed an incomplete response to clomiphene. That is, there was a rise of LH during clomiphene administration but thereafter levels fell to baseline without the appearance of the secondary LH peak. A similar finding has been reported by Russell & Wakeling (1974) and Wakeling et al. (1976). They reported that after the resumption of normal weight the second LH peak and subsequent menstruation were frequently not demonstrated.

Factors other than the nutritional status must presumably be present to account for the incomplete response to clomiphene in patients whose normal body weight has been restored. The finding of an initial LH response to clomiphene but an absent secondary peak suggests that the hypothalamic negative feedback mechanism for oestrogens is functioning and is being effectively blocked by clomiphene. The failure to show a secondary LH peak suggests that non-nutritional factors interfere with the hypothalamic positive feedback mechanism. Marshall et al. (1973) propose that these findings provide support for the proposition that there are two separate hypothalamic feedback centres in women, and shows that in some anorexic patients only the positive feedback mechanism is impaired.

The nature of the disturbance of the positive feedback mechanism is yet to be established. It could be due to an insensitivity of the tonic centre to oestrogens or the absence of the required surge of oestrogens because of ovarian refractoriness (Wakeling et al., 1976). The results of studies employing oestrogen provocation and LHRH administration, to be reviewed shortly, suggest that the defect lies in the hypothalamus and not at the level of the anterior pituitary or the ovaries.

As to the cause of the presumed hypothalamic dysfunction, Wakeling et al. (1976) pointed out that the frequent absence of the clomiphene response after malnutrition has been corrected, taken together with the finding of amenorrhoea antedating weight loss in some patients, suggests that non-nutritional factors are implicated. They speculated that psychological factors may be responsible for the persistent endocrine disorder, observing that it is "true to say that the psychological disturbance is prominent

throughout the course of the illness, and is often the last abnormality to become resolved" (Wakeling et al., 1976, p. 379). As convincing as this argument may appear, such an explanation is still hypothetical.

(b) The oestrogen provocation test:

Another way of investigating the mechanisms underlying the amenorrhoea in anorexia nervosa is to test the capacity of the hypothalamic-pituitary axis to respond with changes in LH release to the negative and positive feedback of exogenously administered oestrogen at different stages of treatment.

In normal women the oestrogen administration initially causes the lowering of the plasma LH levels, presumably as a result of the negative feedback action of the oestrogen. This is followed, 48-96 hrs later, by an acute rise in circulating LH levels, presumably as a result of the positive feedback action of oestrogen acting on the tonic centre in the hypothalamus and possibly on the pituitary as well (Baird et al., 1975; Tsai & Yen, 1971).

The most thorough investigation published to date, of the effects of exogenous oestrogen on the release of LH in subjects with anorexia nervosa, is that of Wakeling et al. (1977). The following review relies heavily on their report.

Wakeling et al. (1977) reported that prior to significant weight gain oestrogen administration produced a lowering of serum LH in those patients with measurable LH levels, but failed to produce a positive feedback release of LH in any patients.

In patients tested after the restoration of normal weight the negative feedback effects were more apparent but only one quarter of their patients showed the complete response to oestrogen with a subsequent positive feedback release of LH. These findings led

Wakeling et al. (1977) to suggest

> that in anorexia nervosa the ability of the hypothalamus to respond to the positive feedback effects of estrogen is more impaired than its ability to respond to the negative feedback effects. Furthermore, the ability to respond to these positive feedback effects is clearly influenced by the patient's weight. They were not demonstrated prior to the resumption of a normal weight and until basal LH levels had risen to normal. However, the capacity of the hypothalamus to respond to the positive feedback effects of estrogen was still impaired in the majority of patients even after these criteria had been fulfilled.
>
> (p. 206)

This finding, Wakeling et al. (1977) observed, is in keeping with the results of the studies using clomiphene which suggest that the failure to menstruate in rehabilitated patients is the result of an impaired hypothalamic response to the positive feedback effects of oestrogen. They suggest that in recovery from anorexia nervosa there is a return to normal HPO activity in a definite sequence which recapitulates puberty i.e. during recovery the hypothalamus first responds to the negative feedback effects of oestrogen, but only at a later stage does it respond to the positive feedback effects.

Although recovery is dependent on weight gain, the fact that in some patients the amenorrhoea persists for long periods following restoration of normal weight suggests that non-weight dependent factors are operating in these cases. Once again the possibility of residual psychological disturbance is raised in order to account for the prolonged hypothalamic dysfunction (Wakeling et al., 1977).

(c) The LHRH test:

The LHRH test is widely used as a test of pituitary function. A positive response is characterized by a rise of LH reaching a peak 15-30 minutes after intravenous injection. FSH elevation is

less marked and maximum levels are usually achieved 60-120 minutes after injection. Before concluding that the response is absent it must be shown that neither conventional doses (100μg), nor larger doses (500μg), nor more prolonged administration (100μg per day for 5 days) of LHRH produces any effect (Katz, 1977).

In order to facilitate a clear discussion the effects of acute or prolonged LHRH administration during the acute phase of anorexia nervosa or during nutritional rehabilitation, will be discussed separately.

Acute phase - acute LHRH administration: The results of the numerous studies employing single intravenous injections of LHRH during the acute phase of anorexia nervosa demonstrate the great variability found in endocrine investigations of this disorder. The response during the acute phase of anorexia nervosa has been described variously as absent, impaired or normal. The variation is not only between investigators but also within the same series of patients being investigated. Furthermore the different findings do not appear to be related, in any consistent way, to the severity or duration of emaciation.

The absence of a LH response to a single injection of LHRH is not found with great regularity but still frequently enough to suggest that this is not a chance finding nor the result of technical errors (Aono, 1975; Mecklenburg et al., 1974; Nillius & Wide, 1977; Warren, 1977; Warren et al., 1975). This finding, in some of their patients, led Mecklenburg et al. (1974) and Nillius & Wide (1977) to suggest either (i) a primary pituitary defect or (ii) a pituitary defect secondary to a relatively severe and chronic hypothalamic deficiency of LHRH which results in decreased pituitary content of gonadotrophins. The results of prolonged LHRH administration

studies tend to support the latter as the most likely explanation.

However, it has been found that the majority of patients do respond to LHRH administration even if the response is an impaired or restricted one (Aono et al., 1975; Beumont et al., 1976; Crosignani et al., 1974; Lundberg et al., 1972; Sherman et al., 1975; Travaglini et al., 1976; Yoshimoto et al., 1975). The LH response of patients below 70% of standard weight (Beumont et al., 1976) or 70% of ideal body weight (Sherman et al., 1975) was strikingly impaired. Nillius & Wide (1977) reported that the LH response of a group of 26 women with a mean body weight of 66% of mean ideal body weight was only one third of the normal response. The finding of an LH response, albeit an imperfect one, provides indirect evidence that the pituitary function of the majority of patients in the acute phase of anorexia nervosa is intact.

Furthermore, a number of investigators have reported finding an LH response to a single administration of LHRH, during the acute phase, that is equal to that found in normal women during the follicular phase of the menstrual cycle (Aono et al., 1975; Mecklenburg et al., 1974; Mortimer et al., 1973; Wiegelman & Solberg, 1972).

A great variability in the FSH response to LHRH administration during the acute phase of anorexia nervosa has also been observed. In some patients the FSH response is found to be impaired (Beumont et al., 1976; Warren, 1977), preserved (Nillius & Wide, 1977; Sherman et al., 1975) or sometimes even increased (Nillius & Wide, 1977). It is difficult to account for these different responses. Beumont et al. (1976) suggested that their patients with impaired FSH responses may either have been more severely emaciated or the duration of emaciation was greater than that of patients

investigated in other series. However, no consistent relationship between severity or duration of emaciation and impairment of the FSH response has been demonstrated.

In the case of those patients showing normal FSH responses to LHRH it is tempting to view the endocrine disturbance in these patients as a regression to a prepubertal-like state. In normal prepubertal girls the FSH response to LHRH is normal while the LH response is minimal (Roth et al., 1972). It has been suggested that the decreased LH response to exogenous LHRH in prepubertal girls is due to inadequate endogenous secretion of LHRH (Roth et al., 1972). This observation adds further support for the view that there is impaired synthesis and/or release of LHRH from the hypothalamus in anorexia nervosa.

Acute phase - prolonged LHRH administration: Studies involving the prolonged exposure of the pituitary to LHRH provide further support for the viewpoint that reduced secretion of pituitary gonadotrophins in patients with anorexia nervosa is secondary to inadequate endogenous secretion of LHRH from the hypothalamus.

To patients showing poor responses to a single LHRH injection, Aono et al. (1975) administered a drip infusion of 400μg LHRH over a period of one hour. The majority of patients showed LH and FSH responses greater than those observed after a single LHRH injection. Further improvement was observed when the patients were tested with a prolonged LHRH test. Six consecutive daily intramuscular injections of 100μg of LHRH were given followed by an intravenous injection of 100μg of LHRH on the seventh day. All patients showed gradual improvement of LH and FSH responses on consecutive days and the responses to the I.V. injection of the seventh day were normal. These findings suggest that the poor

responses to single injections of LHRH shown by some patients is most likely due to a dysfunction at the hypothalamic rather than the pituitary level. As Aono et al. (1975) observed, patients with known organic pituitary lesions do not show enhanced LH or FSH responses following prolonged LHRH tests.

Nillius, Fries & Wide (1975) and Nillius & Wide (1977) were able to induce follicular maturation and ovulation in amenorrhoeic women in the acute phase of anorexia nervosa by means of prolonged treatment with LHRH (500µg LHRH three times daily for about 4 weeks). Nillius & Wide (1977) hypothesised that the decreased LH response to exogenous LHRH, seen in anorexia nervosa patients, like that seen in normal perpubertal girls, is secondary to inadequate endogenous secretion of LHRH. They found that prolonged LHRH administration to anorexic patients resulted in a change from the prepubertal-like response pattern to a normal preovulatory response pattern after about two weeks. This reversal, they suggested, may be due to self priming effects of LHRH on the pituitary acting together with the modulatory feedback effects of oestradiol at the pituitary level. Prolonged treatment with LHRH not only restored the pituitary reserve capacity for gonadotrophin secretion but also induced cyclical changes in the gonadotrophin secretion with subsequent ovulatory menstrual cycles. The induction of ovulation together with restoration of normal basal ovarian steroid secretion indicates that there is no ovarian failure nor refractoriness in anorexia nervosa.

Overall, the results of the studies reported on in this section suggest that reduced secretion of pituitary gonadotrophin in patients with anorexia nervosa is secondary to hypothalamic dysfunction.

During and after rehabilitation - acute LHRH administration:

Recent studies have demonstrated a correlation between LH and FSH responsiveness to LHRH and increasing body weight in anorexia nervosa patients (Beumont et al., 1976; Brown et al., 1977; Palmer et al., 1975; Sherman et al., 1975; Warren et al., 1975). In contrast, Warren (1977) reported that women who had developed amenorrhoea in a setting other than weight loss did not show an LHRH responsiveness which was correlated with body weight. Although the general finding is increased LH and FSH responses to LHRH with weight gain there is little agreement concerning the degree of recovery required before normal responses are observed.

It is not infrequently observed that in some cases there is an exaggerated LH response to LHRH during the recovery period. Although the reasons for the excessive responses are not clear it is interesting to note that a similar hyper-responsiveness to LHRH has been found in children as they enter puberty (Roth et al., 1972). Once again we have the suggestion that recovery from anorexia nervosa is a recapitulation of puberty.

Where abnormal FSH responses to LHRH have been found (Beumont et al., 1976; Warren et al., 1975) the recovery of the FSH response appears to occur earlier in the recovery process than the LH response. Beumont et al.(1976) provided an interesting hypothesis to account for the disparate LH and FSH responsiveness to LHRH during recovery. Their findings and those of Warren et al. (1975) suggest that LH is more sensitive to weight loss than FSH. Beumont et al. (1976) suggested that the sequence of recovery during weight gain may be: an initial recovery of FSH release, resulting in gonadal stimulation with an increase in oestradiol which sensitizes the pituitary to LHRH, resulting in an enhanced

LH response. This hypothesis is consistent with experimental evidence that gonadal steroids exert an effect on the responsiveness of pituitary gonadotrophs to LHRH stimulation (Aiyer et al., 1974; Arimura & Schally, 1971; Jaffe & Keye, 1974; Krey et al., 1973).

In the studies reported on so far it has been observed that some anorexia nervosa patients remain amenorrhoeic for a long while after restoration of normal body weight and that these amenorrhoeic patients presumably have a normal pituitary response to LHRH when normal weight is restored. These observations, taken together, suggest that factors unrelated to weight gain must be operating, possibly by perpetuating a hypothalamic dysfunction.

Conclusions and research objectives

The bulk of the experimental evidence points to the hypothalamus as the primary site of the dysfunction in the secondary amenorrhoea of anorexia nervosa. Pituitary gonadotrophin levels may be low or depleted, but the results of pharmacological tests generally indicate that this is secondary to reduced synthesis or secretion of LHRH from the hypothalamus rather than due to a primary pituitary defect. Even during the acute stage of anorexia nervosa, prolonged LHRH treatment has proved to be successful in restoring normal gonadotrophin responses and even in inducing ovulation. The ability of the ovaries to respond to gonadotrophin stimulation excludes ovarian refractoriness as a significant factor.

While most investigators are in agreement that the hypothalamus is the site of the dysfunction the causes or factors responsible for the dysfunction remain a topic of active debate. It seems likely that different factors operating at different times, individually or in

combination, are responsible for the different patterns of menstrual disturbance. For example, endocrine studies of anorexics during rehabilitation indicate that body weight is a crucial determinant of hormone concentration. It does not, however, seem to be prominent in tonic control of gonadotrophin release which may still be impaired after nutritional rehabilitation.

It was observed earlier that investigations of anorexia nervosa patients are complicated by the fact that nutritional and psychological disturbances are likely to coexist in this disorder. In the present study the research strategy was to investigate the effects of psychological and nutritional factors, at first individually, and then in combination, on the reproductive physiology of the female rat. The use of laboratory animals was necessary because of the obvious limitations on human experimentation. The study to be reported on involves the manipulation of nutritional and psychological variables and an investigation of their effect on the physiological system of a lower animal; namely the reproductive system of the female rat.

The present work represents the first reported attempt at an experimental investigation of the separate and then combined effects of nutritional deprivation and experimentally induced stress on the reproductive physiology of the same strain of rat.

There can be no pretence that a condition equivalent to anorexia nervosa was created in the rat in the laboratory. The intention was to investigate the effects of certain variables on a reproductive system, the hormonal mechanisms of which are essentially the same as those regulating the human cycle. While similarities exist it is recognized that caution needs to be exercised when discussing the results of this study and their relevance to human subjects and anorexia nervosa.

The specific research objectives will be presented in the introduction to each experiment or series of experiments. At this stage the research objectives will be outlined in more general terms. By means of an experimental approach a number of issues related to reproductive disorders occurring as a consequence of malnutrition and/or psychological disturbance were investigated in this research. The findings are discussed in terms of their support for, and opposition to, existing hypotheses about the reproductive disturbance in anorexia nervosa.

The aims of the present research can be summarized as follows:

(i) To investigate the effects of a systematic reduction of the caloric components of the diet on reproductive function. The aim was to determine the severity and duration of caloric restriction required in order to alter gonadotrophin levels and disrupt normal cyclical activity. Is there a relationship between weight loss, reduction of gonadotrophin levels and cessation of cycling?

In broad terms the purpose of these investigations was to determine how sensitive the reproductive system is to changes in caloric intake. Some clinicians claim that relatively minor changes in food intake, without causing significant weight loss, may exert a disruptive effect on cyclical activity. Alternatively it could be proposed that reproductive function will only be disturbed by nutritional deprivation when the latter affects the ability of the organism to support gestation.

(ii) To employ pharmocological tests in order to determine which components of the HPO axis are dysfunctional during nutritionally induced anoestrus and before recovery during rehabilitation. Tests similar to those used in the investigation of the reproductive dysfunction in anorexia nervosa were employed.

(iii) To determine whether nutritional rehabilitation, following severe

caloric deprivation, results in full recovery of the reproductive system. Is there evidence of lasting damage caused by the nature and duration of the malnutrition imposed?

Of particular interest was the duration of nutritional rehabilitation and the extent of weight gain required before resumption of ovulatory cycles was observed. A related objective was to determine whether there is a relationship between the duration of malnutrition and the time taken to recover from the effects of malnutrition. Will resumption of cyclical activity be delayed if the period of malnutrition has been an extended one?

(iv) To establish the nature and duration of experimental stress treatments required to alter gonadotrophin levels and to disrupt cyclical activity in fully fed rats. The aim was to establish the minimum amount of stress treatment required in order to induce anoestrus.

(v) To investigate changes in reproductive function when nutritional deprivation and experimental stress factors are imposed at the same time. The aim was to determine whether inferior nutritional status results in increased susceptibility of the reproductive system to disruption by stress. Stated more specifically the aim was to determine whether stress accelerates cessation of cycling and delays resumption of cyclical activity when rats are subjected to, or are recovering from severe caloric restriction.

(vi) To employ pharmocological tests in an attempt to identify the site(s) of dysfunction in the event of anoestrus persisting in stressed rats after the restoration of normal body weight.

The research objectives summarized above were prompted by a number of unresolved questions concerning aspects of the reproductive disturbance found in anorexia nervosa patients. The research carried out in an attempt to achieve these objectives is described in Chapters 4-6. The relevance of the findings of this research to an understanding of the menstrual disturbance in anorexia nervosa is discussed in Chapter 7.

Chapter 3

METHODOLOGICAL DETAILS

Subjects and housing

Throughout the research BLU:LE Long-Evans descent hooded female rats were used. All animals were 4 day cycling, virgin, adult females bred in the University of Cape Town Medical School animal house. On the day of weaning they were transferred to a rat stock room in the Department of Psychology, University of Cape Town. They were housed in groups of five in a temperature controlled (22±1°C), sound attenuated and artificially illuminated room with a lighting schedule of 10 hours darkness (2000 - 0600h) and 14 hours of light.

Pelleted rat food (Epol, Vereeniging Consolidated) and water were provided ad libitum.

During the experimental phases the rats were housed in a separate room with the same controlled environmental conditions as the stock room. In the experimental room the rats were individually housed in steel cages (Acme) with wire screen floors raised above the dropping tray to prevent copraphagia.

Body weight was measured regularly by placing each rat into a lidded cage on a triple beam balance. Body weight was recorded to the nearest whole gram.

Vaginal smear records were taken each day by inserting a small swab, dampened with distilled water, a short way into the vagina. The smears were allowed to dry on a glass slide, without fixation, and were examined microscopically at a magnification of 100. The various stages of the oestrous cycle were identified on the basis of the relative proportions of epithelial cells, leucocytes and keratin present, as described by J. A. Long and H. M. Evans in 1922. In the present research the days of the 4 day cycle were designated: oestrus, metoestrus,

dioestrus, and pro-oestrus.

Handling and gentling: occurred daily when the vaginal smears were taken and body weights were measured.

Normal control values: The normal growth curve of individually housed Long-Evans female rats fed standard pelleted rat food and water ad libitum from age 5-15 weeks is shown in Supplementary Study No. 1 (Appendix A).

In Supplementary Study No. 8 (See Appendix A) the following control values were obtained from fully fed, non-stressed adult rats at different times of the day on each day of the oestrous cycle.

(i) Serum LH concentration
  (a) of chronically cannulated rats
  (b) of rats sacrificed by cervical dislocation.
(ii) Pituitary LH content and concentration.
(iii) Organ weights - pituitary gland, ovaries, uterus, and adrenal glands.
(iv) Number of surface corpora lutea and follicles reaching criterion size.
(v) Number of tubal ova present.

In Supplementary Study No. 7 vaginal smears were taken daily for a period of 12 weeks from a group of 30 non-stressed fully fed, mature female rats. The incidence and nature of vaginal smear pattern aberrations are presented in Appendix A.

Quantitative feeding

Feeding jars In order to monitor food consumption food was provided in a non-scattering feeding jar similar to that described by Kirsch (1972). Glass ointment jars with a 5 cm diameter mouth were attached to the grill floor of the cage. To reduce spillage the food was

covered by a freely moving aluminium disc, perforated with 5 holes each 12 mm in diameter. This disc, while allowing ready access to food but preventing the scattering of food, could not be moved beyond the neck of the jar once it had been fitted.

Daily (24 hr) food consumption): was measured by weighing the feeding jar before it was placed in the cage and again the following day before refilling with food. Food consumption was recorded to the nearest whole gram.

Spillage The results of Supplementary Study No. 2 revealed that spillage of food amounted to less than 2% of the total amount of food consumed (Appendix A). Since the amount of spillage was so small a correction factor was not employed.

Preparation of experimental diets:

Basal diet: A modification of the casein diet described by Harper (1959b) was used as the basal mixture (hereafter referred to as the basal diet).

The composition of basal diet is shown in Table 1.

Table 1

Composition of Basal Diet (g/100g)	
Casein	20.0
Dextrin	67.1
Mineral Mixture	4.4
Vitamin Mixture	2.2
Maize oil	4.0
Hake oil	1.0
Choline chloride	0.3
DL cystine	1.0

Notes:

1. Milled casein, 90 mesh supplied by the Milk Board, Witwatersrand Area (Certificate of analysis according to International Dairy Federation Specification CE Doc 20 was provided).
2. White dextrin (acid hydrolysed starch) was donated by Glucose & Starch Products Ltd., Bellville.
3. Mineral mixture recommended by National Food Research Institute, CSIR, Pretoria (Dreyer, 1976, pers. comm.).
   For composition refer to Appendix B.
4. Vitamin diet fortification mixture supplied by ICN Pharmaceuticals Inc. Catalog No. 104654.
   For composition refer to Appendix B.
5. Maize oil supplied by Cape Oil, Cape Town.
6. Hake oil supplied by Marine Oil Refineries, Simonstown.
7. Choline chloride: Merck AG.
8. As casein is known to be low in its content of sulphur containing amino acids, DL cystine was added to compensate for a possible deficiency of methionine, the first limiting amino acid in casein (Harper, 1959a).

The results of Supplementary Study No. 2 revealed that the basal diet was capable of supporting normal growth. The amount of basal diet consumed was similar to that of crushed pelleted rat food (Appendix A). The results of Supplementary Study No. 3 suggest that the basal diet was a palatable one.

Dextrin reduction diets In the present research the amount of dextrin in the basal diet was systematically reduced. Hereafter these diets are referred to as dextrin reduction diets. The composition of the dextrin reduction diets are shown in Table 2.

Table 2

Composition of dextrin reduction diets (g/100g)

	% dextrin reduction			
	25	50	75	100
Casein	24.0	30.1	40.3	60.8
Dextrin	60.5	50.5	33.8	-
Mineral Mixture	5.3	6.6	8.9	13.4
Vitamin Mixture	2.6	3.3	4.4	6.7
Maize oil	4.8	6.0	8.0	12.2
Hake oil	1.2	1.5	2.0	3.0
Choline chloride	0.4	0.5	0.6	0.9
DL cystine	1.2	1.5	2.0	3.0

The reduction of dextrin was compensated for by increasing the quantities of all other components, but still maintaining the same relative proportions of all ingredients except dextrin.

The quantities of dextrin reduced diets to be provided were calculated by multiplying the 24 hr intake of basal diet of ad libitum fed control rats by the following factors:

25% dextrin reduction = 0.8322

50% " " = 0.6645

75% " " = 0.4968

100% " " = 0.3290

These factors are derived from the change in the dextrin component proportionate to that found in the basal diet. In this way the absolute quantities of the other components of the diet remained the same as that of the ad libitum consumed basal diet.

For example: Ad libitum fed rats consume 18g of basal diet per day.

∴ Rats on 25% reduction diet receive

18 x .8322 = 14.98 or 15g of 25% dextrin reduction diet.

With the exception of dextrin, both groups of rats received the same absolute quantities of the dietary components, as shown in Table 3.

Table 3

Absolute quantities (g)

	18g basal diet	14.98g25% dextrin reduction
Casein	3.6	3.6
Dextrin	12.08	9.06
Mineral Mixture	0.792	0.792
Vitamin Mixture	0.396	0.396
Maize oil	0.72	0.72
Hake oil	0.18	0.18
Choline chloride	0.054	0.054
DL cystine	0.18	0.18
	18.0g	14.98g

Dextrin and fat free diet (DFF diet): In this diet the dextrin and oil components were excluded. The composition of the DFF diet is shown in Table 4.

Table 4

Composition of DFF diet (g/100g)

Casein	71.7
Dextrin	-
Mineral Mixture	15.7
Vitamin Mixture	7.9
Maize oil	-
Hake oil	-
Choline chloride	1.1
DL cystine	3.6

The quantity of DFF diet provided was computed by multiplying the 24 hr consumption rate of the basal diet by ad libitum fed rats by 0.2790 (e.g. 18 x 0.2790 = 5.02g).

Mixing of dietary components: The procedure of trituration was employed in the mixing of the components of the diet. The dry powdery casein and dextrin were added, in small quantities, to the moist mineral and vitamin ingredients to create a homogenous mixture. The same procedure was repeated when adding the mixture to the oils.

Feeding schedules: The feeding jars containing the measured quantities of experimental diet remained in the cages except when they were removed for re-filling. The procedure of allowing experimental subjects to have 24 hr access to food was adopted after the results of Supplementary Study No. 4 revealed that rats did not adjust to an "interval feeding" regimen in which access to food was restricted to a single daily 2 hr period. After 16 days feeding on the 2 hr interval regimen the mean body weight and mean food consumption rate of basal diet fed rats were significantly lower than those of ad libitum fed rats. The results of Supplementary Study No. 5 revealed that increasing access to food by a further 2 hr interval (i.e. 2 x 2 hr intervals) did not result in food intake reaching control values. These findings are not consistent with those of Stead & Brock (1972) who reported that rats adjusted to a 2 hr interval feeding schedule after 1 week.

Comment on bulk compensation: Mason (1968) raised the possibility that the reduction of bulk intake in nutritional research could result in the animals experiencing stress. In Supplementary Study No. 6 a non-nutritive bulk filler (Vermiculite) was added in different proportions to the basal diet. It was found that, when the filler made up the greater volume of the mixture, normal intake was reduced. It was concluded that, in the present research, the use of the bulk filler

would introduce the problem of regulation of the nutritional content of consumed feeds, (refer to Appendix A).

Stress induction procedures

Restraining devices: Adjustable restraining devices, built to the specifications reported by Crowell & Brown (1973), were used to immobilize the rats in the restraint stress experiments. Two intermeshing sets of 7 parabolic shaped stainless steel rods created an elliptical chamber adjustable to the size of each animal. The device, designed to hold small animals immobile, provided for rapid confinement and release and minimal discomfort apart from the imposed immobilization. The devices, which are electrifiable, were used in the classical fear conditioning experiments.

Conditioned emotional response procedures.

Lick training and lick suppression testing

A 20x20x23 cm operant chamber (Ralph Gerbrands Co. Model C) was used during lick training and lick suppression testing. At one end a hole was centred 8cm above the grid floor through which a drinking tube could be inserted. The end of the drinking tube was positioned .3cm behind the hole in the perspex wall so that all except the rat's tongue was insulated from contact with the drinking tube. Licking was detected by a sensor incorporated into the end of the drinking tube and lick rate was displayed on an electronic counter (Hewlett-Packard 5221B). Other researchers have reported that licking occurs in an all or nothing fashion (Schaeffer & Premack, 1961) and that the mean lick rate for adult rats is 5-6 licks per second (Leaton, 1974). That is, rats either lick at a rate of 5-6 licks per second or do not lick at all. Since the electronic counter was not equipped to measure a lick rate of this magnitude, total licking time in seconds was automatically

multiplied by a constant factor of 6 to give the total number of licks during any given time period.

The operant chamber and drinking tube were housed in a separate air conditioned and sound attenuated room. The electronic counter was located in an adjacent room.

Classical fear conditioning The electrifiable restraining cage has been judged suitable for classical aversive conditioning (Crowell & Brown, 1973). It has the advantage that it does not require removing the animal's hair, or using special electrodes, to present shock.

The unconditioned stimulus (US), produced by a BRS Electronics Shock Generator (Model SGS-001), was an electric shock of 1 sec duration delivered via the coils of the restraining cage. In order to prevent habituation to the CS (Svendsrod & Ursin, 1974) the shock intensity was increased gradually during the conditioning period: Days 1-5, .50ma; days 6-10, .65ma; days 11-15, .80ma.

The conditioned stimulus (CS) was the turning on, for 5 sec, of a lamp, suspended 20cm above the transparent ceiling of the conditioning cage. This increased the illumination at the floor of the cage from 28 to 120 lumens per square foot. In the case of the predictable shock trials the 5 sec CS exposure immediately preceded the US presentation. In the unpredictable shock trials the CS and US were randomly interspersed.

The presentation of the CS and US were controlled by means of a programmable randomizer unit designed and constructed in the workshop of the Department of Psychology, University of Cape Town. The unit consisted of 3 plastic discs each designed to accept 80 actuator pins within 2mm of its outside perimeter, providing up to 80 micro-switching operations per rotation. The 3 discs could be mounted simultaneously on a boss which was driven by a synchronous motor at one-sixth of a

revolution per minute. One disc (A), closest to the shaft base, was used exclusively for self-homing purposes, giving automatic start/stop capability. The other two discs (B and C) were individually programmed by the insertion of actuator pins into the holes near their perimeters. With a cycle duration of 6 min there was a 4.5 sec duration between adjacent pins. On-off times were initiated by the triggering of micro-switches by the actuator pins.

During the predictable shock trials disc B was employed and the switch outputs were interfaced with the CS lamp and the shock generator so that prior to the onset of the shock the lamp went on for 5 sec and the shock was delivered for the ensuing 1 sec.

During the unpredictable shock trials the discs B and C were randomly programmed to independently actuate on and off times of the US and CS respectively. Programming of the discs involved the random placement of 3 actuator pins on each disc. Random numbers tables were used to generate individual programmes for each trial.

Faecal bolus counts: Initially it was intended to use a faecal bolus count as an index of emotionality in rats subjected to restraint or the CS (Brady & Nauta, 1953; Kreezer, 1949). However, the rats invariably defaecated and urinated when placed in the restraining device, and the mean number of faecal boluses remained approximately the same. This gave an indication that stress had been induced when the rats were immobilized but gave no measure of the extent of emotionality. Because the number of faecal boluses remained similar in all restrained groups over a period of time, lengthy summaries of these data are not provided.

Chronic Jugular Cannulation

The technique employed was based on a modification by Querido

(1975) of the procedure described by Steffens (1969). The method allows for frequent sampling of central venous blood and infusion of exogenous hormones without disturbing the conscious rat.

Method

Materials:

Trilene (trichloroethylene) ICI Ltd.

Ether (diethyl)

22 gauge disposable syringe needle shaft; 1.25cm in length

10cm length of silastic tubing (Dow Corning Cat. No. 602-131)

Sterile saline (M.L. Laboratories)

Heparin injection B.P. (Evans Medical Ltd.)

Poly vinylpyrrolidone (PVP) solution (E. Merck A.G.):

1 gm PVP in 2 ml heparinized saline (500 and 1000 units per ml.)

30cm length of polythene tubing (P.E.60-ID 0.030 OD 0.048, Intramedic polythene tubing, Clay Adams division)

22 gauge metal Luer needle attachment with 1 cm length of smoothly polished shaft

¼ solution of tincture of iodine in 70% ethanol.

Terramycin solution - 20mg/ml.

Surgical instruments.

Procedure

After induction and maintenance of anaesthesia, with trilene and ether respectively, the right external jugular was exposed and then pierced and cannulated at a point 5mm caudal to the junction of the anterior jugular, acromiodeltoid, and cephalic veins. The silastic cannula was anchored in position by means of suitably placed ligatures and was passed under the skin to a position in the midline of the back. There it was connected to a metal cannula which was sutured in position

with approximately 1cm projecting vertically from the back. The external entrance to the metal cannula was blocked by a plug fashioned to fit snugly onto the cannula but still removable by the experimenter. The cannuli were kept patent by the infusion of PVP in heparinized saline (1000 units Heparin/ml) into the silastic and metal cannuli. Following surgery all rats were allowed a recovery period of at least 3 days before being used in an experiment.

Blood samples: were aspirated by removing the plug and slipping the polythene tube over the free end of the metal cannula. Blood was withdrawn through the polythene tubing into a 1 ml syringe after disposal of the maintenance PVP solution.

Sampling procedures for radioimmunoassay

Blood samples: were collected in two ways in the present research:

(i) by drawing blood through an indwelling jugular cannula

(ii) by collection of trunk blood immediately following cervical dislocation.

These procedures were adopted since it was believed that they were less likely to cause changes in hormonal balance as a result of stress or of the effects of anaesthetic on LH secretion. Although Campbell et al. (1977) reported that blood values of gonadotrophins in samples obtained by orbital sinus puncture, under light ether anaesthesia, were essentially duplicated in blood collected from decapitated rats, others have demonstrated a significant facilitatory or depressing effect of anaesthesia on serum LH concentration (Dunn et al., 1972; Querido, 1975).

The volume of blood drawn at each sampling in chronically cannulated rats was 0.4 ml. Samples were never drawn more than 6 times from each rat in a single day.

Blood samples were allowed to clot and serum was separated by centrifugation. Serum samples were immediately aliquotted in triplicate, in 0.05 ml quantities in capped Thomas tubes and stored at -18°C until assayed.

A comparison was made between the serum LH values obtained from samples collected by means of the two procedures described above. There was no significant difference between the two differently sampled groups with regard to the serum LH levels at various times of the day on each day of the oestrous cycle.

Pituitary samples: During autopsy the pituitary glands were removed and weighed. Each gland was placed in 1 ml phosphosaline buffer, pH 7.6 and disrupted by means of a Branson Model B12 Sonifier Cell Disruptor. Cellular debris was allowed to settle and the supernatant was diluted 1/400 and 1/800 with phosphosaline buffer, pH 7.6. Each dilution was aliquotted in triplicate and stored at -18°C until assayed.

The pituitary LH content was expressed in terms of µg LH/pituitary.

The pituitary LH concentration was expressed in terms of µg LH/mg pituitary wet weight.

Measurement of rat luteinizing hormone by double autibody radioimmunoassay

The radioimmunoassay for the determination of LH is a well established procedure (Berson & Yalow, 1973) and the microtechnique for the measurement of LH in rats is a tried and proven technique (Naftolin & Corker, 1971; Querido & Beardwood, 1977).

Since the identical procedure of Querido & Beardwood (1977) has been used only the reliability criteria of the assay will be presented here.

Reliability criteria

Sensitivity of the assay: Midgley et al. (1969) have defined the sensitivity of the assay as the least amount of hormone that can be distinguished from no hormone. The sensitivity of the assay was derived by comparing the percentage binding in the 8 ng/ml and 16 ng/ml tubes with the zero binding tube in 11 consecutive standard curves (Table 5).

Table 5

Comparison of percentage binding in the 8 ng/ml and 16 ng/ml tubes with the zero binding tubes in 11 consecutive standard curves (where C = $\bar{x}$ c/m/.05 ml)

	Sample Tube				
Std Curve	0 ng/ml C - NSB	8 ng/ml C - NSB	8 ng/ml B/B_o%	16 ng/ml C - NSB	16 ng/ml B/B_o%
1	5405	5401	99.9	5159	95.4
2	4942	4688	94.9	4896	99.0
3	4865	4999	102.7	4789	98.4
4	5718	5301	92.7	5390	94.3
5	5443	5482	100.7	5408	99.4
6	5175	4199	81.1	4506	87.0
7	6084	6006	98.7	5953	97.8
8	6117	6172	100.9	5747	93.9
9	5847	5285	90.4	5317	90.9
10	5916	5708	96.4	5513	93.2
11	4718	4675	99.0	4526	95.9
$\bar{X}$			96.15		95.02
SD			6.22		3.76
Paired t-test			t = 2.06		t = 4.39
			$p < 0.05$		$p < 0.0025$

It was determined that the sensitivity of the assay was 8 ng/ml since the corresponding percentage binding value (96.2±6.2%) was significantly different ($p < 0.05$) from the zero binding value. The

difference between the mean 16 ng/ml value (95.0±3.8%) and the zero binding value was significant ($p < .0025$).

Reproducibility: The reproducibility of the technique was estimated by measuring the LH levels in rat serum standard in consecutive assays (interassay variation), and by measuring LH levels in replicate samples of rat serum standard in a single assay (intra-assay variation).

The coefficient of inter-assay variation, calculated from 5 consecutive assays, was ±8.1% (Table 6). The coefficient of intra-assay variation, calculated from 10 pairs of rat serum standard in a single assay, was ±8.2% (Table 7). The coefficient of interassay variation (±8.1%) compares well with the reported values of ±7.5% and ±12.7% by Naftolin & Corker (1971) and Seki et al. (1971) respectively. The coefficient of intra-assay variation (±8.2%) was, however, slightly higher than the value of ±5% reported by Naftolin & Corker (1971).

Table 6

Inter-Assay Variation

Coefficient of inter-assay variation calculated from LH values (ng/ml) of rat serum standard samples in five consecutive assays

LH ng/ml
49
45
40
42
42
$\bar{X}$ 43.6
SD 3.51
Coefficient of Variation 8.1%

Table 7

Intra-assay variation

Coefficient of intra-assay variation calculated from sequential LH values (ng/ml) of rat serum standard samples in a single assay

Counts	Mean	Mean-NSB	B/B_o%	ng/ml
5023 5229	5126	4886	89.8	38
5041 4957	4999	4759	87.4	43
4872 5177	5025	4785	87.9	42.5
5167 5128	5148	4908	90.1	37
5012 5047	5029	4789	88.0	42
5064 4961	5013	4773	87.7	42.6
5033 5152	5079	4839	88.9	40
4868 4973	4920	4681	86.0	47
4922 4851	4887	4647	85.4	48
4947 5160	5054	4814	88.4	41
			$\bar{X}$	42.11
			SD	3.47
			Coefficient of variation	8.24%

Administration of pharmacological agents

(a) LHRH administration to cannulated rats

Materials and apparatus:

Stoetling Co. infusion pump.

Stock solution of LHRH: Gonadorelin (Ayerst Laboratories) made up in .01M phosphosaline buffer to contain 50µg LHRH/ml.

Infusion dose: Stock solution was diluted with physiological saline to contain 50 ng LHRH/0.05 ml.

Bolus dose: Stock solution was diluted with physiological saline to contain 50 ng LHRH/.1 ml.

Heparinized saline: containing 250 units of heparin per ml.

Procedure: A length of polythene tubing was connected to the metal cannula and a pre-treatment blood sample was drawn. The polythene tubing was then connected to the infusion pump and volumes of .05 ml heparinized saline or LHRH were infused slowly over a period of 1 hour. Immediately before administering the bolus dose of LHRH a blood sample was drawn (0 min). The polythene tubing was then connected to a syringe and the bolus dose of LHRH was injected. Blood samples were collected at 10, 20, 40 and 80 minute intervals after administration of the LHRH bolus.

(b) Oestradiol benzoate provocation test

Stock solution of oestradiol benzoate: b-Estradiol - 3 benzoate (Sigma E700) was dissolved in absolute ethyl alcohol and then diluted with olive oil to obtain a stock solution containing 200µg/ml.

For injection: the stock solution was further diluted with olive oil to contain 2 µg/0.1 ml.

Control injection: 0.3 ml absolute ethyl alcohol was added to 9.7 ml olive oil and further diluted 1:10 with olive oil.

The experimental group rats were given a single subcutaneous injection of 0.1 ml olive oil containing 2µg oestradiol benzoate. The control animals received a single subcutaneous injection of .1 ml of the olive oil/alcohol mixture.

(c) Gonadotrophin (PMS) test

To an ampoule containing 1000 I.U. of dried serum gonadotrophin obtained from the serum of pregnant mares (Anteron, Schering, AG) 1 ml of physiological saline was added immediately before use. This stock was diluted 1:5 with physiological saline to contain 200 I.U. PMS/ml.

The rats were given a single subcutaneous injection of 0.1 ml saline containing 20 I.U. PMS. Control rats received a single injection of 0.1 ml saline. The biological activity of the PMS used was confirmed by injecting 5 prepubertal rats, 29 days old, with 0.1 ml saline containing 20 I.U. PMS. They were autopsied 65 hours later and examination of the oviducts at the ampullary-isthmus junction revealed that 4 of the 5 rats had ovulated with a mean number of ova per ovulating rat of 34.5. This number is consistent with the "super ovulation" shown by similarly treated immature rats (Reiter et al., 1969).

Investigation of internal organs

Apparatus

Torque balance

Metter H20T balance

Leitz dissecting microscope

Surgical instruments

Organ weights: The rats were autopsied following cervical dislocation

and the following organs were removed, freed of surrounding tissue, and weighed: The pituitary gland; both ovaries, the uterus, and both adrenal glands. In those instances when uterine ballooning was observed, it was recorded and the fluid was expelled before the uterus was weighed.

The ovarian and adrenal gland weights of each rat were summed to give total ovarian and total adrenal gland weight respectively. These and other organ weights were expressed in both absolute terms (mg) and relative to body weight (mg/100g body weight).

Microscopic examination of the ovaries The surface appearance of the untreated ovaries was examined under a dissecting microscope. A useful guide to the state of stimulation or regression of the ovaries may be obtained by counting the number of surface follicles and corpora lutea. In the present research the following criteria were employed:

(i) follicles with a diameter of 0.5mm or greater were counted

(ii) corpora lutea with a diameter of 0.7mm or greater were counted.

The results were recorded in terms of the total number of follicles and corpora lutea counted on the surface of both ovaries.

A satisfactory degree of concordance was found amongst skilled investigators examining the same ovaries.

Tubal ova counts The oviducts were examined under the dissecting microscope for the presence of ova at the ampullary-isthmus junction. It should be noted that failure to detect ova at this site does not exclude their presence in the isthmus of the tubes.

The number of ova present in each oviduct was counted and the total number of ova per rat was recorded. In the experimental sections the mean number of ova of a particular group of rats are expressed in terms of the mean number of ova per ovulating rat.

Statistical procedures

As far as possible parametric techniques of hypothesis testing were used because of their greater power efficiency. The tests used included the Student's t test, one-way analysis of variance, two-way analysis of variance, and two-way analysis of variance with repeated measures on one factor (Kirk, 1968; Winer, 1971).

A significant F ratio in a one-way analysis of variance was followed up using Tukey's honestly significant different (hsd) statistic for pairwise comparisons and the Scheffé F technique for multiple comparisons of means. In the factorial experiments significant main effects were examined further by performing pairwise comparisons on the treatment means. The finding of significant interaction in a factorial experiment was followed by tests on simple main effects. In the event of significant simple main effects pairwise and multiple comparisons were conducted on cell means using Tukey's hsd procedure and Scheffé F technique respectively (Winer, 1971).

Analysis of variance and simple main effects summary tables are provided in Appendix C. Because of the vast numbers of comparisons run summaries of Tukey's hsd and Scheffé F statistics are not provided in an appendix.

In the text significant differences are reported at the .05 and .01 levels of significance. On a few occasions the probability value is reported at the $p < .001$ or $p < .0025$ level of significance specifically to indicate highly significant differences.

The non-parametric analyses, such as Chi square (χ^2). were performed using the procedures described by Siegal (1952).

Chapter 4

THE EFFECTS OF NUTRITIONAL FACTORS ON REPRODUCTION AND ENDOCRINE FUNCTION

In this chapter both clinical studies with humans and experimental work with animals will be reviewed in order to identify the effects of altered food intake on reproductive physiology.

There is an unjustifiable tendency for some authors to ignore or disparage experimental work on animals, presumably because it lacks relevance for humans. As Platt (1968) so cogently argues

> ... the subject should be treated as a problem of comparative nutrition of man and other animals and differences should receive serious consideration in keeping with the late Sir Edward Mellanby's "golden rules" of research - treasure your exceptions.

(p.246)

In the absence of general agreement in the literature on definition of terms it is necessary to specify how terms will be used to describe nutritional status in this review.

"Malnutrition" will refer to the reduction or absence of certain constituents from the diet e.g. protein or calorie malnutrition. In a broader context it would also refer to conditions caused by interference with the digestion, absorption or utilization of specific component nutrients. "Undernutrition" will refer to a reduction in the quantity of a balanced diet. In order to avoid confusion, the term starvation will be confined to instances of a total absence of food intake. The term "semi-starvation", even though it is used widely in the literature, will be avoided because it lacks the desired specificity and preciseness required in this review.

"Inanition" describes the condition of the organism and not the nature of the altered nutritional intake. Inanition may be the result of either malnutrition or undernutrition or a combination of both.

The effects of altered nutrition on human reproductive physiology

The relationship between undernutrition and disturbances of menstrual function and lowered fertility in humans has been recognised for a long time. A further understanding of this relationship is a legacy of the tragedies of modern history. Concentration camp victims as well as war time civilian populations subjected to severe undernutrition were investigated during and following World War 2 (Antonov, 1947; Gillman & Gillman, 1951; Keys et al., 1950; Samuels, 1948; Smith, 1947).

Smith (1947) described the effects of severe undernutrition that existed for a period of 6 months in Dutch cities. During this time half the female population suffered amenorrhoea, while half of the remainder had menstrual irregularities. There was a dramatic drop in birth rate, to one-third of normal, which later rose to unprecedented levels following the restoration of adequate food supplies; revealing the temporary nature of the infertility caused by undernutrition.

The fertility rates of the civilian population during the siege of Leningrad were even more profoundly affected (Antonov, 1947). Amenorrhoea was widespread and the number of births during the latter half of the 18 month long siege dropped to about 4% of the pre-war birth rate. However, the contribution of psychological factors, confounded with undernourishment, may have been greater than in Holland since Leningrad was subjected to frequent bombardment.

In the studies described so far, and other studies on concentration camp victims, the levels of hormone excretion were not determined. In 1953 Zubiran and Gomez-Mont published the results of an extremely important study of patients suffering from chronic undernourishment. They reported finding low levels of urinary oestrogens and low gonadotrophin excretion. In the group of women of menstrual age amenorrhoea was

observed in 50% of the cases and atrophy of the breasts in 76% of cases. A significantly lowered excretion of gonadotrophins was found in 72% of cases of women of menstrual age. Gross examination of the ovaries, at autopsy, revealed small or atrophic gonads. Zubiran & Gomez-Mont (1953) hypothesized that ovarian hypofunction and the disturbance of other endocrine glands must be due, in part, to a lack of pituitary stimulus. Although pathological changes in the pituitary were found at autopsy in some cases, a study of a group of undernourished women during refeeding demonstrated that the hormonal changes observed were reversible. They monitored the increase in gonadotrophin and oestrogen excretion and observed the return of normal menstruation and pregnancy. Gonadotrophin excretion was found to be markedly increased within a few weeks of the start of nutritional rehabilitation.

Further evidence that a relationship exists between weight loss, gonadotrophin excretion and amenorrhoea is found in the work of Beardwood (1974). In a study of three obese patients on a weight reduction diet Beardwood found that the mean output of gonadotrophin diminished during successive menstrual cycles, but the rhythmicity of the gonadotrophic output remained normal. All of his subjects eventually became amenorrhoeic after having lost at least 16kg in weight.

Although human undernutrition has received a great deal of attention in recent decades, little work has been focussed directly on its effects on reproductive physiology. The exception is the studies of anorexia nervosa patients. However, since anorexia nervosa has a characteristic psychological disturbance associated with inanition the findings are not directly comparable with patients suffering from involuntary undernourishment.

The effects of altered nutrition of the reproductive physiology of experimental animals

There appears to be little doubt that undernutrition and malnutrition alter the function of some endocrine glands. It seems that the effects vary with the nature of the dietary restriction and its chronicity.

Before reviewing the more recent studies employing radioimmunoassay procedures some of the early reports on the influence of diet on gonadotrophin secretion and reproductive physiology will be examined briefly. (This has been reviewed more fully by Ershoff, 1952; Follis, 1958; Leathem, 1958; Lutwak-Mann, 1958; Samuels, 1948.) Attention will first be devoted to undernutrition and then to specific forms of malnutrition.

Ovarian and uterine atrophy and anoestrous vaginal smear patterns during inanition have been reported by numerous authors (Marrian & Parkes, 1929; Mulinos & Pomeranz, 1940; Piacsek & Meites, 1967; Rinaldini, 1949). The ovarian hypofunction, with consequent uterine atrophy and anoestrus smears, appears to be due to a decrease in the level of circulating gonadotrophins and not to a refractory state of the ovaries. Werner (1939) found that, in female rats, the atrophy of the genital system could be arrested by means of pituitary implants. By injecting hypophysial or chorionic gonadotrophins other researchers were able to reestablish normal ovarian weights in starved animals (Marrian & Parkes, 1929; Rinaldini, 1949). Since the condition found in malnourished animals was similar to that following hypophysectomy the term "pseudohypophysectomy" was applied to this state (Mulinos & Pomerantz,. 1940).

There was general agreement that gonadotrophin levels were decreased. However, it was not clear whether it was the synthesis or

release of pituitary gonadotrophins that was impaired. Pituitary gonadotrophin content was reported to be reduced (Mason & Wolfe, 1930), unchanged (Marrian & Parkes, 1929), or increased when judged in terms of potency/mg pituitary (Meites & Reed, 1949; Rinaldini, 1949). The finding of consistently low levels of circulating gonadotrophins together with that of normal or increased pituitary gonadotrophin content led Ershoff (1952) to conclude that during inanition the release of pituitary gonadotrophins is impaired and that failure of the secretory mechanism, and not reduction in hormone production, is responsible for gonadal atrophy.

Some investigators assumed that decreased pituitary hormone secretion, during inanition, was a direct consequence of the deficiency of nutrients of anterior pituitary function (Ershoff, 1952; Leathem, 1958). Subsequent research (to be reviewed shortly) supports the notion of impaired release but suggests that the effects of reduced food intake may be due, in part, to a decrease in synthesis and release of hypothalamic releasing factors (Campbell et al., 1977; NegroVilar et al., 1971; Piacsek & Meites, 1967; Root & Duckett, 1973).

Caloric restriction: Ershoff (1952) proposed that the effects of caloric restriction on anterior pituitary hormone release is particularly marked with respect to the gonadotrophins. Ball et al. (1947) reported that the pregnancy rate of mice was reduced by 70% when caloric intake was restricted to one third of ad libitum intake. It has been reported that rats become anoestrous when calorie intake is restricted by one half (Carr et al., 1949). The effect of caloric restriction on the ovulation rate in swine (Zimmerman et al., 1960) and sheep (Memon et al., 1969) has been demonstrated.

Howland (1972) found that glucose supplementation (ad lib access to glucose powder) was sufficient to correct the relative caloric

deficiency resulting from 50% reduction in normal food intake. This observation led Howland to suggest that caloric intake per se is an important factor in determining pituitary and subsequently ovarian function. An alternative explanation, offered by Howland (1972), is that rats on a diet low in protein but adequate in calories utilize the reduced protein more efficiently i.e. use less protein for metabolic fuel. It has long been recognized that if the caloric intake is inadequate then the protein in the diet is utilized to supplement energy resources. Platt (1966) has pointed to the interdependence of protein and caloric deficiencies, since restriction of calories reduces the value of protein in the diet.

Protein deficiencies: Protein restriction leads to the cessation of the oestrous cycle in the rat (Evans & Bishop, 1922; Guilbert & Goss, 1932). These effects are apparently due to reduced gonadotrophin levels since pituitary implants or gonadotrophin extracts stimulated the ovaries (Courrier & Raynaud, 1932 cited by Ershoff (1952)). Guilbert & Goss (1932) found that feeding adult rats diets containing less than 6% protein resulted in anoestrus. Srebnik & Nelson (1963) suggested that the effects of protein deprivation on the reproductive physiology of sexually mature female rats are much more severe than those produced by caloric restriction. Animals fed a protein-free diet became anoestrous in approximately 5 days whereas pair-fed rats receiving the same quantity of a standard diet (46% of ad lib controls) did not become anoestrous until after approximately 3 weeks of food restriction. Bioassays of both protein deficient and pair-fed rats revealed that the gonadotrophin content of the anterior pituitary was approximately twice that of control rats, suggesting impaired release of pituitary gonadotrophins.

Vitamin and mineral deficiencies: Evans & Bishop (1922) were amongst

the first to show that diets deficient in one or more vitamins may impair gonadal function presumably by causing impaired secretion of pituitary gonadotrophins (see Ershoff (1952) for a review).

The effects of mineral deficient diets on reproductive function have not been consistent (Ershoff, 1952) except with regard to zinc deficiency. A deficiency of zinc has been associated with impaired reproduction in several species (Underwood, 1971). The results of a study by Gombe et al. (1973) suggest that a lack of zinc has a direct adverse effect on reproduction in addition to causing a decrease in food consumption.

Before reviewing the more recent research on the effects of inanition on reproductive physiology it is worth noting the different responses of the sexes to complete starvation. Studies by Widdowson (1976) indicate that females are better able to withstand a shortage of food than males. She has found that adult female rats lost less protein from their bodies than male rats when totally deprived of food for 6 days. These sex differences may account for some of the conflicting findings to be discussed in the next section.

Recent endocrine studies employing radioimmunoassay procedures

Circulating gonadotrophins: Very few studies of the effects of food restriction on circulating gonadotrophin levels in the intact female rat have been reported. Furthermore the great majority of studies, using both sexes, have investigated the effects of undernutrition rather than specific forms of malnutrition.

With a few exceptions food restriction (reduction in quantity of intake) caused a significant reduction in circulating levels of LH, and to a lesser extent FSH. Howland (1971, 1972), for example, reported that restricting food intake to 50% of normal for 20 days

resulted in a significant reduction in serum LH levels in female rats. Gombe et al. (1973) also reported reduced plasma LH levels in female rats after 10 and 32 days of feeding on a diet restricted to 40% of normal quantity. Surprisingly, however, they found an elevation of plasma LH after 25 days on the same feeding regimen.

Howland (1975) observed a reduction in serum LH in male rats subjected to a 50% ad libitum intake for 10 and 20 days. Male rats subjected to complete food removal had significantly reduced serum LH levels within 24 hours and these levels remained low after 2 and 7 days of starvation (Howland & Skinner, 1973). In contrast, it has been reported that serum LH levels remain unaltered in male rats starved for a period of 7 days (Root & Duckett, 1973; Root & Russ, 1972; Root et al., 1975). However, the findings of Campbell et al. (1977) are more consistent with those of Howland and his coworkers. Campbell et al. (1977) reported a 75% reduction in serum LH levels in male rats after 7 days of starvation. The low serum LH levels were maintained during a further two weeks of feeding on one quarter of ad libitum intake.

It has been suggested by Critchlow & Bar-sela (1967) that there is, in states of underfeeding, a preferential blockade of LH release, while FSH is little affected. The endocrine changes in female rats on restricted diets (Kennedy & Mitra, 1963, a & b) are consistent with the hypothesis of a specific LH blockade. A number of recent studies have revealed that the reduction in serum LH levels is greater than the reduction in serum FSH levels, thus suggesting that the mechanism controlling LH release may be more sensitive to the effects of inanition. Howland (1971) found that serum LH levels were reduced to about half of control levels in female rats restricted to 50% of normal food intake for 20 days, whereas the serum FSH levels in the same rats

remained unchanged. In a later study with male rats restricted to 50% of normal food intake Howland (1975) found that serum FSH levels were not altered after 10 or 20 days of restriction, whereas LH levels were reduced after 10 days. In contrast both serum LH and FSH levels were significantly lower in male rats after 7 days of complete starvation (Howland & Skinner, 1973). Serum LH levels were reduced within 24 hours and the serum FSH levels had dropped within 48 hours of the start of starvation. Campbell et al. (1977) reported that 7 days of complete starvation resulted in a 75% reduction in circulating LH levels but only a 32% reduction in FSH levels in male rats.

Overall these results suggest that the secretion of LH is more sensitive to moderate states of undernutrition than is the secretion of FSH but that the secretion of both gonadotrophins is impaired in extreme states of undernutrition. However, under conditions of complete starvation LH release appears to be affected to a greater extent, and possibly sooner, than is the release of FSH.

The rapidity of the reduction of circulating gonadotrophins, reported by Howland & Skinner (1973), suggests that more than nutritional factors are implicated in the reduction of gonadotrophin levels. It seems unlikely that, in previously well nourished rats, circulating nutrients are so rapidly depleted that they alone interfere with endocrine mechanisms. A more likely proposition is that stress associated with complete food deprivation, plays a part in the rapid drop in gonadotrophin levels.

Pituitary gonadotrophins: Recent studies employing radioimmunoassay procedures have revealed that restricting food intake, in both male and female rats, results in either no significant change in pituitary gonadotrophin levels (Gombe et al., 1973; Howland, 1975; Howland & Skinner, 1973; Root & Russ, 1972) or increased pituitary LH and FSH

concentrations (Howland, 1971, 1975).

The dichotomy that exists between the normal or increased levels of gonadotrophins in the pituitary and the low levels of circulating gonadotrophins led some authors to postulate that there is a failure of pituitary release mechanisms (Ershoff, 1952). For many years it was assumed that the apparent reduction in gonadotrophin secretion was a direct consequence of inanition on anterior pituitary function. However, the results of a number of studies suggest that the primary fault lies not in the pituitary but in the central nervous system. Piacsek & Meites (1967) reported that underfed female rats exposed to constant illumination for 10 days showed a return to pro-oestrous or oestrous smear patterns and an increase in ovarian and uterine weight; indicating a release of pituitary gonadotrophins. Furthermore ovariectomy has been observed to counteract the inhibitory effects of inanition or pituitary gonadotrophin release (Howland, 1971; Ibrahim & Howland, 1972).

Hypothalamic releasing factors: Further attempts to identify the nature and the site of the dysfunction during states of inanition have included assays of hypothalamic LHRH content and the administration of exogenous LHRH.

Ibrahim & Howland (1972) suggested that the reduction in serum gonadotrophins is, in part, due to a decrease in synthesis and release of hypothalamic releasing factors. Piacsek & Meites (1967) presented findings consistent with the view that the synthesis of LHRH is impaired. They found that the hypothalamic LHRH content of female rats subjected to 50% food restriction was significantly reduced (¼ of ad libitum controls) after 21 days. Complete starvation of male rats for 7 days was reported to be accompanied by a reduction in FSH-RH (Negro-Vilar et al., 1971) and growth hormone releasing factor (Dickerman

et al., 1969). In contrast to the rest of the studies Root et al. (1975) found that total starvation for 7 days did not affect the hypothalamic content of LHRH in either intact or castrated male rats. One possible explanation, they suggested, is that inanition may exert a direct effect at the pituitary level. This viewpoint, however, is not supported by other experimental evidence. Root & Duckett (1973), for example, obtained results indicating that the pituitary of the starved male rat is as sensitive to the LH releasing activity of exogenous LHRH as is the pituitary of the normally fed animal. More recently Campbell et al. (1977) observed that the increase in serum LH in response to LHRH administration was the same in male rats completely starved for 7 days as it was in normally fed controls. The LH response pattern was different in rats starved for 7 days and then maintained on ¼ normal food intake for a further 2 weeks. The serum LH levels in this group were lower 30 mins post-LHRH administration than those of acutely starved and control rats, but the change from pre-treatment levels was greater than for control rats. The results of the Campbell et al. (1977) study suggest that the major effects of inanition are exerted on the hypothalamus rather than on the pituitary, since the responsiveness of the pituitary of underfed rats to stimulation by LHRH remained the same as the pituitary of fully fed rats. Campbell et al. (1977) concluded that

> the reduction in secretion of AP hormones as a result of restricted food intake therefore appears to be due primarily to a decrease in the release of hypothalamic hormones that control AP function.
>
> (p.585)

The mechanisms involved in the impairment of hypothalamic function during food restriction are yet to be elucidated. One hypothesis is that cellular function in areas of the hypothalamus responsible for the secretion of releasing hormones may be impaired by a reduction in

insulin levels (Ibrahim & Howland, 1972). The suppression of insulin secretion in undernutrition is an adaptive mechanism so that blood glucose does not fall to a level incompatible with life (Widdowson, 1976). At present such an explanation of the mechanisms involved in hypothalamic dysfunction remains hypothetical.

The effects of undernutrition on organ weights

The absolute pituitary weight has been reported to be reduced in underfed male and female rats (Campbell et al., 1977; Howland, 1971, 1972, 1975; Leathem, 1958; Piacsek & Meites, 1967; Srebnik & Nelson, 1963). The reduced secretion of pituitary gonadotrophins is accompanied by a significant reduction in the weight of the gonads of both sexes (Campbell et al., 1977; Howland, 1971, 1972) as well as the uteri (Piacsek & Meites, 1967). Ovarian and uterine atrophy is associated with an anoestrous vaginal smear pattern (Srebnik & Nelson, 1963).

Very few researchers have recorded the weight of the adrenal gland during states of inanition. Those who have, provide evidence that, unlike other target organs of the anterior pituitary hormone complex, the adrenal gland increases in weight during states of severe undernutrition but not during moderate states of undernutrition (Chowers et al., 1969; Meites & Reed, 1949). Campbell et al. (1977) suggested that the increment they observed in adrenal weight following 7 days of starvation reflects the stressful nature of acute starvation.

The latency of endocrine changes and effects on target organs during states of undernutrition

The question of how rapidly the effects of undernutrition or malnutrition are manifested at the gonadotrophin and target organ levels has received little attention in the literature. In the research

reviewed earlier most investigators collected blood samples at specified intervals (e.g. 7, 10 or 20 days) after the dietary regimen was started. These results consequently reflect the endocrine status at the end of a given period but provide no indication of the latency of the observed decrements. In one study with male rats Howland & Skinner (1973) report that the decrement of gonadotrophins observed after 7 days was first evident within 24 hrs of starvation in the case of LH and 48 hrs in the case of FSH.

Very few researchers report on vaginal smear patterns in underfed female rats. Since anoestrus is likely to occur only after a decrease in circulating gonadotrophins the vaginal smear patterns provide indirect evidence of reduced gonadotrophin secretion. They do not, however, indicate when a significant decrement of circulating gonadotrophins first occurs.

Srebnik & Nelson (1963) reported that female rats became anoestrous after 5 days of feeding on a protein free diet. In marked contrast, pair-fed rats receiving the same quantity of a standard diet (46% restriction of normal intake) did not become anoestrous until approximately 3 weeks of food restriction. This observation led Srebnik & Nelson (1963) to conclude that the effects of protein deprivation on reproductive physiology are much more severe than those produced by restricted intake of a standard diet. Lamming & Krause (1963) restricted the food intake of mature female rats so that they lost weight at a rate of 2-3 grams/day. This resulted in anoestrus approximately 3 weeks after the start of restriction, by which time the rats would have lost weight to the extent of 18-24% of their previous body weight. Piacsek & Meites (1967) found that female rats subjected to 50% of normal food intake stopped cycling and exhibited a dioestrous type of vaginal smear within 14-21 days after the start of reduced intake.

Neither Srebnik & Nelson (1963) nor Piecsek & Meites (1967) reported the average weight change of the rats at the time of the onset of anoestrus.

The effects of nutritional rehabilitation on reproductive physiology

In the few available reports on the effects of nutritional rehabilitation on the reproductive physiology of the rat, the results indicate that recovery is relatively rapid. Widdowson et al. (1964) underfed young rats of both sexes so that they gained only 20 gm body weight between the third and eleventh weeks of life. Thereafter the rats were rehabilitated by being given unlimited food supplies. Both sexes responded by gaining weight rapidly. As Widdowson et al. (1964) observed, a small part of the weight gain during the first week was due to an increase in weight of the gastrointestinal contents. They estimated that this amounted to approximately 10 gm.

By the time the rehabilitated female rats were 19 weeks old they had almost achieved the weight of littermates that had not been undernourished. Those rats that still had closed vaginae when rehabilitation began showed vaginal opening within 5 days of refeeding. By the end of the first week of rehabilitation the ovaries were as large as those of fully fed controls of the same age, and numerous corpora lutea were formed. The male rats did not achieve the body weight of littermates following rehabilitation.

No reported data are available on the effects of rehabilitation on gonadotrophin levels in female rats. Male rats refed for 7 days, after a period of chronic undernutrition, showed elevations in circulating gonadotrophins to values well above controls; to the extent of 133% in the case of LH and 157% in the case of FSH (Campbell et al., 1977). This rebound effect, they suggested, may be due to increased

release of LHRH following refeeding rather than to increased pituitary sensitivity to LHRH as proposed by Beumont et al. (1976). Campbell et al. (1977) found no indication that the pituitary gonadotrophs were more responsive to LHRH stimulation in refed rats than in the ad libitum fed controls.

Grewal et al. (1971) and Howland (1975) reported that serum testosterone levels rebounded during rehabilitation to levels higher than those found in fully fed controls. In both studies rehabilitation followed a period of undernutrition during which rats were restricted to 50% of normal food intake. In the Howland (1975) study rehabilitation, following 15 days of underfeeding, produced serum testosterone levels that exceeded the control values on days 1 and 2 of refeeding. A rebound of serum LH levels was not observed in the Howland (1975) study.

Conclusion

Research over many decades has revealed that serum gonadotrophin levels are reduced during states of malnutrition and starvation. Associated with this reduction of circulating gonadotrophins is atrophy of ovaries, uteri and vaginal epithelium. The bulk of the available evidence suggests that the reduction in secretion of gonadotrophins as a result of restricted food intake, is due primarily to a decrease in the synthesis and/or release of LHRH. That is, the failure of the pituitary to release gonadotrophins is presumed to be secondary to a hypothalamic dysfunction caused by nutritional deprivation.

The research to be reported in the following sections covers fresh areas in the investigation of the effects of nutritional factors on reproductive function.

Research objectives

In the studies reviewed earlier in this chapter the animals were subjected to partial or total food restriction for different periods of time. To date there have been no published reports on attempts to investigate the effects of controlled and systematic reduction of the caloric content of the diet on reproductive function of the female rat.

In the series of studies to be described in Part A the aim was to establish the minimum level of caloric restriction required to induce anoestrus. Having identified a dietary regimen capable of inducing anoestrus, the changes in reproductive physiology at regular intervals during the period of caloric restriction and during different forms of nutritional rehabilitation were investigated. Changes in the following were examined: vaginal smear patterns; serum and pituitary LH levels; pituitary gland, ovarian, uterine and adrenal gland weights; the surface appearance of the ovaries. In some studies the oviducts were examined for the presence of tubal ova. Of particular interest was the time taken for alterations to be observed, the extent of the alterations, and the body weight changes associated with the alterations.

The aim was to obtain as complete a picture as possible of the progressive changes taking place during both the weight loss and weight gain phases. The series of experiments constitute a longitudinal study of changes associated with caloric deprivation and impairment of reproductive function and subsequent nutritional rehabilitation with recovery of the reproductive system.

In Part B of the experimental section the aim of the research was to use pharmocological tests in order to investigate which component(s)

of the hypothalamic-pituitary-ovarian (HPO) axis are dysfunctional during the caloric deprivation and nutritional rehabilitation phases.

The specific objectives of each study are described in the introduction to each experiment.

Experimental section

Part A: Caloric restriction and nutritional rehabilitation

Experiment 1

The aims of this experiment were:

(i) to determine the extent of caloric restriction required to disrupt normal oestrous cycles.

(ii) to establish how soon the oestrous cycle is disrupted after the start of caloric restriction.

Method

Forty female rats, approximately 7-8 weeks of age, were individually housed and fed the basal diet and water ad libitum. Body weight, food consumption and vaginal smears were monitored daily. At the end of the 10 day adaptation period 24 rats, with regular oestrous cycles, were allocated to one of 3 groups (n=8 each) and 5 rats to a control group, so that the mean body weight of each group was approximately the same.

The mean 24 hr food consumption during the final 3 days of the adaptation period was used to compute the quantities of experimental diets to be provided during the initial stages of the experimental period (see Chapter 3 for details of computing quantities of diet). Four rats in each group received their reduction diet on the morning of pro-oestrus and the other 4 on the morning of metoestrus.

The groups were fed as follows:

25% group: received 13g of a diet per day containing 25% less dextrin than the basal diet.

50% group: received 11g of a diet per day containing 50% less dextrin than the basal diet.

75% group: received 8g of a diet per day containing 75% less dextrin than the basal diet.

Basal control group: continued to receive the basal diet ad libitum. This group provided data about normal growth rate and changes in food consumption during the 3 week period.

Body weight, food consumption and vaginal smears were monitored daily. On day 10 the quantities of the experimental diets were increased in line with the increased food consumption of the control group. During the remainder of the 21 day experimental period the 3 experimental groups were fed 15g of 25%, 12g of 50% and 9g of 75% dextrin restricted diets respectively.

Results

Body weight: The changes in body weight are summarized in Table 8. For convenience the body weights are grouped into 3 day periods. The Pre values represent the mean body weight of each group on the day before the start of the experimental phase.

Analysis of variance revealed no significant difference between the body weights of the 4 groups until the period 10-12 days. At that time the body weights of the 50% and 75% groups were significantly lower than that of the control. It was only during the final epoch that all 3 experimental diet groups were significantly lighter than the basal diet control group.

Both the control group and the 25% group showed a significant

Table 8

Effects of 3 dextrin-reduced diets on body weight

Period of dextrin restriction (days)	Mean body weight (g)±SD			
		Extent of dextrin-reduction		
	Basal diet control (n=5)	25% (n=8)	50% (n=8)	75% (n=8)
Pre	207.8±13.4	208.0±16.8	207.5±20.9	209.3±12.4
1-3	212.6±13.7	215.6±17.2	211.1±20.5	210.3±12.1
4-6	218.2±13.9	212.5±14.9	206.5±20.4	200.0±10.8
7-9	224.0±13.0	213.3±14.2	205.9±19.8	197.3±11.1
10-12	230.4±12.4	215.9±13.2	206.8±19.9[a]	194.7±11.4[b]
13-15	238.1±11.7	221.4±10.9	207.9±18.5[b]	190.7±10.9[c]
16-18	245.1±11.5	226.3±9.3	209.1±18.1[b]	189.3±10.8[c]
19-21	251.2±10.6	227.9±9.1[a]	208.4±17.9[b]	187.8±10.8[c]
Final BW change (%)	+20.9[d]	+9.6[d]	+0.4	-10.3[e]

a significantly lighter than control ($p<.05$)
b significantly lighter than control ($p<.01$)
c significantly lighter than 25% group and control ($p<.01$)
d significantly heavier than Pre weight ($p<.01$)
e significantly lighter than Pre weight ($p<.01$)

increase in body weight over the 3 week period. The body weight of the 50% group did not change significantly, whereas the 75% showed a significant drop in body weight by the end of the 3 week period.

Food consumption: The rats receiving experimental diets invariably consumed all the food provided. The 24 hr consumption rate of basal diet of all groups prior to the start of the experimental phase was 16.2±2.9g. By day 10 the quantity of food consumed by the basal diet

control group had increased to 17.6±3.0g.

The food consumption data were not subjected to statistical analysis since the quantities of experimental diets were controlled and hence observed difference in intake were imposed ones.

Vaginal smear patterns: Although vaginal smear irregularities, in the form of extended cycles, were observed in all experimental groups there was no cessation of oestrous cycling under any of the dietary conditions. It was only in the 75% group that the incidence of extended cycles was clearly greater than that of the basal diet control group and the subjects in Supplementary Study No. 7 (Appendix A). The incidence of 5-6 day cycles in the 75% group increased from 50% during the first week to 88% during the final week.

There appeared to be no relationship between the occurrence of extended cycles and the day of the cycle (metoestrus or pro-oestrus) on which experimental diets were first presented.

Discussion

Only the 75% dextrin reduction group showed a significant decrease in body weight during the 21 day period of restriction. None of the rats showed cessation of oestrous cycling. The results indicate that the mechanisms controlling the regulation of the oestrous cycle, in this strain of rat, are resistant to the levels of caloric deprivation imposed in this experiment.

This finding is not consistent with those of Meites & Reed (1949) who reported that chronic feeding of low calorie diets without causing undernutrition resulted in anoestrus in rats within 3 weeks. Other researchers feeding rats a standard diet restricted to approximately half the normal quantity reported that anoestrus was observed after 21 days (Srebnik & Nelson, 1963) or within 14-21 days (Piacsek &

Meites, 1967). These investigators did not, however, report the weight loss coinciding with the onset of anoestrus. In this experiment the 75% dextrin reduction group received approximately half the amount of food consumed by the control group but showed no signs of anoestrus. The high relative proportion of protein and other nutrients in the 75% dextrin reduction diet may have resulted in weight loss to a lesser extent than that found with rats restricted to half the normal amount of standard diet.

Experiment 2

The aim of this experiment was to determine whether a further reduction in the caloric component of the diet resulted in cessation of oestrous cycles. Serum LH levels were determined at various intervals after the start of the dietary regimens. Two different procedures were employed in the collection of blood samples for serum LH assay: (i) trunk blood samples were collected immediately following cervical dislocation (ii) blood samples were collected from chronically cannulated rats. This procedure allowed for the repeated collection of blood samples from the same rats at different times after the dietary regimen were imposed. Due to the different nature of the blood sampling procedure this experiment has been divided into two parts.

Part A: Non-cannulated rats subjected to 25, 50 and 100% dextrin restricted diets

Method

Sixty female rats were individually housed and fed the basal diet and water ad libitum for 10 days. Body weight, vaginal smear patterns and food consumption were monitored daily during the adaptation and experimental phases. On the final day of the adaptation phase 50 rats

with regular 4 day cycles were allocated to one of 10 groups (n=5 each) so that each group had approximately the same mean body weight.

The mean food consumption rate during the final 5 days of the adaptation phase was determined and this figure (18.01g) was used to compute the quantities of experimental diets to be provided (see Chapter 3 for details). Since there was no significant change in the consumption rate of the basal diet control group the quantities of experimental diets remained constant throughout the 3 week duration of the experimental phase. One group of rats served as the ad libitum fed basal diet control throughout the experimental phase. Three groups of rats were fed 15g of the 25% dextrin-reduced diet; three groups received 12g of the 50% dextrin-reduced diet; and the final 3 groups received 6g of the 100% dextrin-reduced diet.

At periods approximately 1, 2 and 3 weeks after the start of the experimental phase, groups of 5 rats from each dietary condition were sacrificed at 1000h on metoestrus. The rats in each group were not always sacrificed on the same date since smear patterns were not synchronized. They were, however, sacrificed on the day of metoestrus closest to 1, 2, and 3 weeks after the start of experimentation. Rats which had stopped cycling were sacrificed only after showing at least 3 consecutive days of a dioestrous smear pattern prior to the end of the 1, 2, or 3 week periods. At the end of 3 weeks rats in the basal diet control group were sacrificed at the first appearance of a metoestrous smear pattern.

Results

Body weight: The changes in body weight of the 3 experimental groups surviving the entire 3 week experimental period and of the control group are summarised in Table 9. The body weights of rats sacrificed at 1

and 2 weeks are not included since the aim was to perform a composite statistical analysis of body weight data over the entire 3 week period.

Table 9

Effects of 3 dextrin-reduced diets on body weight

Period of dextrin restriction (days)	Mean body weight (g) ± SD			
		Extent of dextrin restriction		
	Basal diet control (n=5)	25% (n=5)	50% (n=5)	100% (n=5)
Pre	233.8±5.8	236.6±7.4	234.8±6.1	234.2±6.0
7	247.0±4.4	241.0±7.7	228.0±5.7[a]	211.2±2.2[b]
14	258.0±4.5	248.4±8.0	229.0±8.7[c]	195.0±2.6[b]
21	267.6±3.0	255.2±6.4[b]	232.6±9.1[c]	181.0±5.2[b]
Final BW change (%)	+14.5[e]	+7.9[e]	-0.9	-22.7[f]

a significantly lighter than control ($p < .01$) and 25% groups ($p < .05$)

b significantly lighter than all groups ($p < .01$)

c significantly lighter than control and 25% groups ($p < .01$)

d significantly lighter than control group ($p < .05$)

e significantly heavier than Pre weight ($p < .01$) (NB O.)

f significantly lighter than Pre weight ($p < .01$)

Analysis of variance revealed no significant difference between the body weights of the control and 3 experimental groups at the start of the experiment. At the end of weeks 1 and 2 the 50% group was significantly lighter than the control and 25% groups and the 100% group was significantly lighter than all other groups. At the end of three

weeks all experimental groups were significantly lighter than the control group.

Both the control group and the 25% group showed a significant increase in body weight over the 3 week period. At the end of 3 weeks the body weight of the 50% group did not differ significantly from its pre-experimental phase weight. The 100% group showed significant weight reduction at each weekly interval ($p < 0.01$).

Food consumption: The differences in food consumption were imposed and consequently these data were not analysed statistically. Without exception rats receiving the experimental diets ate all the food presented to them. Hence the consumption rates, during the experimental phase, of the 25, 50 and 100% dextrin-restricted diets, were 15, 12 and 6g respectively. The mean daily consumption of the basal diet control group remained in the range of 18-19g.

Serum LH: The serum LH values after 7, 14 and 21 days feeding on dextrin-restricted diets are shown in Table 10 and Figure 1. The mean serum LH level of the basal diet control group at the end of the 3 week period is included.

Table 10

Effects of 3 dextrin-reduced diets on serum LH levels

Period of restriction (days)	Serum LH(ng/ml)±SD			
		Extent of dextrin restriction		
	Basal diet control	25%	50%	100%
7	-	30.8± 9.8	31.0±27.3	18.2±17.3
14	-	34.6±17.3	30.8±17.9	18.8±10.9
21	43.6±9.9	37.6±22.3	31.0± 8.4	8.2± .45[a]

a significantly lower than control group ($p < 0.01$) and the 25%&50% groups ($p < 0.05$)

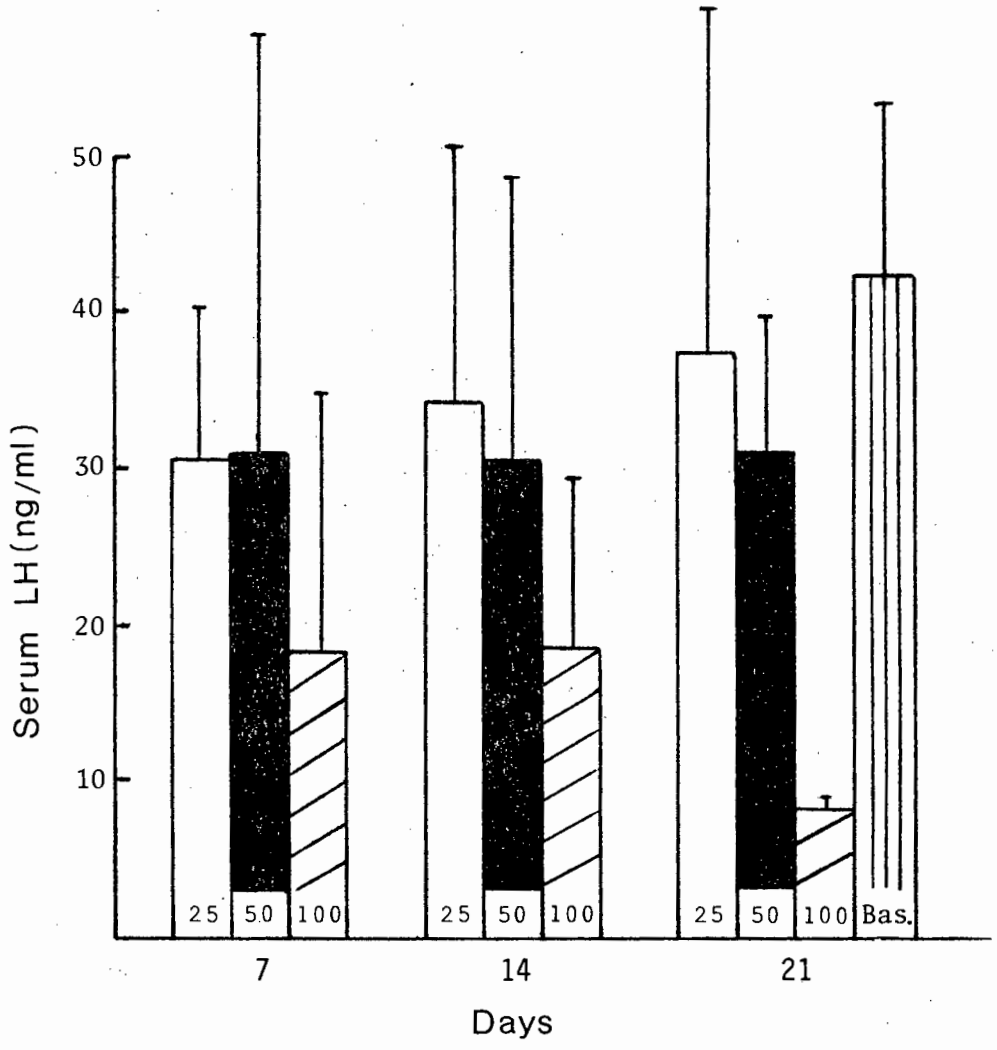

Figure 1 Changes in serum LH concentration (Mean±SD) at weekly intervals after the start of 3 dextrin restricted regimens (25,50 and 100%;Bas.=Basal diet control)

An analysis of variance was performed for each sampling period comparing the serum LH values of each experimental group with each other and the serum LH value of the basal control group, which was sampled only at the end of 3 weeks. These analyses revealed no significant difference between the 4 groups, with respect to mean serum LH level, at sampling periods 7 and 14 days. At the end of the 21 day period the mean serum LH level of the 100% group was significantly lower than that of the basal control, 25% and 50% groups.

Vaginal smear patterns: None of the rats in the basal control, 25% and 50% dextrin-restriction groups became anoestrous during the 3 week experimental period. Occasional 5 day cycles were observed, however,

in all these groups. The incidence of extended cycles in the 25% and 50% groups was so low that they cannot be seen to differ from the basal diet control group.

The majority of the rats in the 100% dextrin-restriction group showed changes in vaginal smear patterns that were consistent with anoestrum. Although a criterion of 3 consecutive days of a dioestrous-type smear pattern was initially used to define anoestrus it was observed that some rats displaying such a pattern exhibited signs of renewed cycling at a later stage. Hence caution needs to be exercised when using the criterion of 3 consecutive days of a dioestrous smear as an indication of complete cessation of cycling.

The latency of onset of anoestrus was highly variable in the rats fed the 100% dextrin-restriction diet. If the day on which the experimental diets were first presented is designated day 1, then the onset of anoestrus was first observed as early as day 6 and as late as day 18, when the rats were 7.7% and 18.5% below their original weights, respectively.

Two rats in the 100% dextrin-restriction group still showed evidence of cyclical activity after 21 days. This rhythmicity was apparent in spite of the fact that they were approximately 23% below their original weight and that they had serum LH levels of <8ng/ml and 9ng/ml respectively.

Comment

At the end of 21 days feeding on the dextrin-restricted diets only the 100% group showed a significant decrease in body weight and a significant reduction in serum LH concentration. The finding of anoestrus was not invariable and there were individual differences in susceptibility of the oestrous cycle to the effects of 100% dextrin

restriction. Some rats became anoestrous much sooner than others while other rats still showed evidence of oestrous cycling at the end of the experimental phase.

Although the mean serum LH levels of the 100% groups were lower than those of the other groups at 7 and 14 days the difference was not significant until sampling at 21 days. This finding suggests that severe caloric restriction does not cause a rapid reduction in serum LH levels.

Part B: Cannulated rats subjected to 25, 50 & 100% dextrin restricted diets

The same dietary regimens were employed as those used in Part A.

The aims of this experiment were to determine, after the start of the dietary regimen:

(i) the serum LH levels of all dietary groups on each day of the first oestrous cycle

(ii) the serum LH levels on selected days of the cycle thereafter; particularly on the day of pro-oestrus.

Method

Twenty five female rats were individually housed and fed the basal diet and water ad libitum for 10 days. Body weight, vaginal smears and food consumption were monitored daily during the adaptation and experimental phases.

Twenty rats with approximately equal body weight and regular 4 day oestrous cycles had jugular cannuli inserted surgically (see Chapter 3 for details). The rats with indwelling cannuli continued to receive the basal diet and water ad libitum during the post-operative recovery period of 7-8 days when the cannuli were cleared (kept patent) every second day. No blood samples were collected during this period. Because

of blockages in the cannuli or other technical mishaps the number of rats available during the experimental phase was reduced to 14.

The final allocation of rats to the different dietary regimens was as follows:

4 rats received the basal diet ad libitum (basal group)

3 rats received 15g of the 25% dextrin restricted diet (25% group)

3 rats received 12g of the 50% dextrin restricted diet (50% group)

4 rats received 6g of the 100% dextrin restricted diet (100% group).

All rats in the experimental groups first received the new diets on day oestrus of the cycle and the first blood samples were collected on met-oestrus, the following day.

Blood sampling: .4ml of blood was drawn from each rat on each day of the first oestrous cycle. Thereafter blood samples were drawn on selected days of subsequent cycles but, as far as possible, never on 2 consecutive days from the same rat. All samples were collected at 1900h in order to coincide with the anticipated LH surge on pro-oestrus.

Results

Body weight: The changes in body weight, at weekly intervals, are shown in Table 11.

The body weight data is provided for visual inspection only. Because of the small sample size statistical analyses were not performed. Analysis of body weights of non-cannulated rats on the same dietary regimens are presented in Part A. As in the case of non-cannulated rats it was only the rats in the 100% group that showed evidence of marked weight reduction. They were 11.7, 19.4 and 25.8% below original weight after 7, 14 and 21 days respectively.

Food consumption: Without exception the experimental group rats ate 15, 12, and 6g of the 25, 50 and 100% dextrin-restricted diets respectively.

Table 11

Effects of 3 dextrin reduced diets on body weight of cannulated rats

Period of restriction (days)	Mean body weight(g)±SD			
		Extent of dextrin restriction		
	Basal diet control (n=4)	25%(n=3)	50%(n=3)	100%(n=4)
Pre	234.3±4.4	232.7±3.5	233.3±4.2	234.5±4.4
7	243.0±3.6	236.7±4.9	231.0±1.7	207.0±6.5
14	255.6±4.2	247.3±1.5	233.7±4.0	189.0±8.1
21	267.0±4.2	254.7±1.5	237.7±7.1	174.0±7.8
Final BW change (%)	+14.0	+9.5	+1.9	-25.8

The mean daily food consumption of the basal diet control group remained relatively constant around 18g. No statistical analysis was performed on these data.

Serum LH levels:

(i) First cycle: The mean serum LH levels for all groups on each day of the first oestrous cycle of the experimental phase are shown in Table 12.

Inspection of the serum LH data during the first cycle reveals no striking difference between any experimental group and the basal diet control group. In all groups the pro-oestrous LH surge was evident.

(ii) Subsequent cycles: For the remainder of the 3 week period blood samples were collected from each rat on every second day. Because of the limitations imposed by this sampling schedule and the occurrence of irregular cycles the number of samples drawn on any designated stage of the cycle were often very small. The serum LH values during the following 4 oestrous cycles or the equivalent of 4 day units in the case

Table 12

Effects of 3 dextrin reduced diets on serum LH levels during the first oestrous cycle of cannulated rats

Stage of first cycle	Mean serum LH (ng/ml)±SD			
		Extent of dextrin restriction		
	Basal diet control (n=4)	25%(n=3)	50%(n=3)	100%(n=4)
Metoestrus	48.5± 19.1	28.3± 18.9	44.7± 13.3	44.0± 15.6
Dioestrus	24.0± 8.0	20.0± 5.0	23.3± 16.6	25.0± 12.8
Pro-oestrus	467.5±291.1	506.7±427.7	573.3±402.7	830.0±171.1
Oestrus	29.8± 6.6	21.7± 3.5	25.7± 6.7	16.5± 1.7

of anoestrous rats are summarized in Table 13. The figures in parentheses indicate the number of observations at that particular stage of the cycle.

The data are presented with full awareness that they represent, at best, possible trends. The sample sizes are too small for statistical analysis and no unequivocal conclusions can be drawn from these data.

Inspection of mean serum LH values reveals little of note except in the case of the 100% dextrin-reduced diet group. All 4 rats in the 100% group had serum LH levels of <8ng/ml by day 11 and they remained as such until the end of the 21 day period. Examination of vaginal smear data revealed that none of the rats were showing evidence of cyclical activity on day 11 and that they continued to be anoestrous for the rest of the experiment.

Vaginal smears: Apart from having occasional 5 day cycles, rats in the basal control, 25% and 50% groups showed no further cyclical aberrations. In contrast all 4 rats in the 100% group became anoestrous during

Table 13

Effects of 3 dextrin-reduced diets on serum LH levels during the second and subsequent cycles of cannulated rats

Stage of Cycle	Mean serum LH(ng/ml)±SD			
	Units of 4 days			
	2nd	3rd	4th	5th
	Basal diet control group			
M	44.0± 11.3(2)	41.0± 2.8(2)	55.5± 20.5(2)	36.0± 5.7(2)
D	17.0± 7.1(2)	24.5± 17.7(2)	20.0± 8.5(2)	14.0± 8.5(2)
P	535.0±162.6(2)	385.0±190.9(2)	340.0±127.3(2)	290.0± 99.0(2)
O	28.5± 2.1(2)	24.5± 6.4(2)	21.5± 5.0(2)	14.0± 1.4(2)
	25% dextrin-restriction group			
M		72.3± 30.0(3)		33.0± 4.6(3)
D	37.3± 7.6(3)		20.3± 2.1(3)	
P	601.0±394.6(2)	381.0±224.9(2)	810.0±268.7(2)	490.0(1)
O	24.0(1)	28.0(1)	32.0(1)	
	50% dextrin-restriction group			
M	27.3± 8.7(3)	42.6± 31.9(3)		43.5± 9.2(2)
D			25.0± 1.4(2)	
P	745.0±360.6(2)	480.0(1)	560.0(1)	560.0±367.7(2)
O	28.0(1)	19.5± 2.1(2)	19.0(1)	20.0(1)
	100% dextrin-restriction group			
M		14.5± 4.9(2)		
D	12.5± 5.9(4)	<8(2)*	<8(3)	
D[1]		<8(4)	<8(4)	<8(4)
P	350.0±99.0(2)			

Figures in parentheses indicate the number of observations
* Day 11 of the experimental period

M Metoestrus D Dioestrus P Pro-oestrus O Oestrus
D[1] Dioestrus-2 or >

the course of the experiment. The onset of anoestrus occurred as early as day 6 in one rat and by day 11 all rats had vaginal smear patterns consistent with anoestrus.

Discussion

The serum LH levels of the cannulated rats in the 100% group dropped to abnormally low levels (<8ng/ml) by day 11 and remained as such until the end of the 21 day experimental period. In contrast non-cannulated rats, fed the 100% dextrin-reduced diet had a mean serum LH concentration of 18.8ng/ml at day 14, which did not differ significantly from that of the control group. It was not until day 21 that significantly reduced serum LH levels were observed. The finding of lowered serum LH levels in the cannulated rats at an earlier time may have been the result of the frequent blood sampling to which these rats were subjected. That is, the combination of nutritional deprivation and blood loss may have accelerated the drop in serum LH levels. On the other hand it is possible, that with the small sample sizes, the observed differences are simply due to chance variation. However, the finding of early onset of anoestrus in all cannulated rats in the 100% group, taken together with the serum LH data, does suggest that caution should be exercised when comparing the serum LH values of rats on reduced food intake, and subjected to repeated blood sampling, with those sampled only once. This may apply only to rats on extreme caloric restricted diets since the rats in the 25% and 50% groups had serum LH values which were similar to those of non-cannulated control rats.

Experiment 3

In the previous experiment it was found that removal of dextrin from the diet of non-cannulated rats did not invariably result in

anoestrus. In contrast, all cannulated rats, fed the dextrin free diet and subjected to repeated blood sampling, became anoestrous within the 3 week experimental period. Furthermore anoestrus was, on average, observed sooner in this group than in non-cannulated rats. It is not known whether this finding is a consequence of the combined effects of dextrin deprivation and repeated blood sampling or whether other factors related to the handling and sampling technique are implicated.

However in the majority of subsequent experiments intact rats were employed. The purpose of this study was to determine the extent of caloric deprivation required to consistently induce anoestrus in intact rats within 3 weeks. The next step in increasing the severity of caloric deprivation was the removal of the fat component of the diet. It was anticipated that this diet, now containing only protein, vitamins and minerals, would induce anoestrus in all rats. Consequently the investigation was extended beyond examining the effects of such a diet on serum LH, body weight and vaginal patterns alone. Attention was now directed to the following as well: pituitary LH content and concentrations; organ weights (pituitary gland, ovarian, uterine, adrenal glands); examination of the surface appearance of the ovaries; and inspection of the oviducts at the ampullary-isthmus junction for the presence of tubal ova.

Method

Twenty-five female rats were individually housed and fed the basal diet and water ad libitum for 10 days. Body weight, food consumption and vaginal smears were monitored daily. At the end of the adaptation phase 20 rats of similar weight and with regular 4 day oestrous cycles were allocated to one of four groups (n=5 each).

Starting on day 1 of the experimental phase, three groups were fed 5g of a dextrin and fat-free diet (DFF diet) daily for periods of 7, 14 and 21 days respectively. The fourth group served as a basal diet ad libitum control group for all the experimental groups. The quantity of DFF diet to be provided was computed according to the procedure described in Chapter 3 using the mean daily consumption of the basal diet during the final 3 days of the adaptation phase as the ad libitum consumption rate.

One group of rats, fed the DFF diet, was sacrificed on dioestrus closest to day 7 of the experimental phase. Not all rats were sacrificed on the same day since some rats were still showing signs of cyclical activity. The other two groups, receiving the DFF diet were sacrificed on days 14 and 21 respectively. All rats in these groups were displaying a constant dioestrous type smear pattern at the time of autopsy. The control group was sacrificed on dioestrus closest to day 21 of the experimental phase. In all cases trunk blood samples were collected for LH assay and autopsies were performed.

Results

Body weight: The changes in body weight of the control group and the group receiving the DFF diet for 21 days are shown in Table 14. Only the body weights of the group surviving the entire 3 week experimental phase are provided since this was the only data used in the statistical analysis. The mean initial and final body weights of the other two groups are shown in Table 16 together with organ weight measurements.

Analysis of variance revealed that the mean body weight of the DFF diet group was significantly lower than that of the basal diet control group from day 7 onwards.

Food consumption: Rats in the basal diet control group continued to

Table 14

Effects of dextrin and fat free (DFF) diet on body weight

Duration of feeding (days)	Mean body weight(g)±SD			
	Dietary regimen			
	Basal diet (n=5)		DFF diet (n=5)	
	Body wt. (g)	BW change (%)	Body wt. (g)	BW change (%)
Pre	266.0±12.1	-	268.2±11.1	-
2	267.2±11.8	+ .5	253.4±11.2	- 5.5
7	275.8±11.5	+ 3.7	224.0± 9.2[a]	-16.5
14	286.6±10.8	+ 7.7	203.0± 9.7[a]	-24.3
21	295.2±13.3	+11.0	177.0± 9.4[a]	-34.0

a significantly lighter than basal diet control group ($p < .01$)

consume approximately 18g of basal diet per day. Consequently the quantity of DFF diet provided was held constant at 5g per day throughout the experimental phase.

Vaginal smear patterns: Rats were judged to be anoestrous if there were more than 3 consecutive days of a dioestrous type smear. The first day of appearance of a constant dioestrous smear pattern was designated the first day of anoestrus.

Three rats in the group sacrificed on day 7 were still showing signs of cyclical activity. The other two rats were sacrificed after 2 consecutive days of a dioestrous type smear, which was not considered sufficient evidence that cycling had ceased.

The time taken to become anoestrous and the body weight changes at the time of anoestrus, of the remaining 10 rats on the DFF diet are

summarized in Table 15.

Table 15

Latency of onset of anoestrus and body weight changes in rats fed a DFF diet (n=10)

	Mean initial BW±SD (g)	Mean latency anoestrum±SD (days)	Mean body weight changes at anoestrus ±SD		
			Body wt. (g)	Absolute BW loss (g)	BW change (%)
$\bar{X}$±SD	266.3±9.4	9.8±1.9	215.7±13.3	50.6±8.2	-19.0
Range	-	7 to 13	-	40 to 65	-14.4 to -24.1

There were marked individual differences with regard to latency of onset of anoestrus and weight loss at the onset of anoestrum. Some rats ceased to show cyclical activity much sooner and after a smaller weight loss than other rats. However, it was established that all rats were anoestrous by day 13 and that the final group remained acyclic until they were sacrificed on day 21.

One rat in the basal diet control group had a single 5 day cycle which was followed by normal 4 day cycles.

Organ weights: The changes in organ weights are summarized in Table 16.

The pituitary glands of all rats fed the DFF diet were significantly decreased in absolute size and the reduction was greatest in the groups sacrificed at 21 days. There were, however, no significant differences between any of the groups when pituitary weight was considered relative to body weight.

The absolute ovarian weights of the 14 and 21 day groups were significantly lower than those of the basal diet control and 7 day groups, but the difference did not reach significance when considered relative to body weight. Both the absolute and relative uterine weights of the

Table 16

EFFECTS OF DFF DIET ON BODY AND ORGAN WEIGHTS

Duration of feeding (days)	No. of rats	Mean initial BW±SD (g)	Mean final BW±SD (g)	BW Change (%)	Mean organ weight ±SD							
					Pituitary		Ovaries		Uterus		Adrenals	
					Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW
7	5	269.4± 7.4	225.0± 9.9	-16.5	10.0± .62[b]	4.4± .36	58.5±8.4[c]	26.0±3.9	336.3±92.6[c]	150.4±44.7[c]	55.4± 7.9	24.7±3.7
14	5	264.4±8.2	193.2± 8.9	-26.9	8.6±1.0	4.6± .57	41.6±2.9	21.5±1.7	190.5±25.8	98.6±16.5	55.3± 5.4	28.9±3.6
21	5	268.2±11.1	177.0± 9.4	-34.0	7.5± .59	4.7± .40	37.1±8.6	21.0±6.0	159.5±12.3	90.1± 7.8	52.0±10.8	29.4±6.1
Basal diet control	5	266.0±12.1	295.2±13.3	+11.1	12.7± .87[a]	4.3± .39	67.7±4.4[c]	23.0±1.6	401.9±28.5[c]	136.3±10.5[b]	50.4± 5.4	17.1±2.4[d]

a significantly greater than all groups ($p < .01$)
b significantly greater than 21 day group ($p < .01$)
c significantly greater than 14 and 21 day groups ($p < .01$)
d significantly lighter than 7 day ($p < .05$) and 14 and 21 day groups ($p < .01$)

14 and 21 day groups were significantly lower than those of the basal control and 7 day groups.

There was no significant difference between any of the groups with respect to absolute adrenal gland weight. However, when expressed in terms relative to body weight, the adrenal glands of the 3 groups receiving the DFF diet were significantly larger than those of the basal diet control group.

Serum and pituitary LH levels: The changes in serum LH concentration and pituitary LH content and concentration are summarized in Table 17 and Figures 2 and 3.

Table 17

Effects of DFF diet on serum and pituitary LH levels

Duration of feeding (days)	No. of rats	Mean Serum LH SD (ng/ml)	Mean Pituitary LH±SD	
			Content (µg)	Concentration (µg/mg wet wt)
7	5	20.8±13.1	164.4±48.3	16.6±4.7
14	5	11.6± 4.2[a]	111.4±42.6[a]	12.7±3.1
21	5	<8 ± 0.0[a]	78.0±29.8[b]	10.4±3.2[c]
Basal control	5	34.3±10.1	219.2±46.2	17.2±2.5

a significantly lower than control group ($p < 0.01$)
b significantly lower than 7 day ($p < 0.05$) and control group ($p < 0.01$)
c significantly lower than 7 day and control groups ($p < 0.01$)

By day 14 the mean serum LH concentration of the rats fed the DFF diet was significantly lower than that of the control group and remained low in the 21 day group. Whereas two rats in the 7 day group had serum LH concentrations of less than 8ng/ml all rats in the 21 day group had levels of less than 8ng/ml.

By day 14 the mean pituitary LH content of the rats fed the DFF diet was significantly lower than that of the control group. After 21 days feeding on the DFF diet the mean pituitary LH content was significantly lower than that of the 7 day DFF diet and basal diet control groups. However, when expressed in terms of μgLH/mg pituitary wet weight, only the 21 day group had a mean pituitary LH concentration that was significantly lower than those of the 7 day DFF diet and basal diet control groups.

Surface appearance of ovaries. The changes in the number of surface corpora lutea and follicles reaching criterion size are summarized in Table 18. The figures recorded represent the total count for both ovaries.

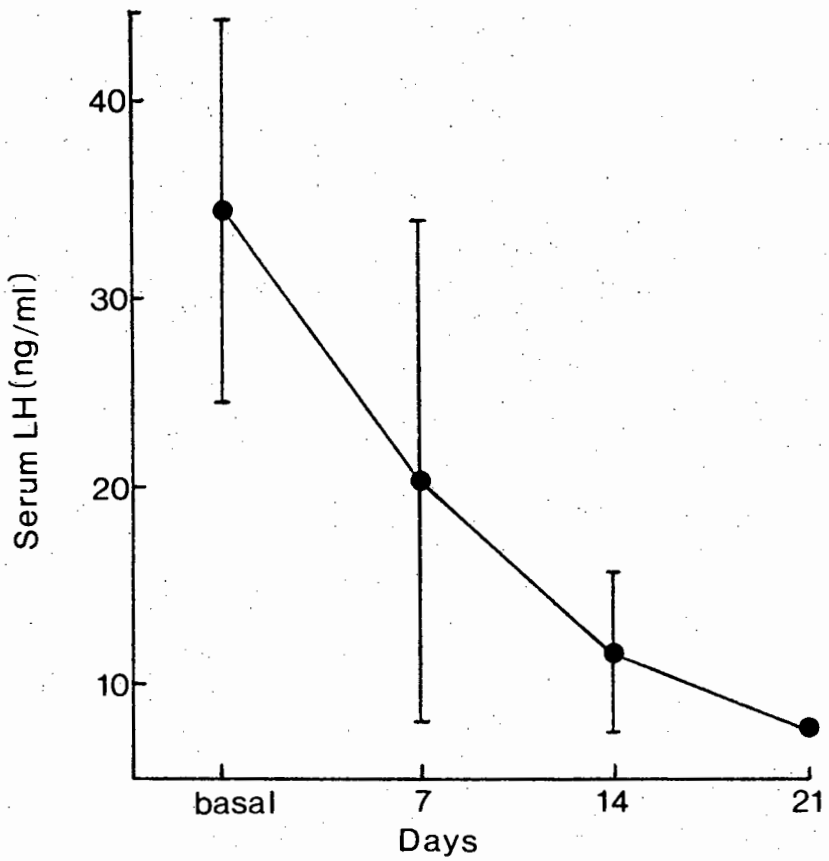

Figure 2 Changes in serum LH concentration at intervals after the start of DFF diet (Means±SD)

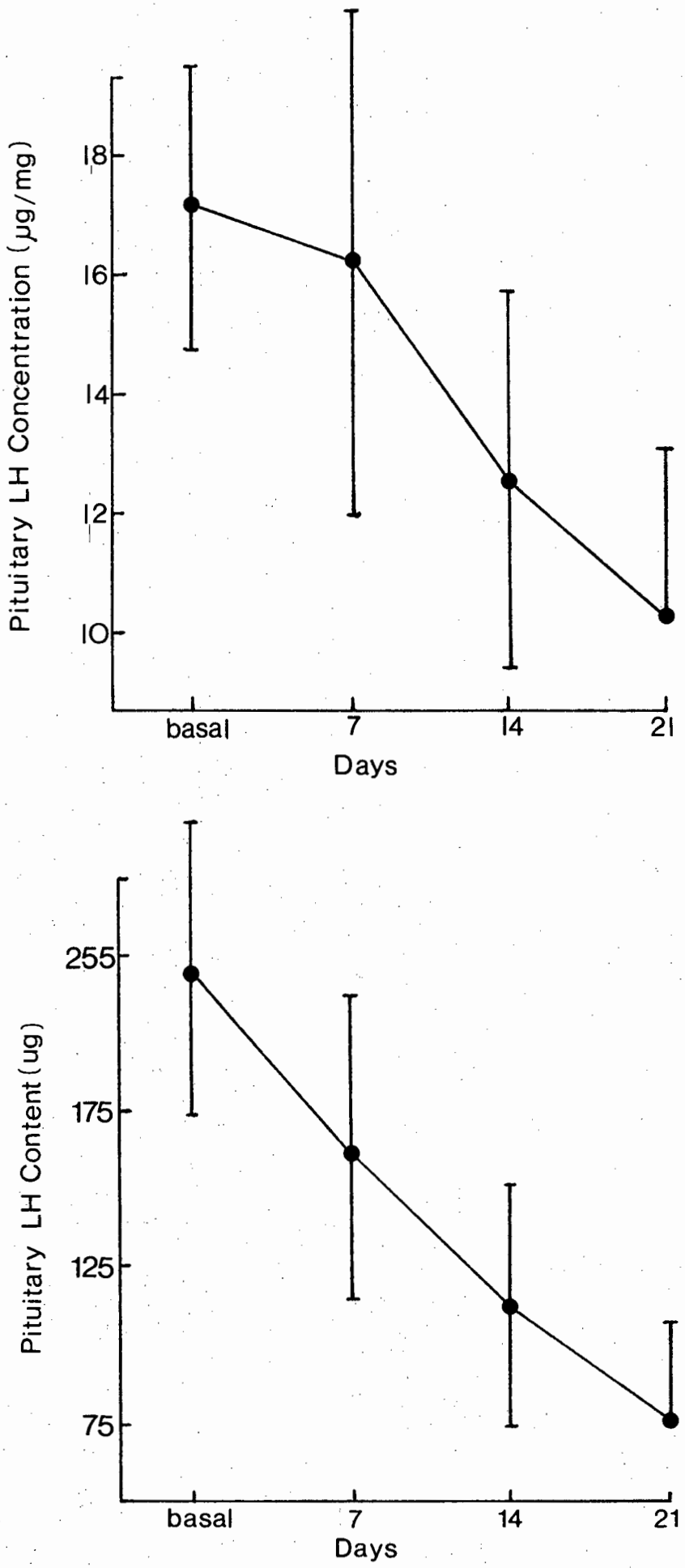

Figure 3. Changes in pituitary LH content and concentration at intervals after the start of DFF diet (Means±SD)

Table 18

Effects of DFF diet on the numbers of surface corpora lutea and follicles

Duration of feeding (days)	No. of rats	Mean number ±SD	
		Corpora lutea exceeding .7mm in diameter	Ovarian follicles exceeding .5mm in diameter
7	5	15.4±2.4	4.2±1.0
14	5	10.2±2.3[a]	0
21	5	8.8±2.6[a]	0
Basal control	5	16.8±2.4	4.8±2.2

a significantly fewer than 7 day and control groups ($p < 0.01$)

The 14 and 21 day groups had significantly fewer corpora lutea than the 7 day and control groups. No statistical analysis was carried out on follicle numbers because of the absence of follicles, exceeding .5mm in diameter, in the 14 and 21 day groups.

Examination of oviducts: revealed no tubal ova at the ampullary-isthmus junction.

Discussion

After 7 days of feeding on 5g of DFF diet per day body weight was significantly reduced but the majority of rats were still showing signs of oestrous cycling. At this stage serum LH and pituitary LH content and concentration were not significantly reduced. Furthermore target organs were functioning normally.

During the second week on the DFF diet all surviving rats became anoestrous. The latency of anoestrus was not consistent across all rats

and there was considerable difference in body weight loss at the onset of anoestrus.

After 14 days on the DFF regimen serum LH levels and pituitary LH content were significantly reduced, but not pituitary LH concentration. These reduced gonadotrophin levels coincided with the invariable onset of anoestrus and a significant reduction in the absolute ovarian weight. The ovaries were small and atrophic, showing no visible evidence of follicular development. The process of atrophy may account for the significant reduction in the number of surface corpora lutea exceeding .7mm in diameter. That is, the overall reduction in ovarian mass may have been accompanied by an accelerated regression of the larger corpora lutea. The uteri were thin and atrophic and the uterine weight was significantly reduced in both absolute terms and relative to body weight.

After 21 days feeding on the DFF regimen gonadotrophin levels and target organs were even more severely affected.

Many of the observed changes in reproductive function confirm the work of earlier investigators. The finding of reduced serum LH concentrations is consistent with the reports of studies employing a variety of restricted dietary regimen with both male and female rats (Campbell et al., 1977; Howland, 1971, 1972, 1975; Howland & Skinner, 1973; Widdowson et al., 1964). However the finding of reduced pituitary LH content and concentration after 21 days feeding on the DFF diet is not consistent with the findings of more recent studies employing radioimmunoassay techniques. Howland (1971, 1972) found no significant alteration of pituitary LH content and concentration after 20 days feeding on a 50% restricted diet. These findings were, in turn, inconsistent with those of Piacsek & Meites (1967) who, using a bioassay procedure, reported a marked reduction in pituitary LH concentration

after 31 days feeding on a 50% of normal intake regimen. It appears that the dietary restriction imposed in the present study was sufficiently severe to cause a significant reduction in pituitary LH content and concentration after 21 days.

The reduction in the absolute pituitary weight of nutritionally deprived rats has been observed by many others (Campbell et al., 1977; Dickerman et al., 1969; Howland, 1971, 1972, 1975; Leathem, 1958; Negro-Vilar et al., 1971; Piacsek & Meites, 1967; Srebnik & Nelson, 1963). A decrease in ovarian weight in underfed rats has also been reported (Howland, 1971, 1972; Piacsek & Meites, 1967; Rinaldini, 1949). Piacsek & Meites (1967) reported that rats fed 50% of normal food intake for 31 days had ovaries in which mature follicles and recently formed corpora lutea were absent. Similar observations were made in the present study.

Consistent with earlier reports was the finding that gonadal atrophy was accompanied by a significant reduction in uterine weight. It is reasonable to assume that the low levels of circulating LH were responsible for the ovarian atrophy and the consequent uterine atrophy.

In the present study the downward changes in serum LH concentration, ovarian and uterine weights had not reached significant proportions 7 days after the start of feeding on the DFF diet. Although there was evidence of a downward trend at 7 days the results indicate that the full consequences of severe caloric restriction were not manifest until the second week. The vaginal smear data indicated that the mean latency of anoestrus in rats fed a DFF diet was about 10 days. Onset of anoestrus occurred at a time when the rats were, on average, 19% below their original weight. Considering the severity of the restriction imposed in the present study it was not an unexpected finding that the latency of anoestrus was shorter than that reported by investigators

restricting food intake to 46% and 50% of normal quantities (Piacsek & Meites, 1967; Srebnik & Nelson, 1963). The small quantity of food provided (5g) could alone account for the more rapid onset of anoestrus. However, it is possible that the mechanisms involved in regulating reproductive function are particularly susceptible to severe caloric restriction as suggested by Ershoff (1952).

Previous researchers have reported an increase in adrenal gland weight during states of severe undernutrition (Campbell et al., 1977; Chowers, 1969; Meites & Reed, 1949). The increase in the relative adrenal gland weight of the rats fed the DFF diet in this experiment presumably reflects the stressful nature of the dietary restriction.

Experiment 4

In the previous experiment the changes in reproductive physiology that occur after 21 days feeding on a DFF diet were established. The aim of this experiment was to investigate the changes occurring during nutritional rehabilitation, following 21 days feeding on a DFF diet. Of particular interest in this study was the time required, after the start of rehabilitation, for the nutritionally induced changes to be reversed. The aim was to establish the time required for the following to occur: (i) return of normal serum and pituitary LH levels (ii) resumption of oestrous cycles (iii) return of organ weights to normal values (iv) evidence of gonadotrophin stimulation of the ovaries and resumption of follicular development.

The time variable was not the only one of interest. Although body weight gain is dependent upon the duration of rehabilitation attention was directed at the body weight changes coinciding with the key events in the resumption of reproductive functioning.

In order to facilitate comparisons with the results of the previous

experiment the rats were sacrificed on dioestrus; the same stage of the cycle as the rats in Experiment 3. In a later experiment rats were autopsied on the day of oestrus in order to determine when ovulation first occurs during nutritional rehabilitation.

Method

After 10 days of adaptation to eating the basal diet from feeding jars 30 female rats of similar weight and with regular 4 day oestrous cycles were allocated to one of 6 groups (n=5 each) so that the mean body weight of each group was approximately equal. Five groups of rats were fed 5g of the DFF diet per day for a period of 21 days while the sixth group continued to consume approximately 18g of basal diet per day on an ad libitum schedule. Food consumption, body weight and vaginal smears were monitored daily.

On day 21 of the experimental phase the 5 groups which had been fed the DFF diet were given access to unlimited quantities of the basal diet and water. On day 23 (48hr after the start of rehabilitation) rats in the 2 day rehabilitation group were sacrificed, blood samples were collected and autopsies were performed. On day 26 the rats in the 5 day rehabilitation group were sacrificed. All animals in this group were sacrificed on the same day since they were all still anoestrous. The rats in the 10 and 15 day rehabilitation groups were sacrificed on the day of dioestrus occurring nearest to days 31 and 36 of the experimental phase respectively. The rats in the fifth rehabilitation group were not autopsied. This group was included in the experiment to provide additional data on vaginal smear changes during ad libitum rehabilitation. The basal diet control group was sacrificed on the day of dioestrus nearest to day 36 of the experimental phase.

Results

Body weight: The changes in body weight of the control group and the group rehabilitated for the full 15 days are shown in Table 19 and Figure 4. The body weight measurements summarized here do not correspond with the day of autopsy of the other groups. The initial and final body weights of the groups autopsied on day 2, 5 and 10 of the rehabilitation phase are summarized in Table 22.

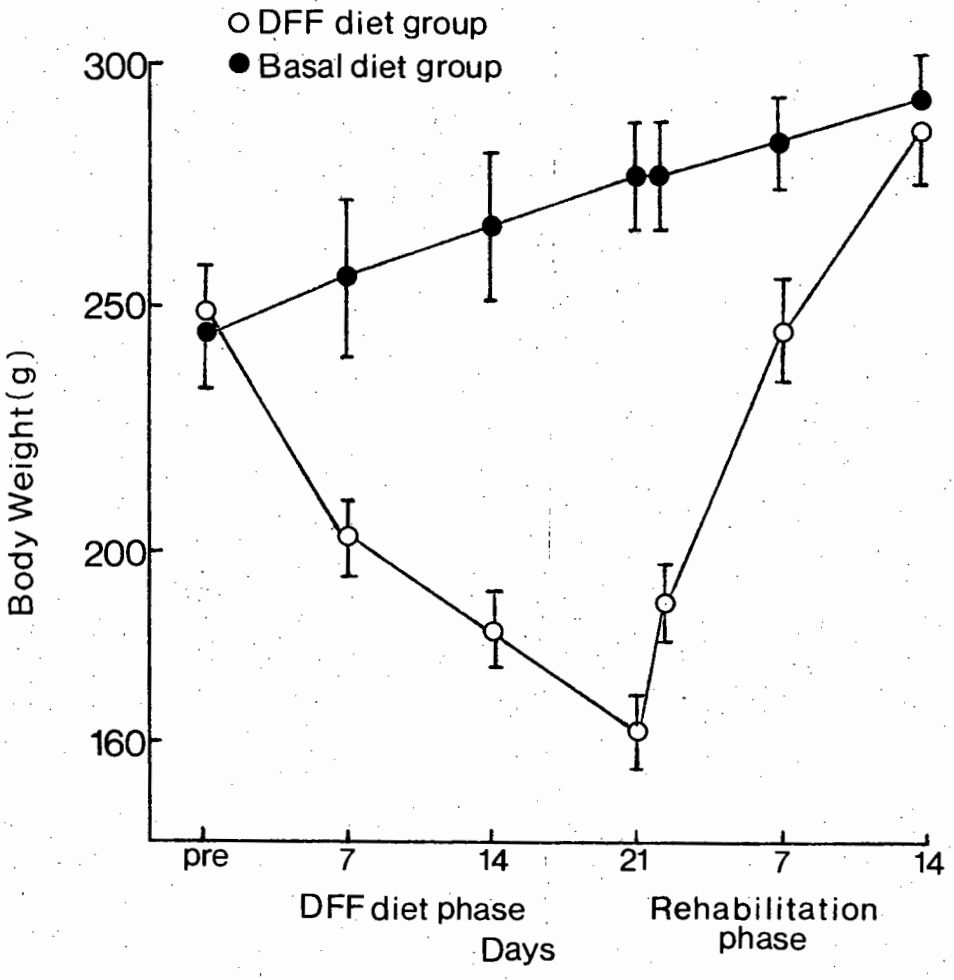

Figure 4 Changes in body weight during caloric deprivation and nutritional rehabilitation phases
(Means±SD)

Table 19

Effects of DFF diet and ad libitum rehabilitation on body weight

Duration since start of experimental period (days)	Mean body weight (g)±SD	
	Basal diet control group (n=5)	DFF diet group (n=5)
Deprivation phase		
Pre	247.8±11.9	248.2± 8.3
7	257.8±14.0	204.0± 6.8[a]
14	268.4±14.6	185.6± 5.9[a]
21	277.0±13.4	163.0± 6.0[a]
Rehabilitation phase		
22 (1 day rehab)	278.2±13.4	192.2± 7.1[a]
28 (7 days rehab)	285.6±11.5	246.8±12.7[a]
35 (14 days rehab)	292.4±11.4	286.6±10.7

a significantly lighter than control group ($p < .01$)

Analysis of variance revealed that the mean body weight of the DFF diet group was significantly lower than that of the control group on days 7, 14 and 21 of the deprivation phase and remained significantly lower for 7 days after the start of rehabilitation.
After 14 days of rehabilitation, however, the body weight of the two groups did not differ significantly. There was a significant increase in body weight of the DFF diet group after 1 day of rehabilitation ($p < .01$).

Food consumption: During deprivation phase: Since the basal diet control group continued to consume approximately 18g of food per day the quantity of DFF diet given to the experimental groups was held constant at 5g. During rehabilitation phase: The 24 hr food consumption rates

of the rats rehabilitated for the full 15 day period were as follows. During the first 7 days of rehabilitation the mean 24hr consumption of the basal diet was 28.3±4.1g. Food consumption, however, was not consistently high during the first week. There was a pronounced drop in food consumption from 28.2g during the first 24hr to 21.6g during the second 24hr. Thereafter food consumption increased again and during the 24hr period ending on day 8 the mean consumption was 29.6g. During the final 7 days of rehabilitation, the mean daily intake was 24.5±4.3g. After 15 days of ad libitum access consumption had decreased but rehabilitated rats were still eating above average quantities of food. The mean consumption on the final day of the experiment was 21.4g.

Vaginal smear patterns:

During deprivation phase: The time taken to become anoestrous and the body weight changes at the onset of anoestrus are summarized in Table 20. The findings summarized in this table are based on the combined data of all rats fed the DFF diet.

Table 20

Latency of onset of anoestrus and associated body weight changes in rats fed a DFF diet (n=25)

	Mean initial BW±SD (g)	mean latency anoestrus ±SD (days)	Mean BW changes at anoestrus±SD		
			Body wt. (g)	Absolute BW loss (g)	BW change (%)
$\bar{X}$ ±SD	247.7±9.2	8.2±2.4	204.1±11.1	43.6±10.4	-17.6
Range	-	5 to 12	-	23 to 58	-8.2 to -24.2

During rehabilitation phase: The time taken for rehabilitated rats to show an oestrous-type smear pattern for the first time and body weight

changes at first oestrus are shown in Table 21. The appearance of a smear in which cornified cells predominated, after an extended period of a dioestrous type smear pattern, was interpreted as resumption of cyclical activity.

The 2 and 5 day rehabilitation groups are not included in Table 21 since they were autopsied at a time when they were still anoestrous. The values in the table are derived from the 10 day rehabilitation group and the 2 remaining groups, rehabilitated for 15 days.

Table 21

Latency of resumption of oestrous cycling and body weight changes at first oestrus during ad libitum rehabilitation following 21 days feeding on a DFF diet (n=15)

	Mean initial BW±SD (g)	Mean latency of resumed cycling (days)	Mean BW at first oestrus ±SD (g)	BW change at first oestrus (%)
$\bar{X}$±SD	247.7±7.9	6.4±1.1	239.1±8.6	-3.4
Range	-	5 to 9	-	-11.2 to +2.1

It was observed that some rats had oestrous type smear patterns that were not preceded the day before by the typical pro-oestrous pattern, but by a smear in which leucocytes predominated. Other rats displayed 2 consecutive days of vaginal cornification.

Organ weights: The changes in organ weights at various intervals during full nutritional rehabilitation are summarized in Table 22.

The absolute pituitary weight of the 2 and 5 day groups was significantly lower than that of the basal diet control and other rehabilitation groups. The absolute pituitary weight of the 10 day group was significantly lower than that of the 15 day and control groups. There were, however, no significant differences between any of the

Table 22

EFFECTS OF NUTRITIONAL REHABILITATION ON ORGAN WEIGHTS AFTER 21 DAYS FEEDING ON DFF DIET

Duration of rehabilitation (days)	No. of rats	Mean initial BW±SD (g)	Mean final BW±SD (g)	BW Change (%)	Mean organ weight ±SD							
					Pituitary		Ovaries		Uterus		Adrenals	
					Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW
2	5	247.8±12.2	196.6±18.3	-20.7	9.1±0.81[a]	4.6±0.38	38.2±4.6[d]	19.5±2.7[d]	180.1±14.0[d]	93.4±4.1[d]	49.3±1.9	25.2±1.4[i]
5	5	247.8±12.1	225.0± 9.8	- 9.2	9.3±0.61[d]	4.1±0.23	61.4±2.9[e]	27.3±1.9	309.5±17.8[f]	137 6±5.3	50.9±0.92	22.7±1.0
10	5	246.8± 4.1	265.4± 9.8	+ 7.5	11.1±0.95[c]	4.2±0.26	65.6±1.5	24.8±1.2	337.7±12.9[g]	127.3±5.1[h]	53.5±3.3	20.1±0.98
15	5	248.2± 8.3	284.6±10.7	+14.7	12.7±1.1	4.5±0.32	69.4±5.8	24.4±1.7	382.0±12.0	134.3±3.5	52.2±9.3	18.3±3.3
Basal diet control	5	247.8±11.9	292.4±11.4	+18.0	13.2±0.45	4.5±0.15	71.8±5.3	24.6±2.1	384.3±35.3	131.3±7.7	56.3±11.1	19.2±3.4

a significantly lighter than 10 and 15 days and control groups ($p < .01$)
b significantly lighter than 10 day ($p < .05$) and 15 day, control groups ($p < .01$)
c significantly lighter than 15 day ($p < .05$) and control groups ($p < .01$)
d significantly lighter than all other groups ($p < .01$)
e significantly lighter than control group ($p < .01$)
f significantly lighter than 15 day and control groups ($p < .01$)
g significantly lighter than 15 day and control groups ($p < .05$)
h significantly lighter than 5 day group ($p < .05$)
i significantly heavier than 10 day ($p < .05$) and 15 day and control groups ($p < .01$)

groups when pituitary weight was considered relative to body weight.

The ovarian and uterine weights of the 2 day group were significantly lower than those of all other groups in both absolute weight and relative to body weight terms. The absolute ovarian weight of the 5 day group was significantly lower than that of the control group. The absolute uterine weights of the 5 and 10 day groups were significantly lower than those of the 15 day and control groups. The relative uterine weight of the 10 day group was significantly less than that of the 5 day group.

There were no significant differences between any of the groups with regard to absolute adrenal gland weight. However, when expressed in terms relative to body weight, the adrenal glands of the 2 day group were significantly heavier than those of the 10, 15 day and control groups.

Serum and pituitary LH levels The serum LH concentration and pituitary LH content and concentration, at various stages during nutritional rehabilitation, are summarized in Table 23.

Serum LH concentration and pituitary LH content and concentration remained significantly lower than normal control values up to and including the tenth day of rehabilitation. There was no significant difference between the 15 day rehabilitation group and the basal diet control group with respect to mean serum and pituitary LH levels.

Surface appearance of ovaries: The changes in the number of surface corpora lutea and follicles, reaching the size criterion, during the course of nutritional rehabilitation are summarized in Table 24.

The mean number of corpora lutea counted in the ovaries of the 2 and 5 day groups were significantly lower than those of the 10, 15 day and control groups. Statistical analysis of follicle data excluded that of the 2 day group which had no follicles exceeding 0.5mm in diameter. The 5 day group had significantly fewer follicles than the control group.

Table 23

Serum and pituitary LH levels at various stages during ad libitum nutritional rehabilitation following 21 days feeding on DFF diet

Duration of rehabilitation (days)	Mean serum LH±SD (ng/ml)	Mean pituitary LH ±SD	
		Content (µg)	Concentration (µg/mg wet wt)
2	8.2± .45[a]	88.0±20.2[a]	9.6±1.5[d]
5	13.4±3.4[b]	67.8±23.8[a]	7.3±2.6[a]
10	16.6±4.8[c]	120.4±35.3[d]	10.8±2.9[c]
15	20.6±5.9	191.0±50.7	15.0±3.6
Basal diet control	26.4±7.4	220.8±24.2	16.8±1.7

a significantly lower than 15 day and control groups ($p < .01$)
b significantly lower than control group ($p < .01$)
c significantly lower than control group ($p < .05$)
d significantly lower than 15 day ($p < .05$) and control groups ($p < .01$)

Table 24

The number of surface corpora lutea and follicles at various stages during ad libitum nutritional rehabilitation following 21 days feeding on a DFF diet

Duration of rehabilitation (days)	Mean number ±SD	
	Corpora lutea exceeding .7mm in diameter	Ovarian follicles exceeding .5mm in diameter
2	7.2±2.9[a]	0
5	8.8±2.9[b]	3.2±2.2[c]
10	15.2±2.3	6.6±2.4
15	14.6±2.1	6.4±1.7
Basal diet control	18.4±2.9	7.8±1.5

a significantly fewer than 10, 15 day and control groups ($p < .01$)
b significantly fewer than 15 day ($p < .05$) and 10 day and control groups ($p < .01$)
c significantly fewer than control group ($p < .01$)

Discussion

The average time taken for rats, fed 5g of DFF diet per day, to become anoestrous was similar to that observed in the previous experiment.

During ad libitum rehabilitation food consumption was extremely high and weight gain was rapid. During the first 7 days of rehabilitation the rats consumed, on average, 28.3g of the basal diet per day. Within 5-9 days after the start of nutritional rehabilitation the rats showed signs of resumed cyclical activity. The mean body weight of the rats at the time of first oestrus was 3.4% below the original mean body weight.

Rapid growth during the period of rehabilitation was observed by Widdowson et al. (1964). Furthermore these investigators found that young rats that still had closed vaginae when rehabilitation began, showed vaginal opening within 5 days of refeeding. At the end of 7 days rehabilitation the ovaries were as large as those of ad libitum fed controls of the same age and numerous corpora lutea were formed (Widdowson et al., 1964).

Because of the large amounts of food consumed the measured body weight included, in part, the increased weight of the gastrointestinal contents. Consequently the organ weights expressed in terms relative to body weight are likely to be slightly lower than they would be in the absence of the additional contents of the gastrointestinal tract.

All rats in the 2 and 5 day rehabilitation groups were still anoestrous at the time of autopsy.

At 2 days serum LH concentration, pituitary LH content and concentration was still significantly below control values. Four of the rats in this group still had serum LH levels of less than 8ng/ml. The ovaries were small and atrophic and showed no evidence of gonadotrophic stimul-

ation. The ovarian and uterine weights of the 2 day group were significantly lower than those of the other rehabilitated groups and the control group in both absolute terms and relative to body weight. In contrast the adrenal gland weight of the 2 day group, when considered in terms relative to body weight, was significantly greater than that of the 10, 15 day and control groups.

At 5 days serum LH concentration, pituitary LH content and concentration was still significantly below control values. The pituitary LH content and concentration values were lower than those of the 2 day group. It is possible that these reduced pituitary LH levels were the result of renewed release of LH into the circulation that preceded the onset of cycling which occurred 5-9 days after the start of rehabilitation. That is, LH release may have outstripped gonadotrophin synthesis, either due to impairment of pituitary function or decreased secretion of LHRH by the hypothalamus.

At 5 days the ovaries were showing distinct signs of gonadotrophin stimulation. New ovarian follicles were visible and the absolute ovarian weight was increased. Evidence of renewed ovarian function was reflected in the increase in the absolute uterine weight.

At 10 days the mean body weight exceeded the group's mean initial body weight. In spite of the rapid weight gain and the reappearance of cyclical activity evidence of the continued effects of the dietary restriction was still apparent. Serum LH concentration, pituitary LH content and concentration were still significantly lower than control values. As proposed earlier the lower pituitary LH levels may be a reflection of LH release that is not matched by gonadotrophin synthesis.

The pituitary and uterine weights, expressed in absolute terms, were still significantly lower than control values. In contrast the ovaries of the 10 day group did not differ from those of the control

group with respect to weight and the number of surface corpora lutea and follicles. Clearly sufficient gonadotrophins were being released to support ovarian development and cyclical activity.

The 15 day group did not differ from the basal diet control group along any of the parameters investigated. By this stage all rats were showing evidence of normal 4 day cycles and had apparently recovered from the period of inanition. The present findings suggest that no permanent damage was caused by the nature and duration of malnutrition imposed. However, since histological examinations of the organs were not performed the possibility that pathological changes occurred in the pituitary, that have been observed in some malnourished humans (Gillman & Gillman, 1951; Zubiran & Gomez-Mont, 1953), cannot be excluded.

There have been no reported studies on the effects of nutritional rehabilitation on gonadotrophin levels in female rats. The present findings are not consistent with the studies of rehabilitation of male rats. Campbell et al. (1977) reported that 7 days of refeeding of male rats, after chronic undernutrition, produced elevations in circulating gonadotrophins to values well above those of controls. Others have reported that serum testosterone levels rebound during rehabilitation of male rats to levels higher than that found in fully-fed controls (Grewal et al., 1971; Howland, 1975). The consistent absence of LH rebound, at various intervals during rehabilitation, in this study may be a function of sex and/or species differences or the severity of disturbance caused by extreme caloric deprivation. Rather than showing a rebound effect, serum LH levels gradually returned to normal. It is possible, however, that a rebound effect may have occurred at a time other than on the days on which blood samples were collected.

Experiment 5

In the previous experiment it was found that rats showed signs of resumed cyclical activity between 5 and 9 days after the start of nutritional rehabilitation. It has yet to be established whether the rats ovulate at the time of the first few appearances of vaginal cornification. The aim of this experiment was to determine whether or not the early cycles during nutritional rehabilitation were ovulatory ones.

Method

After 10 days of adaptation to eating the basal diet from feeding jars 20 female rats, of similar weight and with regular 4 day oestrous cycles entered the experimental phase. Fifteen rats received 5g of the DFF diet per day for 21 days while the remaining 5 rats served as the basal diet control group. Body weight, food consumption and vaginal smears were monitored daily.

On day 21 of the experimental phase the rats which had been feed the DFF diet were allowed access to unlimited quantities of the basal diet. The final allocation of rats, according to smear pattern sequence during rehabilitation was as follows:

(i) 5 rats were autopsied at the first appearance of an oestrous-type smear, preceded the previous day by a typical pro-oestrous smear pattern (Group P_1O_1).

(ii) 3 rats were autopsied on the first day of vaginal cornification preceded the previous day by a smear pattern in which leucocytes predominated (Group D_1O_1).

(iii) 3 rats were autopsied on the second day of the first appearance of two consecutive days of vaginal cornification (Group O_1O_2).

(iv) 4 rats were autopsied on the day of oestrus during the second

cycle (Group P_2O_2).

(v) 5 rats in the basal diet control group were autopsied on the day of oestrus.

The ovaries and oviducts of the rats in all groups were examined.

Results

The results are summarized in Table 25. Because of the small and unequal sample sizes the data collected were not subjected to statistical analysis except in the case of frequency of ovulation. Discussion of other results will be of a descriptive nature only.

The results of greatest interest were the proportion of rats ovulating. All rats in the P_1O_2, D_1O_2 and basal diet control groups ovulated, while the majority of rats in the O_1O_2 and P_1O_2 groups showed evidence of ovulation. Statistical analysis revealed no significant difference between the 5 groups with regard to frequency of ovulation ($\chi^2=0.08$, df=4, $p > .05$). Inspection of the mean number of ova per ovulating rat suggest that the ovulation rate may have been lower in the rehabilitated rats.

Discussion

The first appearance of vaginal cornification during rehabilitation was associated with ovulation in the majority of rats. This was found irrespective of whether the oestrous type smear pattern was preceded by a typical pro-oestrous smear pattern or not.

The findings indicate that the first and second cycles during rehabilitation following anoestrus are generally ovulatory ones.

Table 25

Effects of ad libitum nutritional rehabilitation on body weight, ovulation and surface appearance of ovaries

Group	No. of rats	Mean initial BW±SD (g)	Mean final BW±SD (g)	BW change (%)	Mean duration of rehab. ±SD (days)	Ovaries: Mean number ±SD: Corpora lutea exceeding .7mm in diameter	Ovaries: Mean number ±SD: Follicles exceeding .5mm in diameter	Oviducts: Proportion ovulating	Oviducts: Mean no. of ova per ovulating rat ±SD
P_1O_1	5	249.2±8.9	245.4±14.4	-1.4	6.4±1.1 Range 5-8	15.2±2.6	3.4±1.5	5/5	9.8±3.4
D_1O_1	3	244.0±7.8	240.0±10.0	-1.6	6.7± .58 Range 6-7	14.7±2.5	2.7±1.5	3/3	10.0±2.7
O_1O_2	3	249.3±2.1	249.0± 7.8	- .13	6.3± .58 Range 6-7	13.7±3.8	4.3±1.2	2/3	11.5±2.1
P_2O_2	4	247.8±6.1	262.0± 5.9	+5.7	9.8± .50 Range 9-10	16.3±2.2	4.3±1.5	3/4	9.0±3.0
Basal diet control	5	247.6±3.7	287.8± 6.9	+16.2	-	19.8±1.3	3.0±1.6	5/5	13.2±1.2

Experiment 6

The aim of this study was to determine whether resumption of oestrous cycles, during nutritional rehabilitation is dependent upon weight gain of a critical magnitude or whether intake of small quantities of a high calorie diet is sufficient to initiate renewed cycling.

A second objective was to determine whether an extended period of emaciation, without further weight loss, resulted in a delay in the resumption of cycling during nutritional rehabilitation.

Method

After 10 days of adaptation to eating the basal diet from feeding jars, 15 female rats of similar weight and with regular 4 day cycles were fed 5g of the DFF diet daily for 21 days. At the end of the restriction period the rats were allocated to one of 3 groups (n=5 each) in such a way that the mean weight loss (% of initial weight) of each group was approximately equal. Thereafter the 3 groups were placed on different basal diet regimens.

(i) Full rehabilitation group: Allowed access to unlimited supplies of the basal diet.

(ii) Gradual rehabilitation group: Allowed access to 18g of basal diet per day. This quantity is the average amount of food consumed, during a 24hr period, by fully-fed rats of the same age. However, the quantity is well below the average food consumption rate of rats on full nutritional rehabilitation (approximately 28g during the first week). It was reasoned that limiting basal diet intake to 18g per day would reduce the growth rate of rats during nutritional rehabilitation.

(iii) Low weight maintenance plus gradual rehabilitation group: Allowed access to 10g of basal diet per day for 7 days. It was judged that this quantity of food would not cause further weight loss nor allow significant weight gain in rats previously fed a DFF diet for 21 days. Thereafter the rats in this group were placed on the gradual rehabilitation regimen.

Body weight, food consumption and vaginal smears were monitored daily.

Results

Food consumption: The mean 24hr consumption rates of the full rehabilitation group were as follows: for the first 5 days, 27.7±2.5g; days 6-10, 28.0±3.8g; days 10-15, 22.0±2.9g. The other two groups invariably consumed the full amount of food provided.

Body weight: The changes in body weight of the groups on 3 dietary regimens, over a 20 day period, are shown in Figure 5. For convenience body weights at 5 day intervals were employed in the construction of the graph. The mean body weight of each group at the time of first vaginal cornification (oestrous type smear) is marked with an asterisk. The body weight changes associated with the first day of vaginal cornification are also summarized in Table 26. Statistical analysis revealed no significant difference between the 3 groups with regard to body weight at the onset of renewed cycling ($p > .05$).

Vaginal smear patterns: The time taken by the 3 groups to first show evidence of vaginal cornification is summarized in Table 26. The full rehabilitation group resumed cycling significantly sooner than the other two groups whereas the gradual rehabilitation group showed vaginal cornification significantly sooner than the low weight

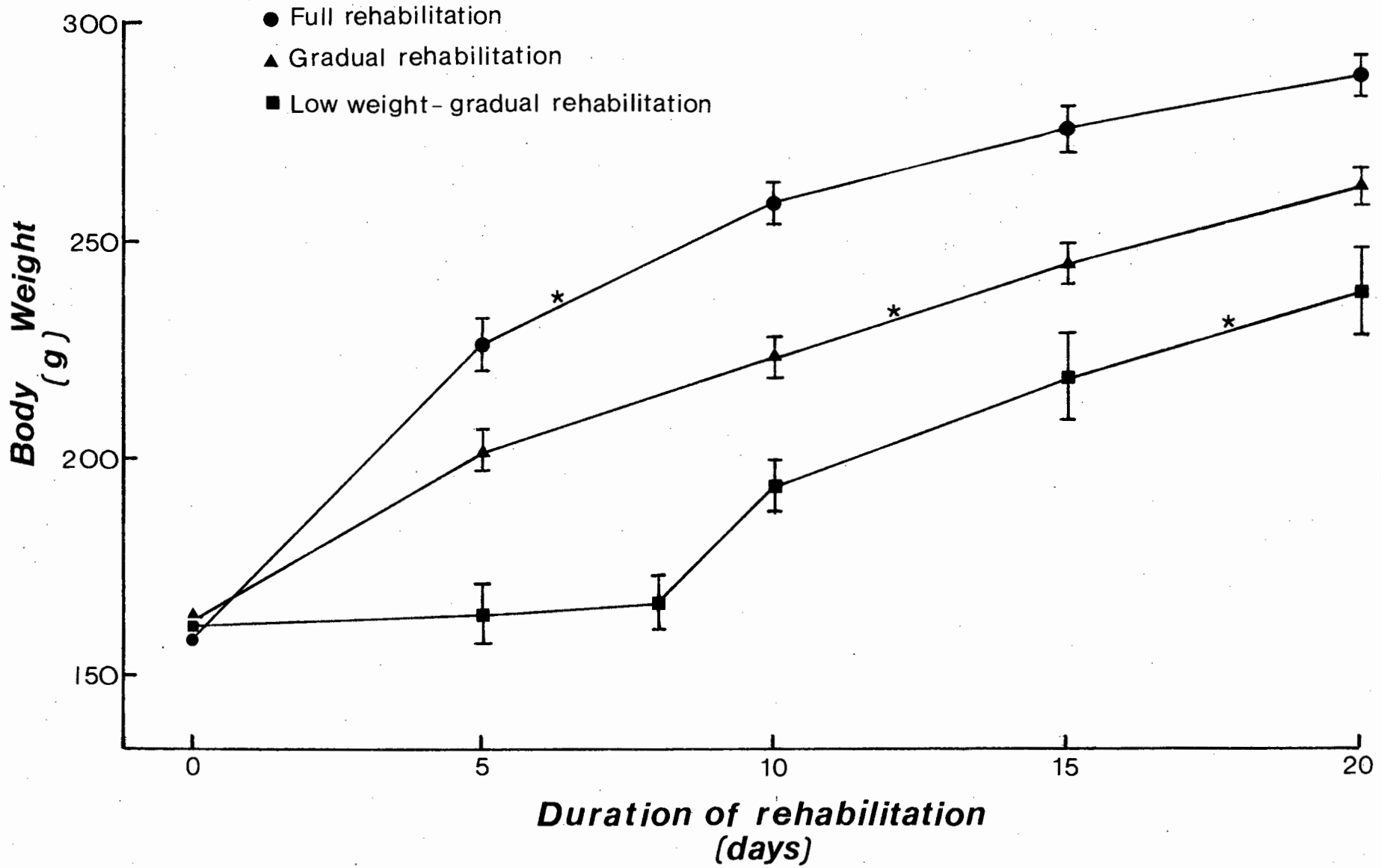

Figure 5 Changes in body weight during different forms of nutritional rehabilitation
* Appearance of first oestrus

maintenance plus gradual rehabilitation group.

The full rehabilitation group first showed evidence of vaginal cornification after 6.4±1.1 days, at a time when the mean group body weight was 1.3% below initial body weight. The gradual rehabilitation group showed evidence of resumed cyclical activity after an average of 12.2± .84 days, at a time when the mean body weight of the group was 3.2% below initial body weight. The onset of oestrous cycling was delayed further in the case of the rats maintained at a low weight and then placed on a gradual rehabilitation regimen. Vaginal cornification was first observed after 17.8±1.1 days, at a time when the mean body weight of the group was 3.4% below initial body weight.

Table 26

Latency of resumption of oestrous cycling and body weight changes of rats on different rehabilitation regimens

Dietary regimen	No. of rats	Mean Initial BW±SD (g)	Mean latency of resumed cycling±SD (days)	Mean BW at first oestrus±SD (g)	BW change at first oestrus (%)
Full rehab.	5	239.8±10.6	6.4±1.1[a] Range 5-8	236.6±5.1	-1.3
Gradual rehab.	5	241.6±13.4	12.2± .84[b] Range 11-13	233.4±3.4	-3.2
Low wt maint. + gradual rehab.	5	240.2± 7.4	17.8±1.1 Range 16-19	232.0±9.0	-3.4

a significantly sooner than other groups ($p < .01$)
b significantly sooner than low wt. maintenance plus gradual rehab. group ($p < .01$)

Discussion

The latency of resumed cycling and associated body weight changes of the full rehabilitation group were similar to those observed in earlier experiments.

The finding of no significant difference between the 3 groups with regard to body weight at the time on onset of renewed cycling, suggests that resumption of oestrous cycling is dependent upon weight gain of a certain magnitude. The present study indicates that provision of limited quantities of a high energy diet is not sufficient to initiate reproductive cycling in rats with nutritionally-induced anoestrus. The similarity of body weight changes at the onset of oestrous cycling, irrespective of the length of time taken to regain weight, suggests that resumption of cycling is not solely a function of the duration of rehabilitation but, more specifically, of weight gain achieved during rehabitation.

The present study indicates that weight gain, to the extent that rats are, on average, about 3% below initial body weight, is critical for the resumption of cyclical activity. Furthermore the results of this study suggest that extending the period of emaciation for an additional 7 days did not have the effect of delaying the resumption of normal cycling after the appropriate body weight had been reached.

Part B: Provocation studies

The purpose of the series of studies to be reported in Part B was to determine which components of the hypothalamic-pituitary-ovarian (HPO) axis are dysfunctional (i) during the state of nutritionally-induced anoestrus (ii) at various stages during the course of nutritional rehabilitation. Use was made of exogenously administered hormones to test the rate of response, capacity and reserve of the

components of the HPO axis.

Most available evidence points to a functional hypothalamic defect as the cause of anoestrus during nutritional deprivation (Campbell et al., 1977; Ibrahim & Howland, 1972; Negro-Vilar et al., 1971; Piacsek & Meites, 1967). However, since the reproductive system is not regulated unidirectionally it is also necessary to examine the responsiveness and the functional capacity of the pituitary gland and the ovaries. It is possible that there is persistent unresponsiveness of the anterior pituitary due to depletion of gonadotrophins. Such depletion may be the result of malnutrition exerting a direct effect on the pituitary or the depletion of gonadotrophins may be due to decreased synthesis and/or release of LHRH from the hypothalamus. Yet a further explanation to be considered is that, due to ovarian refractoriness, there is an absence of sufficient oestrogen to exert a positive feedback effect on the hypothalamus.

The specific purposes of each study will be presented in its introduction.

Experiment 7

To date there are no published reports on the effects of LHRH administration on LH secretion during nutritionally induced anoestrus. Studies with underfed male rats have revealed a normal pituitary response to LHRH administration (Campbell et al., 1977; Root & Duckett, 1973). The only reported studies of the effects of LHRH provocation on emaciated females are those which have been done with anorexic patients. Studies employing single intravenous injections of LHRH during the acute stage of anorexia nervosa have produced inconsistent results. The response to LHRH by anorexic subjects has been described as absent, impaired or normal (see Chapter 2 for more detailed review). In those

patients not showing a response to a single injection of LHRH, it has been found that they do show a normal response pattern after prolonged LHRH treatment (Nillius & Wide, 1977). These findings suggest that LHRH exerts a self-priming effect on the pituitary responsiveness to LHRH, acting together with modulatory feedback effects of gonadal steroids. Nillius & Wide (1977) proposed that the results of prolonged LHRH studies provide support for the hypothesis that there is a deficient production and/or secretion of endogenous LHRH from the hypothalamus.

Further support for the notion that LHRH sensitizes the anterior pituitary to itself comes from studies in which fully fed rats were used. Although a significant increase in circulating LH, following LHRH administration, has been observed on all days of the oestrous cycle it has been found that the maximal response occurs on pro-oestrus (Aiyer et al., 1974; Blake, 1978; Gordon & Reichlin, 1974; Legan & Karsch, 1975; Zeballos & McCann, 1975). This finding led to the suggestion that the enhanced sensitivity of the pituitary to LHRH on the afternoon of pro-oestrus may be, in part, due to LHRH increasing the potential of the gonadotrophs to secrete LH in response to further LHRH stimulation. Alternatively, it has been suggested that LHRH, by raising the levels of circulating LH, stimulates the secretion of sex steroids which, in turn, enhance the sensitivity of the anterior pituitary (Aiyer et al., 1974). It is conceivable, however, that under normal conditions, both mechanisms are in operation. That is, preovulatory oestrogen secretion stimulates the secretion of hypothalamic LHRH as well as directly enhancing pituitary sensitivity to LHRH and that the continuing secretion of LHRH sensitizes the pituitary for further LHRH stimulation.

The purposes of the present study were manifold. Firstly, the aim

was to investigate the functional state of the pituitary gland of rats during nutritionally induced anoestrus by measuring the LH response to a single injection of LHRH. It was reasoned that, in the event of depletion of pituitary stores of LH and/or impairment in the capacity to release LH there would be no significant response to LHRH stimulation.

Secondly, the aim was to determine whether constant infusion of LHRH for 60 mins before a rapid injection of LHRH would result in an enhanced pituitary response. A significantly greater LH response, in these LHRH-primed rats, would indicate a sensitizing effect of LHRH on the pituitary and would add further support for the hypothesis that there is deficient production and/or secretion of endogenous LHRH during states of inanition.

Method

Thirty female rats with regular 4 day oestrous cycles were allowed 10 days to adapt to eating the basal diet from feeding jars. At the end of this period all rats had jugular cannuli inserted surgically and were allowed a recovery period of 3 days. Thereafter they were allocated to one of three groups (n=10 each) so that the mean body weight of each group was approximately equal. On day 1 of the experimental phase 2 groups of rats were placed on a 5g DFF diet per day schedule, which was maintained until day 14. The base of the feeding jars were raised to avoid disturbance of the cannuli while eating. The rats in the third group continued to receive the basal diet ad libitum for the same period. During the recovery and experimental phases the cannuli were cleared every second day. Body weight, food consumption and vaginal smears were monitored daily. By day 14 all rats receiving the DFF diet were showing evidence of anoestrus (constant dioestrus smear pattern). On day 14 five rats from each group, with intact and patent cannuli,

entered the next phase of experimental treatment. In addition, the remaining 2 rats in the basal diet group with intact cannuli were included as an "all saline" control.

The 4 groups of rats were treated on dioestrus as follows:

Dietary regimen	No. of rats	1hr infusion of:	Rapid injection of:
DFF diet (Group 1)	5	.05ml saline containing 50ng LHRH	50ng LHRH in .1ml saline
DFF diet (Group 2)	5	.05ml heparinized saline	50ng LHRH in .1ml saline
Basal diet (Group 3)	5	.05ml heparinized saline	50ng LHRH in .1ml saline
Basal diet (Group 4)	2	.05ml heparinized saline	.1ml saline

Details of the infusion procedure and preparation of the LHRH doses are provided in Chapter 3.

The following blood sampling procedures were employed in this study. In each case samples of .4ml blood were collected.

Pre sample: This sample was collected immediately before the rats were connected to the infusion apparatus

0 min sample: Sample collected at the end of the 1 hour infusion period immediately before the rapid injection of the LHRH bolus or saline

Further samples were collected 10, 20, 40 and 80 minutes after the rapid injection of LHRH or saline.

Results

Body weight: The changes in body weight of groups 1-3, during the 14 day experimental diet period, are summarized in Table 27. The body weights of the groups fed the DFF diet were significantly lower than the basal diet group at days 7 and 14.

Table 27

Effects of 14 days feeding on DFF diet on body weight

Dietary regimen	rats	Mean body weight (g)±SD			BW change at day 14 (%)
		Pre wt.	Day 7	Day 14	
DFF diet (Group 1)	5	250.6±2.4	209.6±6.2[a]	186.4±5.8[a]	-25.6
DFF diet (Group 2)	5	251.0±3.0	206.4±4.3[a]	182.2±4.4[a]	-27.4
Basal diet (Group 3)	5	249.8±4.0	260.2±3.6	271.4±2.6	+ 8.0

a significantly lighter than basal diet group ($p < .01$)

Food consumption: The basal diet group consumed, on average, 17.8±1.5g during the first 7 days and 18.4±1.5g during the second week. The consumption rate of the DFF diet fed groups remained constant at 5g throughout the 14 day period.

Vaginal smear patterns: All rats receiving the DFF diet were showing a constant dioestrous-type smear pattern 8 days after the start of the dietary regimen. Apart from occasional 5 day cycles, largely confined to the period shortly after the installation of the cannuli, the basal diet group continued to display normal 4 day cycles.

Serum LH levels: The changes in serum LH levels at various stages during the LHRH administration procedure are summarized in Table 28 and Figure 6. Only the data of groups 1-3 were employed in the statistical analysis. The basal diet fed, saline infusion-saline injection control group was too small to be included. Analysis of variance indicated that the mean serum LH levels of group 1 were significantly greater than those of group 2 at the end of the infusion period and at all sampling intervals after rapid injection of LHRH, except at the 80 min.

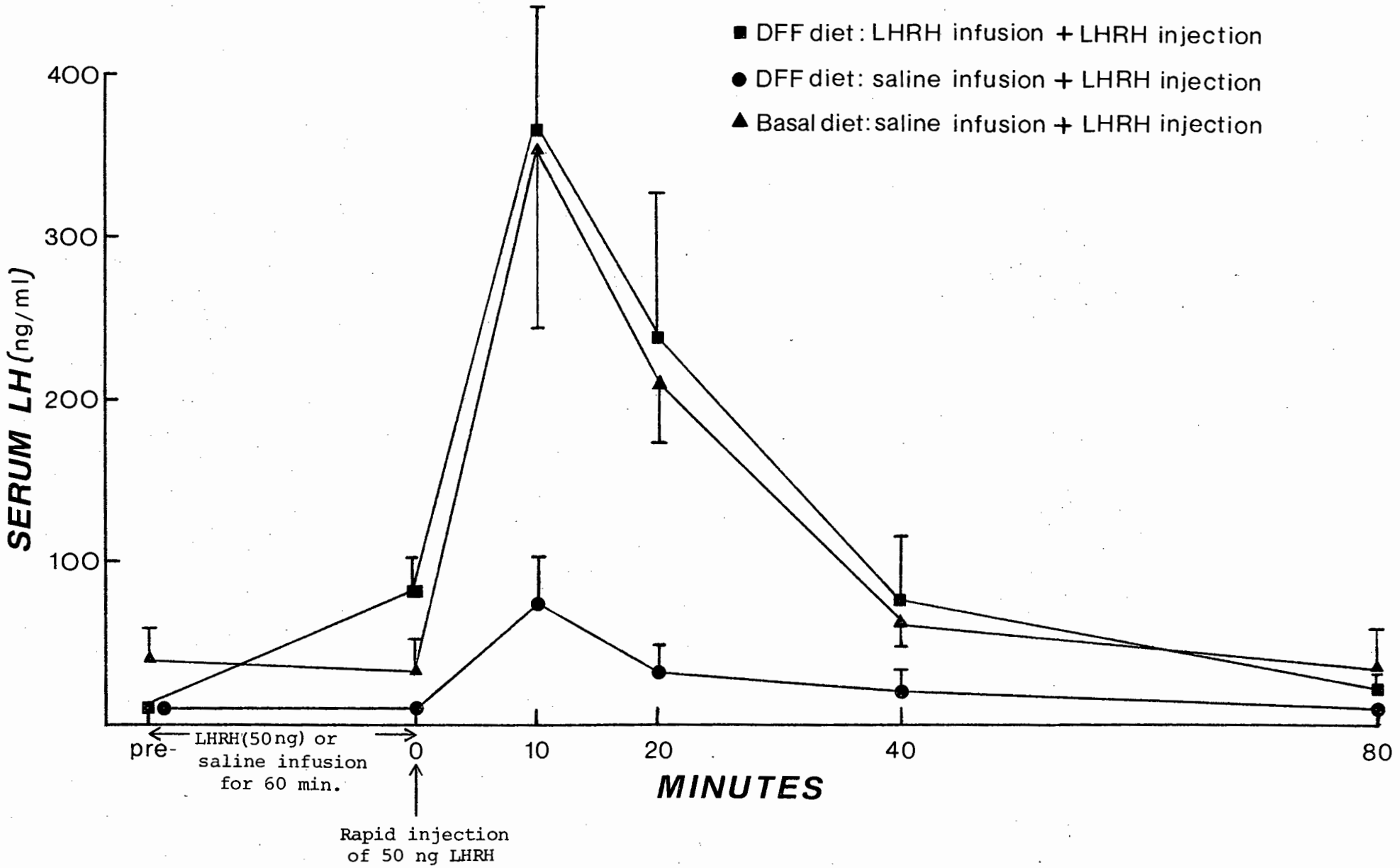

Figure 6 Mean serum LH concentration (± SD) just before and after infusion of LHRH and rapid injection of LHRH

Table 28

Changes in serum LH levels in response to LHRH administration

Group	No. of rats	Mean serum LH(ng/ml)±SD					
		Pre (before infusion)	Time since rapid injection of 50ngLHRH (mins)				
			0	10	20	40	80
1. DFF diet: LHRH infusion+ LHRH injection	5	<8.0± 0	85.6±15.4[a]	365.0± 85.4[a]	242.0± 88.4[a]	79.8±33.3[a]	11.8± 5.5
2. DFF diet: Saline infusion +LHRH injection	5	<8.0± 0	<8.0± 0	82.8± 19.6	30.4±14.9	10.4± 5.4	<8.0± 0
3. Basal diet: Saline infusion +LHRH injection	5	39.8±14.8	28.8±21.0	360.0±105.8[a]	206.0±35.1[a]	69.4±18.2[a]	40.2±28.9
[b] 4. Basal diet: Saline infusion +Saline injection	2	28.5± 3.5	34.0± 5.7	26.0± 4.2	32.5± 6.4	30.0± 4.2	32.5± 3.5

a significantly greater than Group 2 ($p < .01$)
b not included in statistical analysis

stage. The LH peak of group 1, reached after 10 min, was 4 times greater than that shown by group 2. The mean serum LH levels of group

3 at 10, 20, and 40 min were significantly greater than those of group 2

All three groups showed a significant increase in serum LH concentration after LHRH administration (groups 1 & 3 $p < .01$; group 2 $p < .05$). However, the increase was first evident in group 1 at the end of the LHRH infusion period. The LH response was minimal in the case of group 2 with a significant increase only being apparent at the 10 min interval.

Discussion

At the time of LHRH administration rats fed the DFF diet were weighing significantly less than the basal diet group and were showing evidence of anoestrus.

In anoestrous rats the increase in serum LH in response to a rapid injection of 50ng LHRH after LHRH infusion was similar in magnitude and duration to that of the normal weight basal diet group receiving only the rapid injection of LHRH. Although anoestrous rats not receiving prior infusion of LHRH did show a response to a rapid injection of LHRH, the serum LH changes were of a significantly lesser magnitude and shorter duration.

The present results indicate that, in states of emaciation, the pituitary gland of the female rat can synthesise LH and is capable of discharging its pool of LH in response to exogenous LHRH. However, the results further indicate that a normal response occurs only after the pituitary has been exposed to prior LHRH stimulation for at least 60 mins. The present findings, with emaciated female rats, are not consistent with earlier reports on the serum LH response to LHRH administration in underfed male rats. Root & Duckett (1973) and Campbell et al. (1977) have reported that, in underfed male rats, the pituitary response to a single injection of LHRH was the same as that of fully

fed controls. The higher doses of LHRH administered to the male rats could account for the observed differences.

The present results, nevertheless, support the conclusion by Campbell et al. (1977) that the reduced secretion of LH, as a result of restricted food intake, is due primarily to the deficient production and/or secretion of endogenous LHRH. This view, in turn, is supported by earlier reports of reduced hypothalamic content of LHRH (Piacsek & Meites, 1967) and other releasing factors in underfed rats (Dickerman et al., 1969; Negro-Vilar et al., 1971). The finding, in the present study, of an augmented LH response following slow infusion of LHRH suggests a partial pituitary dysfunction as a consequence of reduced LHRH secretion. That is, the results suggest that the responsiveness of the pituitary is influenced by the priming effects of LHRH; a complete pituitary response to LHRH occurring only after a period during which LHRH sensitizes the pituitary to itself. Previous researchers came to similar conclusions following prolonged LHRH treatment of anorexia nervosa patients during the acute phase (Nillius & Wide, 1977).

It is known that gonadal steroids can modulate the gonadotrophin response of the pituitary to LHRH (Schally et al., 1972). However, it would appear that LHRH was capable of exerting a priming effect on the pituitary and inducing LH release in female rats whose gonadal steroid levels were, in all likelihood, greatly reduced. In Experiment 3 it was found that, after 2 weeks feeding on 5g DFF diet, rats had ovaries and uteri that were small and atrophic and there was no visible evidence of follicular development. It is unlikely that the atropic ovaries would have responded, during the experimental period, to gonadotrophin stimulation, initiated by LHRH infusion, given the brief period of time between the start of infusion and the rapid injection of LHRH.

Experiment 8

It has been shown that a single dose of oestradiol benzoate (OB) induces ovulation in immature female rats (Ying, Fang & Greep, 1971; Ying & Greep, 1971a & b). For example, Ying & Greep (1971a & b) found that ovulation occurred in 75-80% of 33 day old female rats given a single injection of .25 or .5µg of oestradiol benzoate at 30 days of age. The ovulation that occurred approximately 72 hrs post-OB injection was associated with an endogenous LH surge which occurred approximately 54-56 hrs following OB administration. Oestrogen administration has also been shown to accelerate vaginal cornification and advance ovulation by about 24 hrs in 5 day cyclic rats receiving 50µg of OB on dioestrus-2 (Everett, 1948).

The presumed mechanism of action of OB in inducing ovulation in immature rats, or advancing ovulation in cyclic rats, is as follows: The exogenous oestrogen exerts a positive feedback effect at the level of the hypothalamus resulting in LHRH release, an endogenous LH surge and eventual ovulation (Baird et al., 1975; Goodman, 1978a & b). It has also been demonstrated that oestrogen exerts a direct effect on LH release by enhancing the sensitivity of the pituitary to LHRH (Aiyer et al., 1974; Arimura & Schally, 1971).

It this experiment OB was administered to anoestrous rats after 3 weeks feeding on 5g DFF diet in order to determine whether they would show a similar response pattern to prepubertal female rats. The aim of the study was to test the functional capacity of the hypothalamic-pituitary axis to respond to the positive feedback action of exogenous oestrogen. A surge of LH and/or ovulation would indicate that oestrogen had exerted a positive feedback effect and would provide evidence of a functional hypothalamic-pituitary axis during nutritionally induced anoestrus.

Method

After 10 days of adaptation to eating the basal diet from feeding jars 35 female rats of similar weight and with regular 4 day oestrous cycles were fed 5g DFF diet per day for a period of 21 days. Body weight, food consumption and vaginal smears were monitored daily. On day 20 the rats were allocated to one of seven groups (n=5 each) so that mean body weight of each group was approximately equal.

At 0800h on day 21 thirty rats received a subcutaneous injection of .1ml olive oil containing 2µg of oestradiol benzoate and the remaining group received an injection of .1ml olive oil containing only ethyl alcohol. The latter group served as a control for the 56hr OB group (see Chapter 3 for details about preparation of injections).

Five rats were sacrificed immediately after receiving the OB injection (0hr group). This group served as a control for all other OB groups. The remaining rats were sacrificed at the following intervals after OB administration.

Day 22	0800h	-	24hr group
	2000h	-	36hr group
Day 23	0800h	-	48hr group
	1600h	-	56hr group
	1600h	-	56hr oil control group
Day 24	0800h	-	72hr group.

All rats continued to receive 5g DFF diet daily until the day of autopsy. In all cases organ weights were recorded, serum and pituitary LH was determined, ovaries and oviducts were examined.

Results

Body and organ weights: The changes in body and organ weights are summarized in Table 29. There were no significant differences between

Table 29

EFFECTS OF 2µg OESTRADIOL BENZOATE ON ORGAN WEIGHTS AFTER 21 DAYS FEEDING ON DFF DIET

Group: Time elapsed since OB injection (hrs)	No. of rats	Mean final BW±SD (g)	BW Change (%)	Mean organ weight ±SD							
				Pituitary		Ovaries		Uterus		Adrenals	
				Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW
24	5	163.2±10.6	-33.9	9.1± .98	5.6± .54	47.2± 9.5	28.9±6.2	266.2±31.7	164.4±28.4	50.2±2.3	30.9±2.4
36	5	161.4±13.6	-36.1	9.3± .49	5.8± .32	45.6± 8.4	28.6±7.2	285.6±65.0	180.1±55.6	50.8±3.2	31.7±4.3
48	5	162.2±10.7	-33.6	9.6±1.4	5.9± .82	36.2± 6.6	22.5±4.8	273.8±38.4	169.9±30.7	50.3±2.6	31.2±3.0
56	5	160.8± 8.6	-37.1	10.7± .62	6.7± .73	43.3±10.0	27.1±6.6	290.2±23.4	181.4±23.5	51.1±2.5	31.8±2.3
72	5	156.0± 6.0	-36.4	10.2± .73	6.5± .30	40.7± 5.9	26.0±2.9	284.8±39.2	182.7±25.1	50.7±2.3	32.6±2.2
Controls 0	5	167.4±12.1	-34.2	8.2± .76[a]	4.9± .79[c]	42.5± 4.0	25.5±3.3	172.3±43.5[e]	102.4±21.6[f]	51.1±2.9	30.6±2.7
Oil control at 56hrs	5	163.2± 8.9	-34.9	7.8± .79[b]	4.8± .57[d]	46.8± 6.5	28.8±4.5	161.1±27.5[e]	99.2±18.9[f]	50.4±1.5	31.0±2.6

a significantly lower than 56hr ($p < .01$) and 72hr groups ($p < .05$)
b significantly lower than 48hr ($p < .05$), 56hr and 72hr groups ($p < .01$)
c significantly lower than 36hr, 48hr ($p < .05$), 56hr and 72hr groups ($p < .01$)
d significantly lower than 56hr and 72hr groups ($p < .01$)
e significantly lower than all other OB injected groups ($p < .01$)
f significantly lower than 24hr, 48hr ($p < .05$), 36hr, 56hr and 72hr groups ($p < .01$)

any of the groups with regard to final body weight. The mean pituitary weights, both in absolute terms and relative to body weight, of the 0hr and oil control groups were significantly lower than those of the 56hr and 72hr groups. Other significant differences between the pituitary weight of the control groups and the OB injected groups are summarized in Table 29. Uterine weights of OB injected rats were significantly increased at 24hrs and remained so up to 72hrs.

Serum and pituitary LH levels: The serum LH concentrations of both the OB injected groups and the control groups remained at undetectable levels (<8ng/ml) at all sampling intervals.

The pituitary LH content and concentration of the OB injected and control groups are shown in Table 30.

Table 30

Pituitary LH levels at various intervals after injection of 2µg OB following 21 days feeding on DFF diet

Group: Time elapsed since OB injection (hours)	No. of rats	Mean pituitary LH±SD	
		Content (µg)	Concentration (µg/mg wet wt)
24	5	112.4±19.8	12.3±1.3
36	5	105.2±21.0	11.3±1.8
48	5	98.6±26.6	10.2±1.5
56	5	90.0±25.0	9.4±2.0
72	5	102.8±28.7	10.0±2.2
0hr control	5	94.2±22.1	11.4±1.8
Oil control	5	88.8±21.8	11.2±1.8

Analysis of variance revealed no significant difference between any of the groups with regard to pituitary LH content or concentration ($p > .05$).

Ovarian appearance No ovarian follicles, meeting the size criterion,

were observed in any of the groups. The changes in the number of surface corpora lutea exceeding .7mm in diameter are summarized in Table 31.

Table 31

The numbers of surface corpora lutea at various intervals after injection of 2µg OB following 21 days feeding on DFF diet

Group: Time elapsed since OB injection(hours)	No. of rats	Mean number of corpora lutea exceeding .7mm in diameter ±SD
24	5	5.8± .84
36	5	5.2±1.3
48	5	4.8±1.3
56	5	3.8± .84
72	5	3.4±1.7
0hr control	5	8.0±1.6[a]
Oil control at 56hrs	5	8.2±1.3[a]

a significantly greater than 36hr group ($p < .05$) and 48, 56 & 72 hr groups ($p < .01$)

The 0hr and oil control groups had significantly more surface corpora lutea than any of the other OB injected groups.

Examination of the oviducts at the ampullary-isthmus junction failed to reveal the presence of tubal ova in any of the groups.

Vaginal smear patterns: Vaginal smears taken just before OB administration were of the dioestrous smear type, with leucocytes predominating. At 24hr after OB injections: all rats continued to display a dioestrous type smear pattern. At 48hr after OB injection: 14 of the remaining 15 rats had vaginal smears in which parabasal cells predominated. Few if any leucocytes were present and 7 rats had smears showing the "cobblestone" appearance of pro-oestrus. One of the OB injected rats

and all oil control group rats had dioestrous type smear patterns. At 72hr after OB injection: all of the surviving rats had smears in which cornified cells were predominant. Some of the cells were anucleated whereas others were of the superficial epithelial type with vesicular nuclei. The overall smear pattern, although showing signs of epithelial proliferation, was not typical of the oestrous-type smear.

Discussion

Administration of 2µg OB to anoestrous rats did not result in ovulation that has been observed in similarly treated immature, female rats. No increase in serum LH nor significant changes in pituitary LH content and concentration were observed at any of the sampling intervals. These findings indicate a failure of this dose of OB to induce an endogenous LH surge in nutritionally induced anoestrous rats.

It is unlikely that an LH increment occurred at times other than when sampled since the ovarian data are not consistent with gonadotrophin stimulation. Contrary to the findings of Ying, Fang & Greep (1971) who reported increased ovarian weight in immature rats at 48hrs, the ovaries of the OB injected rats in this study showed no significant increase in weight and there was no evidence of follicular development. Consistent with the findings of Ying, Fang & Greep (1971) there was a significant increase in uterine weight. In the absence of other evidence of endogenous gonadotrophin stimulation the increase in uterine weight is presumably caused by the direct action of the administered OB.

The finding of increased pituitary weights in OB-treated rats was not an unexpected finding. Flerko & Bardos (1960) demonstrated an increase in pituitary weight in female rats following treatment with oestradiol. Similar pituitary hypertrophy was observed after uninhibited oestrogen secretion following lesions of the ventral anterior

hypothalamus (Flerko & Bardos, 1960). In the present study pituitary hypertrophy was not accompanied by an increase in the pituitary LH content or concentration.

The decrease in the number of large corpora lutea in the OB-treated rats suggests that oestrogen is capable of accelerating luteal regression.

In conclusion, the present findings indicate that oestrogen administration failed to produce a positive feedback release of LH in emaciated, anoestrous rats. The present findings, taken together with those of Experiment 7, suggest that the site of dysfunction in anoestrous rats lies within the hypothalamus. Since it has been demonstrated that the LHRH-primed pituitary is capable of responding to further LHRH stimulation the evidence points to the failure of the hypothalamus to respond to the positive feedback effects of oestrogen.

Experiment 9

The previous experiment demonstrated a failure of the positive feedback effects of oestrogen in emaciated rats which suggested impairment of hypothalamic function. The functional capacity of the other components of the HPO axis have not yet been established. This could be achieved by using the gonadotrophin test.

It has been demonstrated that the administration of pregnant mares serum gonadotrophin (PMS) induces ovulation in immature rats (Longenecker & Gallo, 1971; Ying & Meyer, 1969; Zarrow & Brown-Grant, 1964; Zarrow & Quinn, 1963). Furthermore, Reiter et al. (1969) demonstrated that PMS, administered to immature rats, induced the release of endogenous LH 54hr after the injection, which was followed 12 to 15 hours later by ovulation.

The mechanism of action of PMS is presumed to be as follows:

The gonadotrophins act by stimulating the ovaries directly, promoting follicular development and oestrogen production. As the oestrogen level increases it exerts a positive feedback effect on the hypothalamus triggering the secretion of LHRH, followed by the LH surge and subsequent ovulation (Ying & Greep, 1971b).

The specific aims of this study were to investigate the changes in organ weights and ovarian appearance and to determine whether ovulation occurred at intervals 65, 72 and 89 hours after PMS administration. No attempt was made to detect the anticipated serum LH surge at 54-58 hrs after injection since the PMS used in this study was known to interfere with the LH measurement.

Method

After 10 days adaptation to eating the basal diet from feeding jars 20 female rats of similar weight and with regular 4 day oestrous cycles were fed 5g DFF diet daily for a period of 21 days. On day 20 the rats were allocated to one of four groups (n=5 each) so that the mean body weight of each group was approximately equal.

At 1600h on day 21 three groups of rats received a subcutaneous injection of .1ml saline containing 20IU PMS of proven biological activity. The remaining 5 rats served as the saline control group, receiving a subcutaneous injection of saline. All groups continued to receive 5g DFF diet daily until the day of autopsy. Groups of rats were sacrificed and autopsied at the following intervals after PMS administration:

Day 24	0900h	-	65hr group
	0900h	-	65hr saline control group
	1600h	-	72hr group
Day 25	0900h	-	89hr group.

Throughout the experiment body weight, food consumption and

vaginal smears were monitored daily. At autopsy organ weights were measured and the ovaries and oviducts were examined.

Results

Body and organ weights: The changes in body and organ weights are summarized in Table 32. The mean pituitary, ovarian and uterine weights, both in absolute terms and relative to body weight, of the PMS injected groups were significantly greater than those of the saline group. The mean ovarian weight of the group autopsied 65hr after PMS administration was significantly greater than those of the 72hr and 89hr groups.

Ovarian appearance: The mean numbers of corpora lutea and ovarian follicles counted on the surface of the ovaries are summarized in Table 33. Analysis of variance revealed no significant difference between any of the PMS injected groups and the saline control group with regard to the numbers of surface corpora lutea.

The saline control group, which had no follicles exceeding the size criterion, were excluded from the statistical analysis. No significant difference was found between the 3 PMS-treated groups with regard to number of follicles. The ovaries of the PMS-treated groups were polyfollicular but microscopic examination of the surface revealed no recently ruptured follicles in all except one ovary.

Examination of the oviducts at the ampullary-isthmus junction revealed the presence of ova in only one rat in the 65hr group. In this case ovulation was unilateral and 8 ova were present in the oviduct. There was no evidence of ovulation in the other PMS treated groups or the saline control.

Vaginal smear patterns: Vaginal smears taken just before PMS administration were of the dioestrous smear type in keeping with the anoestrus of at least one week's standing. The saline control group continued to

Table 32

EFFECTS OF 20IU PMS ON ORGAN WEIGHTS AFTER 21 DAYS FEEDING ON DFF DIET

Time elapsed since PMS injection (hours)	No. of rats	Mean initial BW±SD (g)	Mean final BW±SD (g)	BW Change (%)	Mean organ weight ±SD							
					Pituitary		Ovaries		Uterus		Adrenals	
					Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW
65	5	248.8±9.9	165.0±4.6	-33.7	10.5±1.0	6.4± .56	79.4±2.4	48.2±2.7	354.5±32.0	214.7±16.2	50.4±2.7	30.5±0.84
72	5	251.2±6.3	162.4±2.1	-35.4	10.3± .87	6.3± .51	73.3±3.4[c]	45.1±2.5	363.4±25.5	223.7±14.3	50.3±2.6	31.0±1.4
89	5	252.2±7.8	158.8±3.9	-37.0	9.9± .37	6.3± .24	71.7±1.6[d]	45.2±1.2	344.9±22.2	217.3±14.8	50.4±2.0	31.8±1.6
Saline control at 65 hrs	5	251.0±4.0	160.6±1.7	-36.0	7.9±1.0[a]	4.9± .60[b]	34.2±3.2[b]	21.3±1.8[b]	147.5±19.3[b]	91.8±11.3[b]	49.4±2.5	30.8±1.5

a significantly lighter than 65, 72hr ($p < .01$) and 89hr group ($p < .01$)
b significantly lighter than all other groups ($p < .01$)
c significantly lighter than 65hr group ($p < .05$)
d significantly lighter than 65hr group ($p < .01$)

display a dioestrous type smear pattern until autopsy at 65hrs.

Table 33

Effects of 20IU PMS on the numbers of surface corpora lutea and ovarian follicles

Time elapsed since PMS injection (hours)	No. of rats	Mean number ± SD	
		Corpora lutea exceeding .7mm in diameter	Ovarian follicles exceeding .5mm in diameter
65	5	11.2±1.6	17.2±3.9
72	5	9.4±1.1	16.8±2.6
89	5	10.8±1.9	18.2±1.9
Saline control at 65hr	5	9.4± .89	0

At 24hr after PMS injection: all groups were still showing evidence of a dioestrous pattern.

At 48hr after PMS injection: leucocytes were still predominant but there were an increased number of epithelial cells.

At 65-72hr after PMS injection: 13 out of 15 rats had smears containing a mixture of parabasal and superficial cells. Only 2 rats had smears reminiscent of an oestrous-type smear; sheets of cornified cells on a clean background. The one ovulating rat had a smear pattern of this type.

At 89hr after PMS injection: 3 out of 5 rats had oestrous-type smears, whereas 2 rats had smears containing approximately equal numbers of parabasal and cornified cells.

Discussion

Although the ovaries of PMS-treated groups showed evidence of

gonadotrophin stimulation the majority of PMS treated rats failed to ovulate. The significant increase in uterine weight indicates that gonadal steroids were secreted in response to gonadotrophin stimulation. Therefore the failure of the majority of rats to ovulate was due either to oestrogen production of insufficient quantity to exert a positive feedback effect or a failure on the part of the hypothalamus to respond to the positive feedback action of oestrogen.

Although not conclusive the evidence points to adequate secretion of oestrogen. Firstly, there were numerous large ovarian follicles of normal appearance. It is likely that such follicular proliferation was associated with gonadal steroid production. Secondly there was a marked increase in uterine weight, to the extent that the absolute weight was more than twice that of the saline control. Thirdly, an increase in pituitary weight, similar to that found following administration of 2µg OB was observed. These findings do not support the hypothesis of ovarian refractoriness in states of emaciation (Marshall & Frazer, 1971).

The present findings provide further support for the proposition that the hypothalamus is unresponsive to the stimulatory feedback effects of gonadal steroids during states of emaciation. It is presumed to be a functional disturbance since previous studies have revealed that rats readily resumed oestrous cycling when normal eating patterns were reestablished.

Experiment 10

The previous experiment demonstrated a failure by PMS to induce ovulation in emaciated, anoestrous rats. The aim of this study was to determine whether ovulation could be induced, by means of PMS administration, during nutritional rehabilitation but at a time when the rats

were still well below the body weight normally associated with spontaneous ovulation. For this purpose a group of rats fed a DFF diet for 21 days and then fed 18g basal diet daily thereafter (gradual rehabilitation) was employed. In an earlier experiment it was found that rats on gradual rehabilitation regimen first showed signs of ovulation approximately 12 days after the start of rehabilitation. In this experiment PMS was administered and rats were autopsied at first oestrus between the fifth and seventh day of gradual rehabilitation.

A second aim of this study was to determine whether provision of a high calorie diet, in quantities not sufficient to support significant weight gain, would effect the responsiveness of the HPO axis to PMS provocation. For this purpose low weight maintenance groups, receiving only 10g basal diet per day were employed. In an earlier experiment it was found that rats fed this quantity of food showed negligible weight gain and remained anoestrous for the period of feeding on this regimen.

It was reasoned that when using rats on "gradual rehabilitation" and "low weight maintenance" regimens one is presumably dealing with an endocrine system which is relatively dormant, in contrast to one which is beginning to resume the dynamic hormonal interplay of the fully rehabilitated animal. In this way it was hoped to test the responsiveness of the HPO axis to PMS provocation in the absence of the confounding influence of endogenous hormone levels.

Method

After 10 days of adaptation to eating the basal diet from feeding jars 15 female rats, of similar weight and with regular 4 day cycles, were fed 5g DFF diet daily for a period of 21 days. On day 20 the rats were allocated to one of three groups (n=5 each) so that the mean body weight of each group was approximately equal. From day 21 the

rats in Group 1 were given 18g of basal diet per day (gradual rehabilitation), while rats in Groups 2 and 3 were given 10g of basal diet per day (low weight maintenance). At various intervals after the start of the new feeding regimen each group received a subcutaneous injection of .1ml saline containing 20 IU PMS. In this study rats were autopsied at the first signs of oestrus. PMS was administered at 1600h at the following intervals after the start of the new dietary regimens on day 21 (day 1 of new regimens):

Group 1 (gradual rehabilitation): day 4

Group 2 (low weight maintenance): day 5

Group 3 (low weight maintenance): day 9

Throughout the experiment body weight, food consumption and vaginal smears were monitored daily. At autopsy organ weights were measured and the ovaries and oviducts were examined.

Results

Body and organ weights: The changes in body and organ weights are summarized in Table 34. The mean final body weight of the gradual rehabilitation group (1) was significantly greater than those of the low weight maintenance groups (2 and 3). The mean absolute pituitary weight of group 1 was significantly greater than those of groups 2 and 3. However when expressed in terms relative to body weight there were no significant differences between the three groups with regard to pituitary weight.

The mean absolute ovarian weight of group 1 was significantly greater than that of group 3. However, when ovarian weight was expressed in terms relative to body weight the findings were reversed. That is, the relative ovarian weight of groups 2 and 3 were significantly greater than that of group 1. There was no significant

Table 34
EFFECTS OF 20IU PMS ON ORGAN WEIGHTS AT VARIOUS INTERVALS DURING GRADUAL REHABILITATION AND LOW WEIGHT MAINTENANCE

Dietary regimen: day of PMS admin.	No. of rats	Mean initial BW±SD (g)	Mean final BW±SD (g)	BW Change (%)	Mean organ weight ±SD							
					Pituitary		Ovaries		Uterus		Adrenals	
					Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW
Group 1												
18g Basal diet: PMS on day 4	5	252.4±5.4	213.6±7.8[a]	-15.4	11.6± .60[a]	5.4± .31	82.3±3.8[b]	38.6±2.4[c]	442.4±62.4	208.1±35.9	51.6±2.3	24.2±1.5[d]
Group 2												
10g Basal diet: PMS on day 5	5	251.8±5.5	174.2±4.3	-30.8	10.3± .53	5.9± .32	76.8±5.9	44.0±2.6	395.0±46.5	226.5±23.0	53.3±2.7	30.6±1.4
Group 3												
10g Basal diet: PMS on day 9	5	252.2±5.4	174.6±4.7	-30.7	10.0± .33	5.7± .28	74.7±2.4	42.8±1.4	384.0±26.6	220.2±18.7	53.6±2.8	30.8±2.3

a significantly greater than groups 2 and 3 ($p < .01$)
b significantly greater than group 3 ($p < .05$)
c significantly lower than group 2 ($p < .01$) and group 3 ($p < .05$)
d significantly lower than groups 2 and 3 ($p < .01$)

difference between the 3 groups with regard to absolute adrenal gland weight. However, when expressed in terms relative to body weight the mean adrenal gland weight of group 1 was significantly lower than those of groups 2 and 3. It appears that little significance can be attached to this finding because of the greater body weight gain of group 1 since the end of the DFF diet period.

Ovarian appearance: The mean numbers of corpora lutea and ovarian follicles counted on the surface of the ovaries are shown in Table 35. Analysis of variance revealed no significant difference between the 3 groups with regard to the number of surface corpora lutea and surface follicles.

Vaginal smear patterns and evidence of ovulation: The main focus of interest in this experiment was on the occurrence of ovulation. The proportion of rats ovulating and the mean number of ova per ovulating rat is summarized in Table 35. A Chi square test revealed no significant difference between the 3 groups with regard to the frequency of ovulation ($\chi^2(2)=1.2$, $p > .05$). Analysis of variance revealed that the mean number of ova per ovulating rat of the gradual rehabilitation group was significantly greater than that of the low weight maintenance groups.

Group 1 (gradual rehabilitation): Two rats in this group had an oestrous-type smear pattern and showed evidence of bilateral ovulation 65 hrs after PMS administration. Another rat had an oestrous type smear at 65hrs but no ovulation was detected. Of the remaining rats one showed an oestrous type smear and bilateral ovulation at 41hrs and the other showed an oestrous type smear and bilateral ovulation 89hrs after PMS administration.

Group 2 (Low weight maintenance: PMS on day 5): All rats in this group had an oestrous smear pattern for the first time 65hrs after the

Table 35

Effects of 20 IU PMS on surface appearance of ovaries and ovulation at various intervals during gradual rehabilitation and low weight maintenance

			Ovaries		Oviducts	
			Mean number ±SD			
Dietary regimen: day of PMS admin	No. of rats	BW change (%)	Corpora lutea exceeding .7mm in diameter	Follicles exceeding .5mm in diameter	Proportion ovulating	Mean No. of ova per ovulating rat SD
Group 1: 18g basal diet:PMS on day 4	5	-15.4	12.4±1.3	2.2±2.6	4/5	10.3±2.6[a]
Group 2: 10g basal diet:PMS on day 5	5	-30.8	10.0±1.6	5.2±3.7	3/5	5.0±1.0
Group 3: 10g basal diet:PMS on day 9	5	-30.7	10.4±3.6	4.2±2.2	4/5	4.3±2.3

a significantly greater than groups 2 and 3 ($p < .05$)

PMS injection. However, only 3 of the 5 rats had ovulated.

Group 3 (Low weight maintenance: PMS on day 9): All rats in this group first had an oestrous type smear pattern 65hrs after PMS administration. Four of the 5 rats ovulated and in only one case was the ovulation bilateral.

Discussion

The present findings indicate that ovulation can be induced by PMS administration in rats whose body weight is well below that normally associated with spontaneous ovulation. The majority of rats in the gradual rehabilitation group ovulated in response to PMS injection

at a time when they were still, on average, 14.8% below their mean initial body weight.

Furthermore, the present results indicate that significant weight gain was not the important factor in re-establishing HPO responsiveness. The majority of rats on the low weight maintenance regimen ovulated in response to PMS provocation. The ovulating rats in group 2 were still, on average, 30.4% below their initial body weight which was close to their lowest weight on the DFF diet (-33.1%). The ovulating rats in group 3, which were autopsied 12 days after the start of the daily 10g basal diet regimen, were, on average, 31.2% below their initial body weight after having reached a low of -33.2% during the DFF diet period.

The incidence of ovulation in response to PMS injection was consistant with the reported findings of 80-100% ovulation in prepubertal rats (Longenecker & Gallo, 1971; Reiter et al., 1969). However, in all groups the mean number of ova per ovulating rat did not match the superovulation reported by Reiter et al. (1969). The significantly smaller number of ova per ovulating rat in the low weight maintenance groups suggests that either the smaller quantity of available food or the extended period of low weight interfered with the maturation of the ovarian follicles.

The finding of ovulation in the majority of low weight maintenance rats indicates that the ovulation in the gradual rehabilitation group was not a weight dependent phenomenon. Rather, it would appear that the introduction of limited quantities of a basal diet with a relatively high calorie component was sufficient to cause a change in the responsiveness of the HPO axis to PMS stimulation. Howland (1972) reported that rats on a 50% reduction of food intake with ad libitum access to glucose powder had plasma LH levels, body and organ weights that were similar to those of fully fed controls. This led Howland (1972) to

suggest that calorie intake per se was an important factor in determining ovarian function in the rat by altering the activity of higher levels of the HPO axis.

The present findings strongly suggest that caloric intake is an important factor in determining HPO axis functioning. Even when the high basal diet was provided in insufficient quantities to support significant weight gain, it appeared to be sufficient to cause the HPO axis to become responsive to PMS provocation; a phenomenon not observed while rats were fed a DFF diet. It is apparent, however, that the HPO axis does not become fully functional until weight loss is corrected since spontaneous ovulation is not observed until body weight is close to the initial body weight.

The part of the HPO axis that is sensitive to caloric deprivation remains unknown at present. Although the effects of caloric restriction are likely to be pervasive the results of Experiment 7 suggest that it is the hypothalamus rather than the pituitary that is critically impaired. This view is supported by the research of others (Campbell et al., 1977; Negro-Vilar et al., 1971; Piacsek & Meites, 1967).

A hypothesis concerning the mechanisms involved in the presumed hypothalamic dysfunction could be generated from the views of Ibrahim & Howland (1972). They observed that starvation causes a reduction in serum glucose concentration (Herrera & Freinkel, 1968) and a lowering of the blood concentration of insulin (Malaisse et al., 1967; Szepsi & Berdanier, 1971). It is possible, as Ibrahim & Howland (1972) suggested that the areas of the hypothalamus responsible for the synthesis and release of LHRH require insulin for glucose transport into cells, as has been demonstrated for the satiety centre (Debons et al., 1968). The availability of high energy food after an extended period of deprivation may have resulted in an increase in both glucose and insulin

levels and consequently resumption of cellular function in the areas of the hypothalamus critical for LHRH release. At present such an explanation of the mechanisms underlying the presumed hypothalamic dysfunction remains hypothetical.

Summary and conclusions

The results of the series of experiments reported on in this chapter have yielded fresh information about the relationship between caloric deprivation and disturbances of reproductive function in the female rat. In certain respects the results of this research served to confirm earlier research findings on the effects of undernutrition on reproductive function (Ershoff, 1952; Howland, 1971, 1972; Ibrahim & Howland, 1972; Piacsek & Meites, 1969). In other respects new evidence concerning the sensitivity of the reproductive system to changes in the caloric component of the diet has been presented. This was the first systematic investigation of the role of caloric deprivation in the induction of anoestrus and of nutritional rehabilitation in the same strain of rat.

The first significant finding was that the mechanisms involved in the regulation of the oestrous cycle of Long-Evans rats proved to be resistant to relatively severe caloric restriction. It was only after the complete removal of both the carbohydrate and fat components of the diet that cessation of oestrous cycling was observed in all animals. The extent of food restriction required to induce anoestrus was greater than predicted from previous research reports (Meites & Reed, 1949; Piacsek & Meites, 1967; Srebnik & Nelson, 1963).

After 21 days feeding on a DFF diet serum LH concentration and pituitary LH content and concentration were markedly reduced and target organs were small and atrophic. These changes, however, did not occur rapidly. It was only after 7 days that significant reductions were observed. These findings are not consistent with the observation by

Howland & Skinner (1973) that serum LH levels dropped significantly within 24hrs of food deprivation.

A second notable observation was that the nutritionally induced anoestrus was readily reversible. Rats subjected to severe caloric restriction for 21 days and no stress, other than that associated with food restriction, showed a return to normal ovulatory cycles within one week of the start of full nutritional rehabilitation. The resumption of normal cyclical activity during nutritional rehabilition was found to be dependent upon weight gain of a certain magnitude. It would appear that there was no lasting damage caused by the nature and duration of the malnutrition imposed.

The findings of the present research do not provide support for the suggestion (Russell, 1969) that the duration of malnutrition may contribute to the delay in recovery of reproductive function following nutritional rehabilitation. Rats which were kept emaciated for an additional 7 days and then rehabilitated at a reduced pace showed normal cyclical activity when their body weights were similar to the body weight associated with resumption of cycling during ad libitum rehabilitation.

The results of the pharmocological studies provide direct and indirect support for the view that reduced gonadotrophin secretion associated with inanition is due, not to a primary pituitary disturbance, but to impaired synthesis and/or release of LHRH from the hypothalamus. While pituitary LH content and concentration was shown to be reduced in emaciated rats the anterior pituitary gland was found to be capable of responding to LHRH stimulation by releasing LH. This response, however, was even greater when the pituitary had been exposed to prior stimulation by LHRH for 60 mins. Patients in the acute phase of anorexia nervosa have shown similar responses to prolonged LHRH administration (Aono et al., 1975; Nillius & Wide, 1977). The present study, however,

provided the first experimental support for the suggestion that, in states of inanition not accompanied by stress other than that associated with underfeeding, there is impaired synthesis and/or release of hypothalamic LHRH in female rats.

The OB provocation study produced further evidence suggesting a functional disturbance of the hypothalamus. Convincing evidence that the hypothalamus is the primary site at which oestrogen exerts its positive feedback effect has been presented (Goodman, 1978b). The failure of OB to induce ovulation or elevate serum LH levels is consistent with the view expressed by Wakeling et al. (1977) that the ability of the hypothalamus to respond to the positive feedback effects of oestrogen is impaired during states of inanition.

The administration of PMS to emaciated rats produced results consistent with ovarian responsiveness to gonadotrophin stimulation. The absence of ovulation in the majority of PMS treated rats was interpreted as a failure on the part of the hypothalamus to respond to the stimulatory effects of oestrogen and not as a result of inadequate oestrogen production. The overall pattern of findings was not consistent with ovarian refractoriness.

The administration of PMS to rats with limited intake of a high calorie diet, after a 21 day period of caloric restriction, produced unexpected findings. It would appear that the dysfunctional areas, presumably in the hypothalamus, are sensitive to the availability of high energy food. This is seemingly inconsistent with the earlier observation that the endocrine regulation mechanisms are resistant to acute deprivation of dietary calories. However, as caloric restriction became more chronic and bodily reserves were depleted the effects were pronounced, with reduced LH secretion and anoestrus. The re-introduction of a high calorie diet had the effect of increasing the

responsiveness of higher centres to PMS provocation in the absence of significant weight gain. Although the intake of a high caloric diet initially caused increased responsiveness to exogenous stimulation, the reproductive system did not become fully functional until the rats had attained a body weight capable of supporting viable gestation. This finding is consistent with the view that body weight is a crucial determinant of phasic control of gonadotrophin secretion in normal cyclical activity.

Chapter 5

THE EFFECTS OF PSYCHOLOGICAL FACTORS ON REPRODUCTION AND ENDOCRINE FUNCTION

In earlier chapters the notion that psychological factors may be implicated in disturbances of reproductive function was introduced. Clinical studies with humans seem to provide the most pressing evidence that emotional factors exert an influence on reproductive function under certain circumstances. The influence of environmental factors, such as light, temperature, sound etc., on the reproductive system of lower animals is well documented (Bohanan, 1939; Piacsek & Hautzinger, 1974; Zondek & Tamari, 1960). However, the effect of emotional factors on reproductive functioning in lower animals has not been investigated fully. In both the clinical and animal studies there is a notable lack of agreement about the patterns of gonadotrophin and gonadal steroid secretion that are associated with states of emotional arousal or disturbance.

The effects of psychic trauma or emotional stress on the menstrual cycle

The relationship between psychological disturbance and menstrual aberrations has been recognised for many years (Balint, 1937). The menstrual irregularities associated with emotional disturbance include oligomenorrhoea and secondary amenorrhoea. It is the latter condition which will receive the greatest attention in this review. In the review little attention will be devoted to the literature dealing with the investigation of the higher pathways and mechanisms by which psychological factors influence reproductive function in humans. This important work has been reviewed by Rakoff (1968) and Ihalainen (1975).

Klinefelter et al. (1943) were the first to coin the term "hypothalamic amenorrhoea" to refer to those instances when the patient had

no organic disorder but refractory amenorrhoea, presumably of psychogenic origin. These investigators reported finding normal gonadotrophin levels together with evidence of oestrogen deficiency. Klinefelter et al. (1943) suggested that psychological factors exert their effect by blocking the normal release of LH resulting in an inadequate response by the ovaries to FSH. They hypothesized that the failure of LH release was the result of psychological factors acting at the level of the hypothalamus.

Rakoff (1968) presented 6 categories into which clinical observations linking psychogenic factors and disorders of menstruation could be grouped. Russell (1972a), however, proposed that the main sources of clinical observation could be classified into "studies in time of war" and "studies in time of peace".

Studies in time of war: The investigation of the war amenorrhoeas marks the start of scientific interest in the role of emotional factors in secondary amenorrhoea (Ihalainen, 1975). The studies of war amenorrhoea do not provide direct evidence of psychogenic causation since the stress of internment or siege seldom occurred in the absence of nutritional deprivation and it was seldom possible to assess the subject's mental or endocrine status except for simple clinical observations (Russell, 1972b). Nevertheless, clinical observations do suggest that psychological factors were prominent in a significant number of cases. Mazer & Israel (1959) reported that 50% of women in concentration camps developed amenorrhoea which persisted throughout their detention. Although undernutrition was undoubtedly an important factor the amenorrhoea was present in a large number of women long before undernourishment became evident (deNeef, 1965). The study of Sydenham (1946) of internees revealed that menstruation ceased in 60% of women before any weight loss was apparent. Her study does suggest, however, that both

nutritional and psychological factors were operating; with the psychological component presumably playing an important role in the onset of the amenorrhoea and nutritional factors in perpetuating the menstrual disorder. Bass (1947) reported that the threat of extermination of concentration camp victims was associated with a much higher incidence of amenorrhoea than was found amongst concentration camp internees who had less reason to fear for their lives.

Other studies suggesting a psychogenic causation in some cases of war amenorrhoea are those of women exposed to bombing raids (Loeser, 1943; Whiteacre & Barrera, 1944). Loeser (1943) described a study of 4 women who suddenly developed amenorrhoea following exposure to bombing raids. Histological dating of endometrial arrest by an independent histopathologist corresponded with the very day of the cycle on which each patient had been exposed to shock.

Studies in time of peace: Clinical observations suggesting a relationship between psychological factors and disorders of menstruation include aberrations occurring immediately after "psychic trauma", the high incidence of menstrual dysfunction in young girls with long standing emotional problems, as well as in neurotic and psychotic women (Gregory, 1957; Rakoff, 1968; Russell, 1972 b)

A number of reports suggest that relatively minor emotional upheavals such as leaving home and going to strange environments can cause amenorrhoea (Drillien, 1946; Drew & Stiftel, 1968; McCormick, 1975; Winter, 1946). Jeffcoate (1965) claimed that psychogenic factors are the most common causes of secondary amenorrhoea and that amenorrhoea occurred in 50% of young girls living an institutional life.

While many clinicians argue that psychogenic amenorrhoeas are the most common variety the pathogenesis of this disorder is not fully understood. It is not known, for example, why some women develop

amenorrhoea and others not, when placed in the same situation or exposed to the same upheaval. Presumably these differences are due to the fact that some women are more psychologically predisposed to being upset or have a menstrual cycle that is more readily disrupted by emotional arousal.

The absence of consistent hormonal patterns associated with the psychogenic amenorrhoeas has not contributed to an understanding of this disorder. Russell (1972b) observed that "there have been no detailed and reliable studies of the urinary output of gonadotrophins in amenorrhoea of emotional origin" (p.12). In a review of the literature Rakoff (1968) observed that three patterns of urinary gonadotrophin excretion had been reported in women with psychogenic amenorrhoea; decreased, normal and increased titres of urinary gonadotrophin. Rakoff and his coworkers had found all three patterns of urinary gonadotrophins in their samples of amenorrhoeic women (Rakoff, 1968).

The manner in which psychological factors exert an effect on reproductive function by altering gonadotrophin secretion is still open to speculation. The suggestion by Klinefelter et al. (1943) that psychological stimuli, acting at the level of the hypothalamus, block the release of LH remains an appealing but unsubstantiated hypothesis. Others have invoked Selye's "shift theory". Igarashi et al. (1965), for example, suggested that emotional stress induced hypersecretion of corticotrophin releasing factor at the expense of secretion of FSH and LH-releasing factors at the hypothalamic level, resulting in hyposecretion of FSH and LH at the pituitary level. They proposed that the amenorrhoea is the result of reduced secretion of gonadotrophins from the pituitary and suggested that their results provided supportive evidence for Selye's "shift theory"; that is, that stress increased ACTH secretion from the pituitary at the expense of gonadotrophin secretion (Selye,

1946, 1950). However, the finding of normal or increased gonadotrophin excretion in some women with psychogenic amenorrhoea and increased LH secretion in stressed rats (Morishige & Rothchild, 1974) does not provide support for this theory.

Anatomical considerations have led most investigators to propose the hypothalamus as the site at which psychological factors exert a disruptive effect on the reproductive cycle. As Bajusz (1967) observed, the hypothalamus is a nodal point in a vast neural pathway extending from the medial wall of the cerebral hemisphere to the lower boundary of the mesencephalon. It seems logical, he proposed, that the functional state of the hypothalamus is influenced by higher brain circuits and especially by the prevailing activity in the limbic-forebrain-midbrain complex (Bajusz, 1967). The mechanism by which stimuli emanating from higher centres alter gonadotrophin secretion is yet to be elucidated.

Although the gonadotrophin secretion patterns associated with stress are not well established it is clear that stress is not only equated with the release of ACTH but also with alterations in secretion of growth hormone, prolactin, and LH (Donovan, 1974). Some researchers, however, have reported that serum gonadotrophin levels were unaffected by medical stress and surgery. Geuvara (1970) reported that there were no changes in FSH and LH levels during myocardial infarction. Charters et al. (1969) found no statistically significant changes in serum FSH and LH during the course of various types of surgery.

The effect of stress on the reproductive physiology of the female rat

The concept of stress: is an ambiguous one and consequently the literature is fraught with problems of interpretation. On the one hand the term "stress" is used to refer to the complex endocrine and

morphological changes described by Selye (1946, 1950). On the other hand the term is used to refer to a powerful stimulus (Lazarus, 1966). Thus the concept of stress is an ambiguous one since it can refer both to the physiological response and to a property of the stimulus situation (Kollar, 1961). Nevertheless in psychophysiological terms a stressful procedure is usually taken to mean one in which there is both unpleasant emotion and physiological change (Malmo et al., 1948).

It has been argued that a procedure is stressful only if the organism is emotionally aroused. Bush (1960), for example, cited research which demonstrated that severe exercise, cold and fasting produced little or no effect on the secretion and metabolism of cortisol in man unless they were a part of a situation that provoked emotion. Others have also opposed Selye's claim that the organism responds in a non-specific manner to many different stimuli as part of the "general adaptation syndrome". Mason (1968) proposed that the so called general adaptive endocrine response to many different noxious stimuli is not a non-specific response, but rather a specific response to a single type of stimulus (psychological) which the various unpleasant situations have in common.

The nature of stressors: Comparisons of the results of human and animal studies are complicated because of the differences in the nature of the stressful situations. In the case of the human studies the emotional upheaval and associated endocrine changes are rarely a response to physical stressors. In animal studies, bowever, stress is most frequently induced by employing a physical stressor. The problem here is that physical stressors may indeed induce unpleasant emotion but they may also be exerting a direct effect on endocrine activity. It is not known, for example, if the depressed LH levels found after ether anaesthesia (Naftolin, Brown-Grant & Corker, 1972; Querido, 1975) is the

result of the direct effects of the anaesthetic or the stress associated with its administration. As Weiss (1972) observed:

> when experimental animals are exposed to an environmental stressor (stress inducing agent) the effects of psychological variables may be confounded with the effects of the physical stressors (p.105)

In the section that follows the term "stress" will be used to refer to the state that is induced by a specified stressor. It has already been observed that caution is needed when comparing and interpreting the results of these studies.

The effects of experimentally induced stress on reproductive physiology:

There is considerable evidence to suggest that stress can increase prolactin release in the rat (Ajika et al., 1972; Neill, 1970), except on the afternoon of pro-oestrus (Morishige & Rothchild, 1974; Riegle & Meites, 1976).

The reports on the effects of stress on LH secretion are far less consistent. Indeed, it is a hazardous task to attempt to describe a typical LH response pattern to stress. It would appear that the effects of stress on LH secretion in the intact female rat depend on (a) the time of the oestrous cycle when the stress is administered (b) the nature of the stress (c) and the intensity of the stress.

Neill (1970) found that serum LH levels were not affected by laparotomy and blood sampling under ether anaesthesia in intact female rats on any day of the oestrous cycle, including the afternoon of pro-oestrus. Similarly Wuttke & Meites (1970) found that the high levels of serum LH on the afternoon of pro-oestrus were not affected by ether vapour stress. While Morishige & Rothchild (1974) found that ether-laparotomy had no effect on LH or FSH levels during the late afternoon of pro-oestrus they reported that the same procedure resulted in increased LH levels on dioestrus-1. However, etherization three times at 5

minute intervals caused the LH levels to rise significantly on the late afternoon of pro-oestrus (Morishige & Rothchild, 1974). In contrast, surgical stress on the morning of pro-oestrus resulted in the advancement of the surge of plasma LH by several hours (Lawton, 1972).

To add to the confusion Euker, Meites & Riegle (1973) reported that the low serum LH values in dioestrous and oestrous female rats were not affected by 2 hour restraint stress. However, the same procedure caused a sharp reduction in the high initial levels of LH in pro-oestrous females. They found that 2hrs of restraint stress administered between 1530 and 1730h blocked the pro-oestrous LH surge for the duration of the sampling period (up to 4hrs after stress administration). Further evidence that the mid-cycle LH surge may be blocked by stress has been presented by McKay et al. (1975). Using several stress regimens, including restraint stress, they found that stress interfered with the LH surge on the afternoon of pro-oestrus (a) by reducing circulating LH to basal levels or (b) by maintaining basal LH levels. In most cases inhibition of the LH surge was accompanied by inhibition of ovulation the following day. Ovulation did occur, however, in some cases where restraint stress had apparently inhibited the LH surge. Riegle & Meites (1976) reported that 20 consecutive days of 2hr restraint stress blocked the regular ovarian cycle.

The results of studies with ovariectomized (OVX) females and intact male rats are as inconsistent as those in which intact females were used. Ajika et al. (1972), for example, reported that LH levels were elevated in OVX rats within 2 mins. of the administration of ether and bleeding. In contrast Seyler & Reichlin (1973) reported that the high LH levels in OVX rats were not affected by etherization and bleeding.

The results of studies, in which various forms of stress were administered to intact male rats, range from findings of no alteration

of LH levels (Amatayakul, 1971), to decreased LH levels (Howland, Beaton & Jack, 1974), to elevation of LH levels (Dunn et al., 1972; Euker et al., 1975; Seyler & Reichlin, 1973).

In conclusion, it is apparent that there is a lack of consensus regarding the patterns of LH response to stress. At present there is insufficient information available to account for the diversity of observed responses.

Research objectives

The aim of the research to be reported in this chapter was to investigate the effects of conditioned emotional response procedures and unpredictable shock administration on serum LH levels and other aspects of reproductive function. The specific research objectives are presented in the introduction to each experiment.

Comment

Before describing this research a brief comment about an alternative form of stress induction is warranted. In a series of preliminary studies the effects of immobilization in the restraining device (described in Chapter 3) were investigated. It was found that immobilizing groups of rats for 2hrs at overlapping intervals between 0900-1700h on pro-oestrus did not result in blocking of the pro-oestrous LH surge and did not inhibit ovulation the following day. These findings are not consistent with those of Euker et al. (1973) and McKay et al. (1975). In another study it was found that daily 2hr immobilization over a period of 10 days failed to induce anoestrus. This finding is inconsistent with those of Riegle & Meites (1976) who reported that chronic restraint was sufficient to block regular ovarian cyclicity.

The failure to replicate the earlier findings could be related to species differences. It is possible that the Long Evans strain used in

the present research was more robust and less sensitive to restraint than the rats used by other researchers. An alternative explanation is that the method of restraint used by other workers was experienced by the rats as more stressful. For example, the procedure of restraining rats in the supine position by strapping their links onto a board (Riegle & Meites, 1976) may induce greater stress than that induced by the restraining cage used in the present research.

In conclusion this method of stress induction was abandoned in favour of conditioned emotional response procedures and unpredictable shock administration.

Experimental section

The effects of conditioned emotional response procedures and unpredictable shock on reproductive function

Although the procedures used in the present study are best described as Pavlovian or classical conditioning and extinction procedures it was Estes & Skinner (1941) who were the first to perform a quantitative study of conditioned anxiety or conditioned emotional response (CER). The CER is acquired by pairing a painful electric shock (unconditioned stimulus) with a previously neutral stimulus (conditioned stimulus). During conditioning trials the conditioned stimulus (CS) is presented before the unconditioned stimulus (US) which is administered concomitantly with the termination of the CS.

A widely used method of obtaining a measure of conditioned emotionality involves recording the amount of suppression of water licking induced by the presentation of the CS (Leaf & Muller, 1965; Nageishi & Imada, 1974; Shipley, 1974). Initially this method appeared to have the advantage of being both simple, without loss of the same quantitative precision as operant behaviour, and one in which food intake would

not be altered. However, the results of a pilot study, in which rats were placed on a 23hr50min water deprivation schedule, revealed that the lick suppression procedure could not be used without causing considerable weight loss. These findings are consistent with those of Verplanck & Hayes (1953) who demonstrated that food and water intake of rats is interdependent and that deprivation of one results in self-imposed deprivation of the other.

This observation led to the decision not to employ lick suppression procedures with rats from which blood samples were to be collected and which were to be autopsied for measurement of organ weights. It was reasoned that reduced food intake and subsequent weight loss might alone, or in interaction with the conditioned anxiety, alter serum LH levels in ways which could not be controlled.

Instead of excluding a lick suppression measure it was decided to conduct a control study in which rats would be exposed to the conditioning and extinction procedures and measures of conditioned emotionality would be obtained. However, because of their inferior nutritional states measures of serum LH and organ weights were not recorded. It was reasoned that the results of the control study would provide an indication of whether fully fed rats respond emotionally to reinforced, and subsequently to non-reinforced CS exposures.

Experiment 1

The aim of this control study was to determine whether exposure to a conditioned stimulus during fear conditioning and extinction trials would result in suppression of licking consistent with conditioned fear. No measures apart from lick responses were recorded.

Method

Subjects: Twenty five female rats were individually housed and fed pelleted food and water ad libitum. All rats were handled and gentled daily for 5 mins at times of weighing and taking vaginal smears. After 14 days 20 rats with regular 4 day oestrous cycles were allocated to one of two groups (n=10 each) so that the mean body weight of each group was approximately equal.

Procedure: The procedure was divided into 4 phases: lick training; conditioning period; post-conditioning period; extinction period. The fear conditioning schedule described by Nageishi & Imada (1974) was employed in this study.

Lick training Both the experimental and control groups were individually housed and fed pelleted food ad libitum. A water deprivation schedule of 23hr50min was imposed on day 1 and maintained throughout the study. On day 2 of the lick-training period, and each day thereafter, rats in both groups were given 10 min. access to water in the operant chamber. The following lick responses were recorded: latency of first lick (secs); time to reach 100 licks; total number of licks during each 1 min. interval; total number of licks during the 10 min. interval.

It was found that the drinking curve had reached its apparent maximum level by the fourth day of lick training. After day 4 the lick rate remained at a relatively constant level for the following 10 days. Consequently lick training was stopped after 15 days.

Conditioning phase For 15 days immediately following the preliminary training of drinking experimental group rats were subjected to daily fear conditioning trials of 5 min. duration. The rats were transferred from their home cages to the conditioning cage (restraining device) in an adjacent room. During the 5 mins. in the conditioning cage the rat

received 3 shocks, each of which was signalled by the prior presentation of the CS (increased illumination) for 5 sec. Thus, over the 15 day period, each rat received a total of 45 paired CSs and USs. Shock intensity was increased at various intervals during the conditioning phase. Following each conditioning trial the rats were placed in the operant chamber and allowed a 3 min recovery period. The drinking tube was then inserted and the rats were allowed to drink for 10 mins.

The control group rats were treated the same way except they did not receive any shocks. On days 1, 5, 10 and 15 of the conditioning period 3 CS test probes for lick suppression were run at random intervals while the rats were in the operant chamber. That is, during the 10 min. drinking period rats from both the experimental and control groups were subjected to three separate 5 sec presentations of the CS. A criterion of 100 licks was required before the first CS presentation. The lick rate average for the three 5 sec segments before CS presentation was recorded as well as the lick rate during the 5 sec of CS presentation.

A suppression ratio was calculated for each presentation using the formula described by Shipley (1974):

$\frac{B}{A + B}$ where B was the number of licks during a 5 sec CS segment and A was the average number of licks during the three 5 sec pre-CS segments. With this measure a ratio of .500 indicates no difference in lick response rate between the pre-CS and CS periods. A ratio of .333 reflects a response rate during the CS which is half the pre-CS rate and a ratio of .000 indicates the subject failed to respond at all during the CS presentation.

Post-conditioning phase Following the end of the conditioning period both groups of rats were maintained on ad libitum access to food and a 23hr50min water deprivation schedule for 5 days. All rats were

allowed daily access to water for 10 min. in the operant chamber, without exposure to the CS or US.

Extinction trial phase Rats from both groups were placed in the conditioning cage for 5 min without US or CS presentation. After a 3 min recovery period in the operant chamber they were allowed 10 min. access to water during which time the 5 sec CS was randomly presented 3 times. As before, a criterion of 100 licks was required before the first CS presentation. The lick rate average for the three 5 sec segments prior to the CS presentation was recorded as was the lick rate during the 5 sec of the CS presentation. A suppression ratio was calculated for each CS test probe.

Results

Lick suppression ratios: The mean suppression ratio, during CS presentation, of the experimental and control groups is shown in Figure 7. Analysis of variance revealed that the mean suppression ratios of the experimental group were significantly lower than those of the control group on days 5, 10, 15 and 21 ($p < .01$).

Discussion

The present findings indicate that after 5 days of conditioning the presentation of the CS resulted in a significant suppression of licking during test probes. Suppression of licking was also apparent at days 10 and 15 of the conditioning period and during extinction trials on day 21. These findings are consistent with conditioned fear (anxiety).

It was reasoned that after 5 days of fear conditioning the presentation of the CS would elicit the fear response in animals subjected to the same conditioning and extinction procedures but not to lick

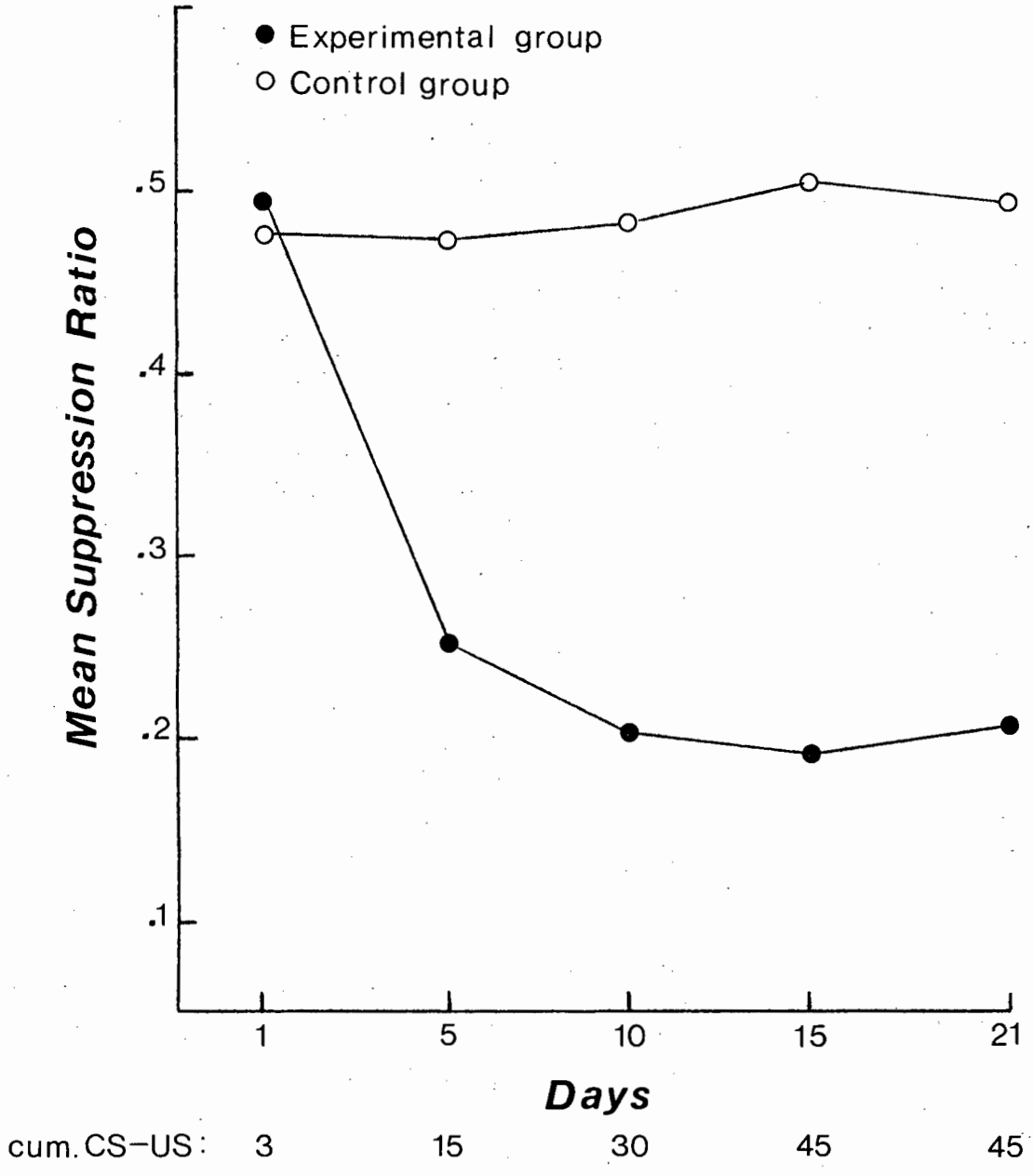

Figure 7 Mean suppression ratio for experimental and control groups during CS presentation: days 1-15 conditioning period; day 21 extinction trial period.

training and lick suppression testing.

The baseline lick rate (lick rate between CS presentations) of the experimental group was similar to that of the control group. Indeed, baseline lick rates of both groups reflect almost constant licking from the drinking tube. The finding of normal baseline licking in the experimental group is consistent with Seligman's safety-signal hypothesis (Seligman, 1968; Seligman et al., 1971) since all shocks were

predicted by the CS and the absence of the CS presumably predicted safety.

Experiment 2

No research on the effects of fear conditioning or the presentation of the CS after conditioning on gonadotrophin secretion or other aspects of reproductive function has been reported in the literature.

A number of researchers have provided experimental evidence that unpredictable shocks are more successful in inducing chronic fear (Seligman, 1969) as well as greater somatic stress reactions and more stress-induced pathology (Weiss, 1970, 1972) than predictable shock. According to Seligman's safety-signal hypothesis:

> When shock is reliably predicted by a CS, the absence of CS reliably predicts safety. When CS and shock are randomly interspersed, however, there can be no signal which predicts absence of shock. In the absence of such a safety signal, the rat is in chronic fear...
>
> (Seligman, 1969, p.487)

In the present study predictable shock was used specifically since it has been reported that the CER is an increasing function of the probability of CS-Shock pairings (Brimer & Dockrill, 1966; Nageishi & Imada, 1974). It was the intention to establish the association between the CS and the shock so that, during extinction trials, the CS would elicit the fear response.

The aim of the present study was to investigate certain aspects of reproductive physiology at the end of a conditioning phase in which predictable shock was administered, at the end of a recovery period, and after CS presentation during an extinction trial . The procedures were duplicated in order to obtain measurements from both dioestrous and pro-oestrous rats. One of the aims of this study was to determine whether experimental treatment would result in a blocking or reduction of the LH surge on pro-oestrus.

Method

Subjects: Fifty female rats were individually housed and fed pelleted food and water ad libitum. All rats were handled and gentled daily at the time of weighing and the taking of smears. After 14 days 40 rats with regular 4 day oestrous cycles were allocated to one of eight groups (n=5 each) so that the mean body weight of each group was approximately equal.

Procedure: In this study the rats were subjected to the same conditioning procedures as the rats in Experiment 1. However, in keeping with the rationale outlined earlier, lick suppression procedures were not employed. The fear conditioning schedule described by Nageishi & Imada (1974) was employed. The various groups used in this study are identified in Table 36.

Conditioning phase All rats, except those in the non-conditioned control group (group 3), were subjected to daily conditioning trials of 5 min. duration during which time they received 3 shocks, on a variable schedule, each shock being immediately preceded by the CS. At various intervals during the course of the conditioning phase shock intensity was increased in order to avoid habituation. The daily conditioning trials were continued for 15 days, at the end of which each rat had received 45 CS-US pairings. During this phase physical stressors were being employed and the effects of these on the oestrous cycle were monitored by examination of vaginal smear patterns. Furthermore three groups were sacrificed and autopsied at the end of the 15 day conditioning phase. Group 1 was sacrificed at 1500h on dioestrus, immediately after being subjected to a conditioning trial. Group 2 was sacrificed at 1900h on pro-oestrus after being subjected to a conditioning trial at 1500h earlier that day. Group 3 was subjected only to 5 min.

Table 36

Experimental design: Experiment 2, Chapter 5

15 day conditioning phase: Daily US-CS pairings during 5min trials				5 day post conditioning phase: No conditioning				Extinction trial phase: Presentation of non reinforced CS			
Group	Day of cycle	Treatment on day of autopsy	Autopsy at	Group	Day of cycle	Treatment on day of autopsy	Autopsy at	Group	Day of cycle	Treatment on day of autopsy	Autopsy at
1 (n=5)	Di	Conditioning trial at 1500h	1500h	4 (n=5)	Di	Undisturbed	1500h	6 (n=5)	Di	Extinction trial at 1500h	1505h
2 (n=5)	Pro	Conditioning trial at 1500h	1900h	5 (n=5)	Pro	Undisturbed	1900h	7 (n=5)	Pro	Extinction trial at 1500h	1900h
3 (n=5)	Di	No conditioning 5min in restraining cage at 1500h	1505h					8 (n=5)	Di	5 min in restraining cage at 1500h	1505h

immobilization in the restraining device used in the conditioning trials. After approximately 15 days of such treatment this group was sacrificed at 1500h on dioestrus to provide non-conditioned control values.

Groups 1 and 2 provided data which could be compared with those of animals not exposed to physical stressors, either at the end of the recovery period or following CS presentation during the extinction phase.

Post conditioning recovery phase Instead of proceeding immediately with extinction trials the remaining animals were allowed a 5 day period in which to recover from any physical effects which might have been induced during the conditioning phase.

On or about the fifth day after the end of the conditioning phase group 4 rats were sacrificed at 1500h on dioestrus and group 5 rats were sacrificed at 1900h on pro-oestrus. These groups were included to provide data which might indicate changes since the end of the conditioning phase and which could be compared with groups exposed to the non-reinforced CS presentation during extinction trials.

Extinction trial phase On the appropriate day of the cycle (dioestrus or pro-oestrus) two groups of rats received three 5 sec exposures to the CS, in the absence of shock, during a 5 min. extinction trial at 1500h. Group 6 was sacrificed immediately after the extinction trial on dioestrus and group 7 was sacrificed 4hrs later, at 1900h on pro-oestrus.

On the basis of the findings of Experiment 1 it was presumed that the presentation of non-reinforced CSs would elicit fear responses. It is well established that conditioned fear, like other classically conditioned responses, is reduced by the repeated or extended presentation of the CS in the absence of the US (Kalish, 1954). However,

considering the small number of non-reinforced CS exposures it is unlikely that there was a significant reduction in conditioned fear.

A final group of rats (group 8), which had been exposed to the same 15 days conditioning and allowed 5 days of recovery, were placed in the restraining cage for 5 min. at 1500h on dioestrus. Neither the CS nor the US was presented. This group was included to determine whether the restraining device acted as an extraneous CS. That is, whether the device alone elicited fear, and hence altered serum LH levels, through its association with US presentations. Wolpe (1952) reported that animals subjected to noxious stimulation responded later with marked anxiety to the environment in which the aversive stimulus was experienced.

During all phases the rats were sacrificed by cervical dislocation, trunk blood samples were collected for serum LH assay and organ weights were measured.

Results

Vaginal smears: Aberrations of oestrous cycling were observed in about one half of the rats during the conditioning phase. The atypical cycles were in the form of extended cycles with additional days of dioestrous-type smears or vaginal cornification. In some rats the cycles were extended over 8 days. Anoestrus was not observed in any of the rats. During the post-conditioning recovery period persistent atypical smear patterns were observed in about one half of the surviving rats.

Serum LH levels: Serum LH values are summarized in Table 37. The serum LH data of rats sacrificed on dioestrus were statistically analysed separately from those obtained from pro-oestrous rats.

Analysis of variance revealed no significant difference between any of the dioestrous groups with regard to serum LH levels ($p > .05$).

Table 37

Effects of fear conditioning and presentation of non-reinforced CS on serum LH levels of dioestrous and pro-oestrous rats

Group[a]	Mean serum LH concentration (ng/ml)±SD
Dioestrous groups	
1	30.0± 11.6
3	22.8± 13.1
4	22.8± 17.2
6	25.0± 14.0
8	28.4± 7.3
Pro-oestrous groups	
2	675.8±136.6
5	784.4±110.4
7	845.8±155.8

a all groups, n=5

Similarly there was no significant difference between the three groups of pro-oestrous rats with regard to serum LH concentration at 1900h ($p > .05$).

Normal control groups were not included in this experiment since this would have necessitated including a control for each of the 8 groups. However, a further analysis of the serum LH data was done including normal control values from Supplementary Study No. 8. This revealed that the dioestrous and pro-oestrous serum LH values, of the groups used in this study, did not differ significantly from normal values ($p > .05$).

Body and organ weights: The body weight and organ weights are summarized in Table 38. None of the groups differed significantly with regard

Table 38

BODY AND ORGAN WEIGHTS AT VARIOUS INTERVALS DURING THE CONDITIONING, RECOVERY AND EXTINCTION TRIAL PHASES

Group	No. of rats	Mean initial BW±SD (g)	Mean final BW±SD (g)	Day of cycle at autopsy	Mean organ weight ±SD							
					Pituitary		Ovaries		Uterus		Adrenals	
					Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW
Conditioning Phase												
1	5	256.6±4.9	272.6±5.7	Di	13.3± .76	5.0± .10	79.4±5.9	29.1±2.0	432.5±31.6	158.7±11.3	60.6±1.3	22.2± .74
2	5	255.4±5.1	273.0±3.4	Pro	13.7± .46	5.0± .15	83.2±3.7	30.5±1.1	590.1±25.7[a]	216.2± 8.4[a]	59.9±1.9	21.9± .72
3	5	255.2±5.1	275.2±7.2	Di	14.2± .64	5.2± .13	84.2±5.1	30.6±1.9	435.6±41.1	158.5±16.7	58.7±1.8	21.4± .69
Post-Cond. Phase												
4	5	254.6±5.0	276.4±4.9	Di	14.9± .87	5.4± .25	83.9±5.6	30.3±1.5	436.3±45.9	156.3±16.9	60.5±2.5	21.9±1.1
5	5	254.8±4.9	276.8±4.2	Pro	13.9± .81	5.0± .28	81.6±5.2	29.5±1.8	572.3±40.3[a]	206.8±15.5[a]	61.9±1.2	22.4± .10
Extinction Phase												
6	5	254.0±5.3	278.2±3.6	Di	14.0±1.1	5.0± .35	85.6±5.7	30.8±1.7	410.8±29.2	147.6± 9.4	62.0±2.5	22.3± .85
7	5	254.6±4.4	276.0±5.0	Pro	13.6± .58	4.9± .15	82.8±7.3	29.9±2.2	575.9±51.3[a]	208.7±18.7[a]	60.6±2.8	22.0±1.1
8	5	255.6±5.3	275.8±5.5	Di	13.7 .58	5.0± .18	81.1±8.9	29.4±2.9	423.1±50.3	153.2±15.4	60.6±2.5	22.0± .81

a significantly greater than all oestrous groups

to body weight at the time of autopsy. Analyses of variance revealed no significant difference between any of the groups with regard to pituitary, ovarian and adrenal gland weights, expressed either in absolute terms or relative to body weight. Not unexpectedly, all groups autopsied on pro-oestrus had mean uterine weights that were significantly greater than those of dioestrous rats in both absolute and relative body weight terms. The greater uterine weight of pro-oestrous rats was consistent with the normal serum LH values of the pro-oestrous groups.

Discussion

Since similarly treated rats showed evidence of lick suppression (Experiment 1, Chapter 5) it was presumed that presentation of the US and the CS, after a period of conditioning, would elicit a fear response in the rats in the present study.

The results indicate that daily conditioning trials exerted an effect on the oestrous cycle of half of the rats by causing extended cycles. However, none of the rats became anoestrous. Furthermore the conditioning trials had no effect on basal serum LH levels on dioestrus and the CS-US pairing did not block the LH surge when presented at 1500h on pro-oestrus. Similarly the presentation of the non-reinforced CS during extinction trials had no significant effect on serum LH levels on dioestrus and pro-oestrus.

With the finding of normal serum LH levels it was not surprising to find that pituitary, ovarian and uterine weights did not differ from normal values. However, the finding of no significant increase in adrenal gland weight following 15 consecutive days of conditioning trials was unexpected. It would appear that the stress induced by this treatment was not sufficient to cause adrenal hypertrophy.

It is likely that a greater number of CS-US administrations per day

over a longer period of time would have eventually produced alterations in gonadotrophin secretion patterns and consequently target organ weights. However, it was neither technically feasible nor personally desirable to subject rats to the extreme conditions known to cause somatic stress reactions in rats. In order to induce gastric ulceration Weiss (1970) subjected rats to a variable shock schedule over a period of 19hrs.

In conclusion, the administration of 3 shocks per day for 15 days or the presentation of a non-reinforced CS to fully fed rats failed to alter serum LH levels or induce anoestrus.

Experiment 3

Since daily exposure to 3 predictable shocks for 15 days failed to induce anoestrus an alternative procedure was employed in the present study. It has been reported that giving unsignalled shocks had a greater suppressive effect upon the basal rate of licking than signalled shocks (Imada & Soga, 1971; Miyashita, 1971; Nageishi & Imada, 1974). That is, the suppression of lick rate in the absence of the CS suggests that the rats are in a state of more "chronic fear" during the period of the conditioning trial (Seligman, 1969).

The aim of the present study was to determine whether the daily administration of 3 unpredictable shocks would be more successful in disrupting the oestrous cycle than signalled shocks.

Method

The subjects in this experiment were 10 female rats with regular 4 day cycles. They were individually housed and fed food and water ad libitum.

All rats were subjected to daily 5 min. periods in the restraining

cage, during which 3 unpredictable shocks were randomly presented. Daily shock administration was continued for 21 consecutive days. As in the previous experiment shock intensity was increased gradually at various intervals. Vaginal smears were examined daily during the shock administration phase and for 14 days thereafter.

Results

Vaginal smear patterns: A criterion of 5 or more consecutive days of a leucocytic smear pattern was used to define anoestrus. The first day of the constant dioestrous smear pattern sequence was designated the first day of anoestrus.

All rats showed some aberration of oestrous cycling in the form of extended cycles. Two rats became anoestrus, both on the ninth day of the shock administration phase. Anoestrus persisted in these rats throughout the remaining period of the shock administration phase. The anoestrous rats showed signs of renewed cycling, 5 and 6 days respectively, after the end of the conditioning phase. At the end of the 14 day recovery period 9 out of 10 rats were showing normal 4 day oestrous cycles.

Discussion

The findings of this study indicate that unpredictable shock resulted in more widespread disturbance of oestrous cycling than that observed following administration of predictable shock (Exp. 2, Chap 5). Furthermore anoestrus was induced in two of the rats and persisted for as long as the unpredictable shock was administered. Recovery of cyclical activity followed soon after shock administration was stopped.

These results suggest that unpredictable shock, administered during daily trials of 5 mins. duration, exerted a more marked effect on the

on the oestrous cycle than predictable shock. However, it was not successful in inducing anoestrus in the majority of rats. Nevertheless, the unpredictable shock schedule appeared to be the most potent of the stress regimens imposed so far in this research.

Concluding discussion

The results of the lick suppression study indicate that the presentation of the CS, after a period of conditioning, elicited a fear response. However, the degree of emotionality aroused during conditioning (CS-US pairings) and extinction phases was not sufficient to exert a demonstrable effect on serum LH levels and organ weights. Indeed, the daily presentation of 3 signalled shocks for 15 days appeared not to be stressful enough to cause an increase in adrenal gland weight.

When viewing the different conditioning and shock schedules, used in this section, with respect to their effect on vaginal smear patterns, it would seem that unpredictable shock exerted the most disruptive effect on the oestrous cycle. It was the only procedure that induced anoestrus; albeit in only 20% of rats. These findings are consistent with reports that unpredictable shocks are more successful in inducing stress related pathology than predictable shocks (Weiss, 1970, 1972).

Since the administration of unpredictable shock proved to be the most successful in disrupting the oestrous cycle this procedure was adopted in later experiments.

Chapter 6

THE COMBINED EFFECTS OF ALTERED NUTRITIONAL STATUS AND STRESS INDUCTION ON REPRODUCTIVE AND ENDOCRINE FUNCTION

The aim of the research reported in this chapter was to determine whether inferior nutritional status resulted in an increase in the susceptibility of the reproductive system to the effects of stress. To date, no such effect has been demonstrated experimentally. The objective was to determine whether stress induced during the caloric deprivation phase hastened the onset of anoestrus and whether similar stress treatment during nutritional rehabilitation delayed resumption of oestrous cycling.

It was proposed in Chapter 2 that the aetiology of amenorrhoea in anorexia nervosa may be multifactorial. For example, nutritional and psychological stress factors acting together may be responsible for the onset of amenorrhoea in some patients. Admittedly the onset of amenorrhoea precedes weight loss in many cases (Kay & Leigh, 1954), thus suggesting that psychological factors play a dominant role in those instances. In other patients, however, the onset of amenorrhoea coincides with the manifestation of both weight loss and psychological disturbance. The question as to whether these factors interact in precipitating the onset of amenorrhoea has yet to be resolved. Furthermore it has been suggested that persistent amenorrhoea after restoration of normal body weight may be due to residual psychological disturbance (Wakeling et al., 1977).

The present research was carried out in order to cast more light on the possible interactive effects of caloric deprivation and stress factors on reproductive function.

Experimental section

Unpredictable shock administration during caloric restriction and nutritional rehabilitation

The results of previous experiments (see Chapter 5) indicated that unpredictable shock was the most potent stressor used in the present research. Daily administration of 3 unpredictable shocks failed to induce anoestrus in all fully-fed rats, but it was the only procedure that resulted in anoestrus in some of the rats so treated.

In the studies described in this section the aim was to determine whether unpredictable shock would exert a more disruptive effect on reproductive functioning when animals were subjected to, or were recovering from, severe caloric restriction.

Experiment 1

The aim of this study was to investigate the combined effects of caloric restriction and shock administration (stress) on reproductive function. The combined effects of caloric deprivation and stress during both the caloric deprivation phase and the rehabilitation phase were investigated.

Stated more specifically the aims of this study were:

(i) to determine whether the daily administration of 3 unpredictable shocks during a 5 min. period in the restraining cage would accelerate the onset of anoestrus in rats fed 5g of the DFF diet per day.

(ii) to determine whether the exposure to unpredictable shocks during the caloric restriction phase exerted a persistent effect by preventing or delaying the resumption of oestrous cycles during full nutritional rehabilitation.

(iii) to determine whether the daily administration of 3

unpredictable shocks during full nutritional rehabilitation, following 21 days feeding on the DFF diet, would prevent or delay the resumption of oestrous cycles.

(iv) to determine whether 3 unpredictable shocks administered daily during gradual rehabilitation (18g basal diet/day), following 21 days feeding on the DFF diet, would prevent or further delay the resumption of oestrous cycles.

Method

Thirty female rats had free access to the basal diet from feeding jars for a period of 14 days. Thereafter 25 rats, with regular 4 day oestrous cycles, were allocated to one of 5 groups (n=5 each) so that the mean body weight of each group was approximately equal. All groups were fed 5g of the DFF diet daily for 21 days. Body weight and vaginal smears were monitored daily.

Table 39

Experimental design: Experiment 1, Chapter 6

		Caloric restriction phase	Rehabilitation phase	
Group	No. of rats	Daily admin. of 3 unpredictable shocks for:	Dietary regimen	Daily admin. of 3 unpredictable shocks for:
1	5	First 15 days	Full rehab.	None
2	5	None	Full rehab.	None
3	5	None	Full rehab.	First 10 days
4	5	None	Gradual rehab.	First 15 days
5	5	None	Gradual rehab.	None

The different groups identified in Table 39 were treated in the following ways:

Group 1 For the first 15 days of the caloric restriction (DFF diet) phase the rats were subjected to daily 5 min. periods in the restraining cage, when 3 unpredictable shocks were administered. Shock intensity was increased gradually (days 1-5, .50ma; days 6-10, .65ma; days 11-15, .80ma).

No shocks were administered during the remainder of the DFF diet phase; nor during rehabilitation. After 21 days feeding on the DFF diet the rats were allowed free access to the basal diet (ad libitum or "full rehabilitation"). Body weight and vaginal smears were monitored daily and the rats were sacrificed on the first day of vaginal cornification during full nutritional rehabilitation.

Group 2 served as a non-stressed control for groups 1 and 3. Twenty one days of feeding on the DFF diet was followed by full nutritional rehabilitation. Rats in this group were not subjected to shock stress. Like the rats in group 1 they were sacrificed on the first day of oestrus during full rehabilitation.

Group 3 Following 21 days of feeding on the DFF diet the rats were allowed free access to the basal diet (full nutritional rehabilitation). Starting on day 1 of the rehabilitation phase each rat was subjected to daily 5 min. periods in the restraining cage, when 3 unpredictable shocks were administered. Daily shock administration was continued for the first 10 days of the rehabilitation phase or until the first appearance of vaginal cornification (oestrous type smear). Shock intensity was increased from .50ma after the first 5 days to .65ma during the second 5 days.

Group 4 Following 21 days of feeding on the DFF diet the rats in this group were allowed access to 18g of basal diet per day ("gradual rehabilitation"). Starting on day 1 of the gradual rehabilitation phase each rat was subjected to daily 5 min. periods in the restraining cage,

when 3 unpredictable shocks were administered. Daily shock administration was continued for the first 15 days of the gradual rehabilitation phase or until the appearance of vaginal cornification. Shock intensity was increased gradually (days 1-5, .50ma; days 6-10, .65ma; days 11-15, .80ma).

Group 5 served as a non-stressed control for group 4. Twenty one days of feeding on the DFF diet was followed by gradual rehabilitation on 18g of basal diet per day.

During the caloric deprivation and rehabilitation phases body weight, food consumption and vaginal smears were monitored daily. Rats from all groups were sacrificed on the day of the first appearance of vaginal cornification during the rehabilitation phase. Trunk blood samples were collected, organ weights were measured, and the ovaries and oviducts were examined.

Results

Of greatest interest was the effect of shock administration on the onset of anoestrus during caloric deprivation and on the resumption of oestrous cycles during full and gradual rehabilitation.

Vaginal smear patterns:

Latency of anoestrus (groups 1 and 2): Group 1 rats, subjected to daily shock administration from the start of the DFF diet phase, became anoestrus after 5.2±1.8 days. In contrast, group 2 rats fed the same diet but not subjected to shock administration became anoestrous after 11.6±2.3 days. The difference between the two groups was statistically significant ($t(8) = 4.92$, $p < .01$, one tailed).

Latency of resumption of cycling The time taken for the different groups to first show evidence of resumed cycling during rehabilitation is summarized in Table 40.

Table 40

Effects of unpredictable shock administration on the latency of resumed oestrous cycling during rehabilitation

Group	No. of rats	Rehabilitation regimen	Treatment during rehabilitation	Mean latency of resumed cycling ±SD (days)
1	5	Full	No shock	6.4± .89 Range 5-7
2	5	Full	No shock	6.2± .84 Range 5-7
3	5	Full	Daily shock for first 10 days	15.2± 1.5[a] Range 13-17
4	5	Gradual	Daily shock for first 15 days	20.6±1.2[b] Range 19-2
5	5	Gradual	No shock	12.4±1.1 Range 11-14

a significantly longer than group 2 ($p < .01$)
b significantly longer than group 5 ($p < .01$)

Group 1 vs 2 - shock administration during the caloric deprivation phase: As shown in Table 40 groups 1 and 2 took approximately the same time to resume oestrous cycles during full nutritional rehabilitation. These results indicate that the administration of shocks during the first 15 days of the DFF diet phase did not cause a delay in the resumption of cyclical activity during full rehabilitation.

Group 2 vs 3 - shock administration during full nutritional rehabilitation phase: The rats in group 3 did not show evidence of vaginal cornification during the course of the 10 days of shock administration. Termination of daily shock administration was followed, about 5 days later, by resumption of oestrous cycles. The time taken by group 3 rats to show evidence of resumed cycling was significantly longer than the 6.2±.84 days taken by group 2 rats ($t(8)=11.84, p < .01$, one-tailed). These results indicate that shock administration during full nutritional rehabilitation inhibited the resumption of oestrous cycles.

Group 4 vs 5- shock administration during gradual rehabilitation phase: The rats in group 4 did not show evidence of cycling for the duration of the 15 days of shock administration. Termination of daily shock administration was followed, about 5 days later, by resumption of oestrous cycles. The time taken by group 4 rats to show evidence of resumed cycling was significantly longer than the 12.4±1.1 days taken by group 5 rats ($t(8)=13.28, p < .01$, one-tailed). As was the case with full rehabilitation rats, shock administration during gradual rehabilitation inhibited resumption of normal cyclical activity.

Ovulation rate and tubal ova count: The proportion of rats ovulating and the mean number of ova per ovulating rat are summarized in Table 41. The data of the full rehabilitation and gradual rehabilitation groups were analysed separately.

Table 41

Effects of unpredictable shock administration on ovulation and surface appearance of ovaries

		Oviducts		Ovaries	
				Mean number ±SD	
Group	Rehabilitation regimen	Proportion ovulating	Mean no. of ova per ovulating rat ±SD	Corpora lutea exceeding .7mm in diameter	Follicles exceeding .5mm in diameter
1	Full	5/5	10.2±1.4	13.8±1.9	2.2±0.83
2	Full	5/5	10.0±2.8	15.2±1.9	1.4±1.1
3	Full	4/5	6.0±2.2[a]	14.4±1.1	2.0±1.0
4	Gradual	4/5	5.3±1.7[b]	10.4±2.1	2.8±1.9
5	Gradual	5/5	10.2±1.6	8.2±2.5	1.4±0.89

a significantly fewer than groups 1 and 2 ($p < .05$)
b significantly fewer than group 5 ($p < .01$)

Full rehabilitation groups: There was no significant difference between the 3 full rehabilitation groups with regard to the frequency of ovulation ($\chi^2(2)=.2, p > .05$). The majority of rats showed evidence of ovulation on the day of the first appearance of vaginal cornification during the rehabilitation phase. However, group 3 which was subjected to shock administration for the first 10 days of rehabilitation had a mean number of ova per ovulating rat that was significantly lower than those of groups 1 and 2.

Gradual rehabilitation groups: There was no significant difference between the two groups with regard to the frequency of ovulation (χ^2 with Yates' correction$(1)=.1, p > .05$). The majority of rats ovulated on the first day of vaginal cornification during the rehabilitation phase. However, a Student's t test revealed that the mean number of ova per ovulating rat of group 4 was significantly lower than that of group 5 ($t(7)=4.88, p < .01$, two-tailed).

Surface appearance of ovaries: The mean numbers of surface corpora lutea and ovarian follicles are shown in Table 41. The data of the full rehabilitation and gradual rehabilitation groups were analysed separately.

Full rehabilitation groups: Analysis of variance revealed no significant difference between the 3 groups with regard to the number of corpora lutea and the number of surface follicles ($p > .05$). Normal control values from Supplementary Study No. 8 were not included in the analyses since groups 1 and 3 had a non-stressed control; and, furthermore the effects of the DFF plus rehabilitation on ovarian appearance have already been determined.

Gradual rehabilitation groups: Student's t tests revealed no significant difference between groups 4 and 5 with regard to the numbers of corpora lutea ($t(8)=1.24, p > .05$) and follicles ($t(8)=1.46, p > .05$, both

two-tailed). Visual inspection of the corpora lutea data suggests that both groups had fewer corpora lutea of criterion size than fully-fed, non-stressed rats from Supplementary Study No. 8 (Appendix A).

Serum LH levels: The serum LH values on the first day of oestrus during rehabilitation are summarized in Table 42.

Table 42

Effects of unpredictable shock administration on serum LH levels on the first day of oestrus during rehabilitation

Group	Treatment during rehabilitation	Mean serum LH (ng/ml)±SD
Full rehab:		
1	No shock	24.4±8.3
2	No shock	21.2±5.3
3	Daily shock for first 10 days	25.8±5.0
Gradual rehab:		
4	Daily shock for first 15 days	19.0±4.1
5	No shock	23.8±6.2

The data of the full rehabilitation and gradual rehabilitation groups were analysed separately. In both analyses normal control values on oestrus from Supplementary Study No. 8 (19.4± 8.1 ng/ml) were included for comparison.

Analysis of variance revealed no significant difference between the serum LH levels of the 3 full rehabilitation groups and the normal control group. Similarly there was no significant difference between the serum LH levels of the gradual rehabilitation groups and that of the normal control group ($p > .05$).

Body weight: The changes in body weight are summarized in Table D1 in Appendix D.

In an earlier study (Experiment 6, Chapter 4) it was found that, irrespective of the nature and duration of rehabilitation, resumption of cycling occurred at a time when the rats were approximately 1-3% below their initial body weight. The present findings, with rats not stressed during rehabilitation (groups 1, 2 and 5), are consistent with earlier observations. However, in the case of the groups subjected to unpredictable shock stress during full and gradual rehabilitation, anoestrus persisted in spite of restoration of initial body weight. Indeed ovulation was delayed until the body weight was 8.6% (full rehabilitation group) and 9.4% (gradual rehabilitation group) greater than initial body weight.

Organ weights: The changes in organ weights are summarized in Table D1 in Appendix D. Since the different groups were autopsied at widely discrepant times after the start of rehabilitation, differences in body and organ weights were expected. Consequently these data were not subjected to statistical analysis and are not described in detail here. There were, however, no significant differences between any of the groups with regard to adrenal gland weight when expressed in terms relative to body weight.

Discussion

The findings indicate that unpredictable shock administration during 5 min. periods in the restraining cage exerts a pronounced effect on cyclical activity when rats are subjected to, or are recovering from severe caloric restriction.

Shock administration during the caloric deprivation phase hastened the onset of anoestrus. Exposure to shock stress during the caloric

deprivation phase, however, did not have a long lasting effect. Rats on full rehabilitation, following shock administration during the caloric deprivation phase, resumed cycling at approximately the same time as non-stressed rehabilitated rats.

During both full and gradual rehabilitation daily shock administration was successful in inhibiting the resumption of cycling for the duration of the shock administration phase. In both rehabilitation groups resumed cycling was first observed about 5 days after the last shock was administered. It is possible that anoestrus would have been maintained for a longer period if shock administration was continued. It is unlikely that it was mere coincidence for both groups to take approximately 5 days after the last shock administration to show evidence of ovulation. It is probable that this is the time span required for restoration of hormonal equilibrium between the components of the functional HPO axis, following termination of stress.

In both the full and gradual rehabilitation groups exposed to stress, oestrus was first observed at a time much later than that of non-stressed controls. Consistent with earlier findings the rats not stressed during rehabilitation first resumed cycling when their body weight was 1-3% below their initial weight. In the present study rats stressed during rehabilitation (full or gradual) were about 9% heavier than before the start of the DFF diet phase. In an earlier section (Experiment 6, Chapter 4) it was concluded that weight gain of a critical magnitude was necessary for resumption of cyclical activity. The present findings indicate that continued shock stress is capable of preventing resumption of cyclical activity even after the nutritional disturbance has been corrected.

Since the shock schedule alone was not sufficient to induce anoestrus in the majority of rats (Experiment 3, Chapter 5) it would

appear that caloric deprivation for 21 days had the effect of causing the rats to be more susceptible to the stress induced by the shock schedule. That the shock schedule itself does not induce extreme stress is suggested by the fact that there was no increase in adrenal gland weight when considered relative to body weight.

Recent exposure to the shock stress appeared to have an effect on ovulation. Although the majority of rats ovulated on the day of first oestrus the groups subjected to shock stress during rehabilitation shed fewer ova than did the non-stressed groups. There appears to be a recency effect since the group subjected to shock stress during the caloric deprivation phase shed a normal quota of ova.

Regardless of earlier shock stress or the nature and duration of rehabilitation the serum LH levels, associated with the first day of vaginal cornification, were within the normal range.

Experiment 2

The aim of the present study was to use the PMS test to investigate the functional capacity of the HPO axis of rats subjected to shock stress while on 3 different dietary regimens, following 21 days of caloric restriction.

Results of previous experiments in this series have revealed (i) that resumption of normal cycling during full and gradual rehabilitation was prevented by subjecting rats to shock stress during rehabilitation (Experiment 1, Chapter 6); (ii) that ovulation was induced, by PMS administration, in rats on gradual rehabilitation and low weight maintenance regimen at times when they were still well below the body weight normally associated with ovulation (Experiment 10, Chapter 4).

The specific aims of this study were:

(i) to determine whether ovulation, shown to be blocked by

administration of shock stress during full rehabilitation, would be induced by means of PMS injection at various intervals during rehabilitation and shock administration

(ii) to determine whether ovulation, induced by PMS administration while rats are still below normal weight during gradual rehabilitation and low weight maintenance, would be blocked by means of shock administration.

Method

Fifty female rats were allowed to eat the basal diet ad libitum from feeding jars for a period of 14 days. Thereafter 45 rats, with regular 4 day cycles, were allocated to one of 9 groups (n=5 each) so that the body weight of each group was approximately equal. All groups were fed 5g of the DFF diet daily for 21 days. Body weight and vaginal smears were monitored daily. At the end of the caloric restriction phase 3 groups each were placed on one of 3 dietary regimens:

Full rehabilitation - basal diet ad libitum

Gradual rehabilitation - 18g basal diet per day

Low weight maintenance - 10g basal diet per day.

Treatment of the various groups is summarized in Table 43.

Full rehabilitation groups:

Group 1. Starting on day 1 of the full rehabilitation phase each rat was subjected to daily 5 min. periods in the restraining cage, when 3 unpredictable shocks were administered. Daily shock administration was continued until the day before autopsies were performed, approximately 6 days after the start of rehabilitation. On the third day of rehabilitation each rat received a subcutaneous injection of .1ml saline containing 20IU PMS and were autopsied 65 hours later at 0900h on the sixth day of rehabilitation or at the first appearance of an oestrous

type smear pattern.

Table 43

Experimental design: Experiment 2

Caloric deprivation phase All subjects fed DFF diet for 21 days					
New dietary regimen phase					
Group	No. of rats	Dietary regimen	Experimental Treatment: Daily admin of 3 unpredictable shocks for:	PMS injected on day:	Anticipated day of autopsy (65hr post-PMS)
1	5	Full rehab	First 5 days	3	6
2	5	Full rehab	First 9 days	7	10
3	5	Full rehab	None	None	First vaginal cornification
4	5	Grad rehab	First 5 days	3	6
5	5	Grad rehab	None	3	6
6	5	Grad rehab	None	None	6
7	5	Low wt main.	First 10 days	8	11
8	5	Low wt main.	None	8	11
9	5	Low wt main.	None	None	11

Full rehab = Ad lib access to basal diet
Gradual rehab = 18g basal diet per day
Low wt main. = 10g basal diet per day

Group 2. This group was treated in the same way as group 1 except that daily shock administration was continued for a longer period. That is, until the day before autopsies were performed, approximately 10 days after the start of rehabilitation. Twenty IU of PMS was administered on the seventh day of rehabilitation and autopsies were performed 65hrs later at 0900h on day 10 or at the first appearance of an oestrous type smear.

Group 3. The rats in this group received neither shock treatment nor PMS injections during the rehabilitation phase. They were autopsied on the day of the first appearance of an oestrous type smear. This group was included as a non-stressed control group.

Gradual rehabilitation groups:

Group 4. Starting on day 1 of the gradual rehabilitation phase each rat was subjected to daily 5 min. periods in the restraining device, when 3 unpredictable shocks were administered. Daily shock administration was continued until the day before autopsies were performed approximately 6 days after the start of gradual rehabilitation. On the third day of rehabilitation each rat received a subcutaneous injection of .1ml saline containing 20IU PMS and was autopsied 65hrs later, at 0900h on the sixth day of rehabilitation or at the first appearance of an oestrous type smear pattern.

Group 5. This group served as a non-stressed control group. The rats in this group were treated in the same way as those in group 4 with the exception that shock was not administered.

Group 6. This group served as a non-stressed, non-PMS test control. Gradual rehabilitation was continued until autopsy on the sixth day of rehabilitation. It was anticipated that spontaneous cycling would not occur within this period of gradual rehabilitation (Experiment 6, Chapter 4).

Low weight maintenance groups:

Group 7. Starting on day 1 of the low weight maintenance regimen phase each rat was subjected to daily 5 min. periods in the restraining cage, when 3 unpredictable shocks were administered. Daily shock administration was continued until the day before autopsies were performed approximately 11 days after the start of the low weight maintenance regimen. On the eighth day after the start of the new regimen each rat

received a subcutaneous injection of .1ml saline containing 20IU PMS and were autopsied 65hrs later, at 0900h on the eleventh day or at the first appearance of an oestrous type smear.

Group 8. This group served as a non-stressed control group treated in the same way as group 7 rats with the exception that shock was not administered.

Group 9. This group served as a non-stressed, non-PMS test control. The low weight maintenance regimen was continued until the eleventh day when autopsies were performed. It was anticipated that spontaneous cycling would not occur while the rats were on this dietary regimen.

In all groups body weight, food consumption and vaginal smears were monitored daily during the caloric deprivation phase and while the rats were on the new dietary regimen. At autopsy, body and organ weights were measured and the ovaries and oviducts were examined. Blood samples for serum LH assay were not collected since the PMS used in this study was known to interfere with the LH measurement.

Results

Since 3 different dietary regimens were employed the data were analysed separately.

Vaginal smear patterns

Full rehabilitation groups:

Group 1: All rats continued to display a constant dioestrous smear pattern until days 5-7 (48-89hrs after PMS injection) when evidence of vaginal cornification was first apparent.

Group 2: All rats remained anoestrous for the duration of shock administration. Vaginal cornification was first observed between 65 and 89 hrs after PMS injection which was given on day 7.

Group 3: The rats in this group showed spontaneous resumption of

cycling after 5.8±.84 days (range: 5-7 days).

Gradual rehabilitation groups:

Groups 4 and 5: All rats in these groups continued to display a dioestrous type smear pattern until approximately 65hrs after PMS injection which was given on day 3.

Group 6: All rats were still anoestrous when autopsied on day 6 of gradual rehabilitation.

Low weight maintenance groups:

Groups 7 and 8: Vaginal cornification was first observed between 48 and 89hrs after the PMS injection which was given on day 8.

Group 9: All rats were still anoestrous when autopsied on day 11 of the low weight maintenance regimen.

Ovulation rate and tubal ova count:

The proportion of rats ovulating and the mean number of ova per ovulating rat are shown in Table 44.

Full rehabilitation groups:

Since it was anticipated that the two groups receiving PMS and the group showing spontaneous vaginal cornification might ovulate the observed frequency of ovulation was subjected to a χ^2 test with an expected frequency of 5 for each group. There was a significant difference between the groups with regard to frequency of ovulation ($\underline{\chi}^2(2)=8.2$, $\underline{p} < .01$). Nine out of 10 rats exposed to shock stress failed to ovulate in response to PMS provocation. All the rats in the non-stress-non PMS group ovulated at first oestrus.

Gradual rehabilitation groups:

Shock stress inhibited ovulation in the gradual rehabilitation group given PMS. All the non-stressed rats receiving PMS ovulated.

Low weight maintenance groups:

Shock stress inhibited ovulation in the low weight maintenance

Table 44

Effects of unpredictable shock administration and 20IU PMS on ovulation and surface appearance of ovaries

Group	No. of rats	Shock stress for	PMS injected on day	Proportion ovulating	Mean no.of ova per ovulating rat ±SD	Ovaries: Mean number ±SD: Corpora lutea exceeding .7mm in diameter	Ovaries: Mean number ±SD: Follicles exceeding .5mm in diameter
Full rehab.							
1	5	First 5 days	3	0/5	0	14.8±2.4	9.4±2.1
2	5	First 9 days	7	1/5	3.0	17.2±1.9	11.2±1.5
3	5	None	None	5/5	9.8±1.3	15.8±1.6	3.2±1.3[a]
Gradual rehab.							
4	5	First 5 days	3	0/5	0	8.8±1.9	10.8±1.3
5	5	None	3	5/5	10.2±1.8	10.0±1.4	4.4±1.1[b]
6	5	None	None	0/5	0	9.2±1.3	0
Low wt. Main.							
7	5	First 10 days	8	0/5	0	9.4±1.1	9.8±1.5
8	5	None	8	5/5	5.2±1.9	11.2±2.8	4.8±1.6[d]
8	5	None	None	0/5	0	6.4±1.1[c]	0

a significantly fewer than groups 1 and 2 ($p < .01$)
b significantly fewer than group 4 ($p < .01$)
c significantly fewer than groups 7 and 8 ($p < .01$)
d significantly fewer than group 7 ($p < .01$)

group given PMS. All non-stressed rats receiving PMS ovulated.

Surface appearance of ovaries:

The mean number of surface corpora lutea and ovarian follicles are shown in Table 44. The data of the full rehabilitation, gradual rehabilitation and low weight maintenance groups were analysed separately.

Full rehabilitation groups:

Analysis of variance revealed no significant difference between the 3 groups with regard to the number of surface corpora lutea ($p > .05$). However, group 3 had significantly fewer ovarian follicles than the PMS injected groups.

Gradual rehabilitation groups:

Analysis of variance revealed no significant difference between the 3 groups with regard to the number of surface corpora lutea ($p > .05$). No follicles were visible on the surface of the ovaries of group 6 rats and a Student's t test revealed that group 5 rats had significantly fewer follicles than group 4 rats ($t(8)=8.27, p < .01$, two-tailed).

Low weight maintenance groups:

Analysis of variance revealed that group 9 had significantly fewer corpora lutea than groups 7 and 8. No follicles were visible on the surface of the ovaries of group 9 rats and a Student's t test revealed that group 8 rats had significantly fewer follicles than group 7 rats ($t(8)=5.06$, $p < .01$, two-tailed).

Body and organ weights

The changes in body and organ weights are summarized in Table D2 in Appendix D. Since these data are not directly pertinent to the objectives of the present study they will not be described in detail.

Under all 3 dietary regimens the pituitary, ovarian and uterine weights of the PMS injected groups were significantly greater than

those of the non-injected groups, in both absolute terms and relative to body weight. There were no significant differences between the 3 groups, within each dietary regimen, with regard to adrenal gland weight, either in absolute terms or relative to body weight.

Discussion

The results confirm the earlier findings (Experiment 10, Chapter 4) that ovulation may be induced by means of PMS administration in non-stressed rats on the gradual rehabilitation and low weight maintenance regimen, at times when they were still well below the body weight normally associated with ovulation.

PMS failed to induce ovulation in the gradual rehabilitation and low weight maintenance groups subjected to daily shock stress during rehabilitation. Similarly treated, non-stressed control rats all responded to PMS provocation by ovulating. Stress treatment inhibited ovulation in 9 out of 10 full rehabilitation group rats given PMS.

These findings indicate that the inhibition of ovulation caused by shock administration persisted despite PMS injection. The failure by stressed rats to ovulate in response to PMS provocation was, presumably, due to the absence of an ovulatory surge of LH. This does not appear to be related to ovarian refractoriness since ovarian weight was increased and the uteri showed changes consistent with adequate gonadal steroid stimulation.

It is reasonable to propose that the hypothalamus failed to respond in the normal way to the positive feedback action of oestrogen. This being the case, the hypothalamus would not have secreted sufficient LHRH to prompt the endogenous LH surge. It is proposed that the functional state of the hypothalamus was disturbed as a result of shock induced stress. Anatomical considerations have led most

investigators to propose the hypothalamus as the site at which psychological factors (stress) exert a disruptive effect on the reproductive cycle (Bajusz, 1967).

It would appear that the effect of the stress treatment was not dependent upon the stringency of the dietary regimen imposed following caloric restriction. Inhibition of PMS-induced ovulation was observed under all dietary regimens, ranging from full rehabilitation to intake restricted to 10g basal diet per day. Common to all groups was the caloric restriction phase, weight loss and the subsequent anoestrus (induced by this restriction). It would appear that, in rats recovering from nutritionally induced anoestrus, the hypothalamus is more sensitive to the effects of stress than in fully fed animals. That is, the hypothalamus remains relatively resistant to the effects of stress when imposed on the animal that is fully fed. However, when rats are recovering from nutritionally induced anoestrus it appears that a hypothalamic dysfunction persists due to the influence of stress factors. In conclusion, the results of the present research suggest that a period of emaciation is likely to render the hypothalamus more sensitive to the effects of stress both during caloric deprivation and during recovery from the nutritional disturbance.

Concluding discussion

The results of the experiments described in this section indicate that shock stress exerts a more disruptive effect on the reproductive cycle when presented while rats are being subjected to or are recovering from caloric restriction.

Shock stress induced by the daily administration of 3 unpredictable shocks accelerated the onset of anoestrus during caloric deprivation and delayed the resumption of cycling of rats for the

duration of stress administration, despite restoration of normal weight. These results suggest that the caloric deprivation treatment had the effect of increasing susceptibility to stress, presumably at the hypothalamic level.

The results of the final experiment indicate that there was a persistent dysfunction, probably at the hypothalamic level, as a result of stress administration during rehabilitation. Since the stress treatment alone was not successful in inhibiting ovulation in fully fed rats, the results suggest that the period of caloric restriction was the important factor in establishing the enhanced susceptibility of the hypothalamus to stress factors. The proposition that the earlier period of caloric restriction was the crucial determinant of the dysfunction, rather than the nutritional status at the time of PMS administration, is supported by the observation that ovulation was blocked after 10 days rehabilitation in rats fed the basal diet ad libitum (±28g/day) as well as in rats fed only 10g basal diet/day. These findings add support for the hypothesis that the failure by stressed rats to ovulate in response to PMS provocation was the result of a hypothalamic dysfunction, initiated by caloric restriction but prolonged by stress.

Chapter 7

SUMMARY AND CONCLUSIONS

The present research was prompted by the number of unresolved issues still surrounding the aetiology of secondary amenorrhoea in anorexia nervosa.

By means of animal experimentation it was possible to carry out investigations which would have been impractical and unethical with human subjects. A number of investigators have argued for the place of experimental animals in the study of malnutrition (Platt, 1968; Widdowson, 1968). Although there are differences in the reproductive cycles of humans and rats the hormonal mechanisms regulating the oestrous cycle of the rat are essentially the same as those regulating the human menstrual cycle (Bentley, 1976; Tepperman, 1973).

Using animal subjects, the changes in reproductive physiology from the beginning of caloric deprivation through to the period of emaciation and cessation of oestrous cycles were investigated. The physiological changes associated with nutritional rehabilitation and recovery of the reproductive system were then established. A laboratory approach also allowed for the separate investigation of the effects of caloric deprivation and stress induction on reproductive function. This was followed by an investigation of the combined effects of caloric deprivation and stress induction on reproductive function during the weight loss and rehabilitation phases. To date there have been no published studies on work of this nature.

The investigation of the effects of systematic reduction of the caloric component of diets produced a number of pertinent findings. The results indicated that the mechanisms controlling the regulation of the oestrous cycle were resistant to relatively severe caloric deprivation. It was only after the complete removal of both the carbyhydrate and fat components of the diet that anoestrus was consistently

observed. This finding does not provide support for the notion that changes in eating patterns without causing significant weight loss may be responsible for the onset of amenorrhoea in human females. Shearman (1969) observed that teenagers dieting for cosmetic reasons showed an abrupt onset of amenorrhoea preceding significant weight loss. A more likely explanation for this observation is that psychological factors related to their needs for cosmetic change are implicated. A high incidence of menstrual dysfunction has been observed in adolescent women with emotional problems (Fries et al., 1974; Jeffcoate, 1965; Rakoff, 1968).

The results of the present study indicated that, in non-stressed rats subjected to involuntary food restriction, the reduction in serum LH levels and the onset of anoestrus was related to weight loss. Although there were within group differences the average body weight of the rats at the onset of anoestrus was 18-19% below initial body weight. Investigations of patients with anorexia nervosa have revealed a similar relationship between reduced plasma LH levels and weight loss (Beumont et al., 1976; Brown et al., 1977). In one of the few studies of endocrine changes occurring during a period of voluntary weight loss Beardwood (1974) found that obese patients only became amenorrhoeic after losing 16kg in weight.

Contrary to the findings of Howland & Skinner (1973), who reported a dramatic reduction in serum LH levels within 24hrs, severe dietary deprivation did not cause a rapid depletion of serum LH. It took more than 7 days feeding on the dextrin and fat free(DFF) diet before a significant reduction in serum LH concentration was observed. In the absence of stress apart from that associated with reduced food intake, severe caloric restriction did not exert a direct and immediate effect on the mechanisms regulating the oestrous cycle. Rather, the sustained

observation that correction of body weight is a pre-requisite for resumption of menstrual cycles in human females.

In the present study weight gain during rehabilitation was accompanied by a progressive increase in serum LH concentration. Within 2 weeks after the start of rehabilitation normal serum LH levels were observed. In some cases of anorexia nervosa weight gain during rehabilitation is accompanied by an increase in gonadotrophin and gonadal steroid levels and the return of menstrual function (Beumont et al., 1976; Crisp et al., 1973; Marshall & Fraser, 1971; Wakeling et al., 1976, 1977). In those instances in which gonadotrophin levels do rise to normal levels there appears to be a relationship between weight gain and increased gonadotrophin levels. Beumont et al. (1976) found a linear correlation between gonadotrophin levels and body weight. In other cases, however, basal LH levels remain low even after restoration of normal weight (Nillius & Wide, 1977; Wakeling et al., 1976) and some females may remain amenorrhoeic for years after regaining weight (Russell, 1969). The patients who fail to menstruate continue to show an absence of cyclical changes in gonadotrophins and oestrogens that characterize the normal menstrual cycle (Bell et al., 1966; Crisp et al., 1973; Russell & Beardwood, 1968).

A number of explanations have been offered to account for the persistent amenorrhoea found in some anorexia nervosa patients after the restoration of normal body weight. Mecklenburg et al. (1974) proposed that the failure to resume normal rhythmical changes of gonadotrophin and gonadal steroid secretion may be a consequence of damage to the hypothalamus caused by starvation. They suggested that persistent defects of water balance and thermoregulation in some patients may be due to irreversible changes in hypothalamic function caused by the earlier malnutrition. In the present study there was no apparent

evidence of permanent damage caused by the nature and duration of the malnutrition imposed. It should be conceded that only the reproductive system was investigated and that other hypothalamic functions were not examined. However, during rehabilitation studies it was found that the rectal temperature of rats returned to normal when body weight was restored to normal levels.

Russell (1969) suggested that the severity and duration of emaciation may account for the different secretory patterns found in rehabiliated anorexia nervosa patients. Brown et al. (1977), however, found no relationship between basal LH levels and duration of amenorrhoea. The results of the present research indicated that prolonged emaciation did not result in a delay in the recovery of reproductive function during nutritional rehabilitation. Rats which were subjected to extended emaciation and then rehabilitated at a reduced pace resumed oestrous cycles when they reached the body weight normally associated with spontaneous resumption of oestrous cycles.

In the present study there was no evidence that nutritional factors caused prolonged disturbance of reproductive function of non-stressed rats previously subjected to involuntary malnutrition. However, more than phylogenetic differences distinguish the experimental animals from anorexia nervosa patients. Apart from differences in the voluntary/involuntary nature of food restriction there is also the prominent psychological disturbance characteristic of anorexia nervosa. Whereas the emaciated rats ate voraciously until normal weight was restored anorexia nervosa patients are known to resist attempts to rehabilitate them (Bruch, 1965; Crisp, 1967; Dally, 1969). Furthermore restoration of normal body weight is not always accompanied by resolution of the psychological disturbance. Mecklenburg et al. (1974) claimed that the traditional view to which most psychiatric

investigators subscribe is that the endocrine disorder is due to a hypothalamic dysfunction induced by psychological conflict. As Beumont (1970) observed social, sexual and emotional adjustment may remain unsatisfactory despite improvement of the physical state. Dally & Sargent (1966) suggested that normal menses return in anorexia nervosa patients only after psychological disturbance has been resolved, in addition to them maintaining normal body weight. A number of other investigators have proposed that residual psychological disturbance may be responsible for the prolonged menstrual dysfunction observed in some anorexia nervosa patients after the nutritional disturbance has been corrected (Beardwood, 1975; Russell, 1972a; Wakeling et al., 1976, 1977). The possible role of psychological factors in prolonging the amenorrhoea will receive further attention later in this chapter.

Pharmocological studies aimed at determining which component(s) of the hypothalamic-pituitary-ovarian (HPO) axis were dysfunctional produced results providing additional support for the assertion that the hypothalamus is the primary site of dysfunction during inanition (Campbell et al., 1977).

Administration of LHRH to non-stressed, emaciated rats resulted in an abnormally low LH response. A normal response, however, was observed in rats whose pituitaries were primed with LHRH before the bolus injection of LHRH. There is physiological evidence that, under normal conditions, LHRH exerts a priming effect on the pituitary (Aiyer et al., 1974; Blake, 1978; Gordon & Reichlin, 1974; Legan & Karsch, 1975; Zeballos & McCann, 1975). The normal LH response shown by emaciated rats after LHRH priming suggests a failure by the hypothalamus to secrete sufficient LHRH to maintain normal pituitary gonadotrophin secretion. These findings suggest that the disturbance of pituitary function is secondary to a hypothalamic dysfunction involving decreased

synthesis and/or release of LHRH. The finding of reduced hypothalamic content of LHRH in underfed rats (Piacsek & Meites, 1967) suggests that synthesis is impaired.

The LH response to a single injection of LHRH during the acute phase of anorexia nervosa has been found to be absent in some patients (Nillius & Wide, 1977; Mecklenburg et al., 1974) and normal in others (Aono et al., 1974; Mortimer et al., 1973; Wiegelman & Solberg, 1972). However, the majority of patients, when below 70% of standard weight, show an impaired LH response similar to that found in emaciated rats (Beumont et al., 1976; Crosignani et al., 1974; Nillius & Wide, 1977; Sherman et al., 1975).

Treatment of patients, during the acute phase of anorexia nervosa, with prolonged LHRH administration has produced findings similar to those found in emaciated rats primed with LHRH (Aono et al., 1975; Nillius et al., 1975; Nillius & Wide, 1977). The effectiveness of prolonged LHRH treatment in inducing ovulatory menstrual cycles led Nillius & Wide (1977) to propose that reduced secretion of pituitary gonadotrophins in anorexia nervosa is secondary to a dysfunction at the hypothalamic rather than at the pituitary level. Patients with organic lesions of the pituitary do not show enhanced LH responses following prolonged LHRH tests (Aono et al., 1975).

The administration of 2μg oestradiol benzoate (OB) to emaciated, anoestrous rats failed to induce ovulation or elevate serum LH levels. These findings are similar to those of Wakeling et al. (1977) who reported that anorexia nervosa patients, during the acute phase, failed to respond with positive feedback release of LH to oestrogen administration. In their patients positive feedback effects were observed only after the restoration of normal body weight.

Convincing evidence that the hypothalamus is the primary site at which oestrogen exerts its positive feedback effect has been presented (Goodman, 1978b). The present failure of OB to induce ovulation or elevate serum LH levels provides strong support for the proposition by Wakeling et al. (1977) that the ability of the hypothalamus to respond to the positive feedback effects of oestrogen is impaired during states of inanition.

The administration of 20IU of pregnant mares' serum (PMS) gonadotrophin to emaciated, anoestrous rats produced results consistent with ovarian responsiveness to gonadotrophin stimulation. It is proposed that the inability of PMS to induce ovulation was due to the failure by the hypothalamus to respond to the positive feedback effects of endogenous oestrogen rather than to inadequate oestrogen production. The overall pattern of findings was not suggestive of refractoriness of the ovarian follicles.

PMS was administered to rats being rehabilitated nutritionally with the basal diet, but at a reduced pace. PMS-induced ovulation occurred at times when rats were well below the body weight normally associated with spontaneous resumption of oestrous cycles. These results suggest that the dysfunctional areas, presumably in the hypothalamus, are sensitive to the availability of a diet containing a relatively high proportion of carbohydrate and fat. The HPO axis became more responsive to PMS provocation after small quantities of basal diet were made available. Earlier results, however, indicated that the HPO axis does not become fully functional until weight loss is corrected. Sponteneous resumption of ovulatory cycles during rehabilitation occurred only when the rats had attained a body weight almost equal to their initial body weight.

Clinical observations indicate that psychological factors are

prominent not only in initiating weight reducing strategies, but that a complex psychopathology may persist in anorexia nervosa patients even after restoration of normal body weight (Beumont, 1970; Bruch, 1965; Crisp, 1967; Dally, 1969; Dally & Gomez, 1969). Clearly it was not possible to induce a state of stress in rats that could be equated with the psychological disturbance of anorexia nervosa. An attempt was made, however, to determine the nature and duration of stress treatments capable of altering serum LH levels and disrupting the reproductive cycle of fully fed female rats.

A variety of stressors were employed in an attempt to alter basal serum LH levels, block the pro-oestrous LH surge, and induce anoestrus. In contrast to the findings of Euker et al. (1973) and McKay et al. (1975) restraint stress administered at overlapping intervals between 0900-1700h on pro-oestrus did not block the LH surge or inhibit ovulation the following day. Contrary to the findings of Riegle & Meites (1976), who reported that chronic restraint was sufficient to block ovarian cyclicity, chronic restraint failed to induce anoestrus in the strain of Long-Evans rat used in this research. It is likely that the discrepant findings were due to species differences and/or the nature of the stress treatment.

Of the other stress treatments investigated, unpredictable shock appeared to be the most potent stressor. However, the daily administration of unpredictable shock alone was incapable of causing widespread anoestrus. In the final series of experiments unpredictable shock was administered to rats during the caloric deprivation and nutritional phases in order to determine whether the inferior nutritional status resulted in an increase in the susceptibility of the reproductive system to the effects of stress.

A significant finding was that the stressor which proved to be

unsuccessful in inducing widespread anoestrus in fully-fed rats, exerted a profound effect on cyclical activity when rats were subjected to, or were recovering from severe caloric restriction. The onset of anoestrus was hastened during the weight reduction phase by daily stress treatment. This suggests that nutritional and stress factors interacted in accelerating the disruption of the oestrous cycle. A similar interaction may occur in precipitating the onset of amenorrhoea in some anorexia nervosa patients after moderate weight loss.

Imposition of stress during the rehabilitation phase prevented the resumption of oestrous cycles even after normal body weight had been restored. It was only after the termination of stress treatment that oestrous cycling resumed. The results indicate that 5 days were required before the HPO axis became fully functional again after the termination of stress.

It has been emphasised that the psychological disturbance is prominent throughout the course of anorexia nervosa, persisting even after full nutritional rehabilitation (Beumont, 1970; Bruch, 1965; Crisp, 1967). As Wakeling et al (1976) observed it is often the last abnormality to become resolved. A number of investigators have proposed that psychological factors may be responsible for the persistent amenorrhoea found in some patients after recovery of normal body weight (Beardwood, 1974; Dally & Sargent, 1966; Russell, 1972a; Wakeling et al., 1976). Beardwood (1974), for example, suggested that the phasic secretion of gonadotrophins in anorexics may be more readily affected by psychological stress than by weight factors. The present findings provide strong experimental support for the assertion that stress is capable of grossly prolonging the resumption of reproductive cycling after successful nutritional rehabilitation.

It has also been suggested that residual psychological disturbance

could account for the atypical responses to oestrogen provocation and clomiphene citrate stimulation obtained with some anorexic patients (Wakeling et al., 1976, 1977). In the present study stress treatment inhibited induction of ovulation by PMS in rats whose body weights were both above and below initial body weight. Since PMS administration succeeded in inducing ovulation in non-stressed rats the findings indicate that the stress contributed to or exacerbated an existing dysfunction of the ovulatory mechanisms. With evidence indicative of adequate gonadal steroid secretion, the results suggest that the hypothalamus failed to respond to the positive feedback action of oestrogen. The absence of a secondary LH peak in response to clomiphene stimulation led Marshall et al. (1973) to propose that the hypothalamic positive feedback mechanisms are impaired in some anorexia nervosa patients.

That prolongation of anoestrus by stress treatment was dependent upon a prior period of caloric deprivation and weight loss was evident from the finding that the stressor alone was incapable of inducing extensive anoestrus. Furthermore the earlier inanition, and not the body weight at the time of PMS injection, appeared to be the determinant of the enhanced susceptibility to stress. Inhibition of ovulation was found in PMS-injected rats weighing more than their initial body weight as well as in rats still 30% below initial body weight. It is proposed that the functional state of the hypothalamus was initially disturbed by caloric deprivation and weight loss and that imposition of stress acted to prolong this dysfunction. That is, the stress had the effect of preventing the restoration of normal HPO function that typically follows nutritional rehabilitation.

It is possible that a similar stress related dysfunction occurs in anorexia nervosa patients who fail to resume menstruation or who

show atypical response to provocation tests after restoration of normal body weight. The persistence of amenorrhoea due to residual psychological disturbance may not necessarily be contingent upon a pre-existing, nutritionally induced hypothalamic dysfunction. It has been established that some patients become amenorrhoeic prior to the onset of dieting or before significant weight loss has occurred (Kay & Leigh, 1954; Kanis et al., 1974; King, 1963).

The questions of where and how the aetiological factors are operating in producing disturbances of reproductive function have yet to be answered to the satisfaction of all investigators. At the present stage we are in a better position to address ourselves to the question: Where?

The results of this and other studies in which laboratory animals were employed indicate that the reduced gonadotrophin secretion associated with involuntary inanition is due, not to primary pituitary disturbance, but to a functional hypothalamic defect (Campbell et al., 1977; Piacsek & Meites, 1967). Endocrine investigations of anorexia nervosa patients have led researchers to arrive at a similar conclusion that these patients have a hypothalamic defect (Aono et al., 1975; Marshall et al., 1973; Mecklenburg et al., 1974; Nillius & Wide, 1977; Roth et al., 1972; Wakeling et al., 1976, 1977). However, as Mecklenburg et al. (1974) observed, the origins of the hypothalamic defect remain obscure. Whether it antecedes, coincides or is a consequence of the illness remains undetermined. While it is possible that the weight loss in anorexia nervosa is a consequence of a primary hypothalamic defect the present evidence indicates that malnutrition along can cause a disturbance of hypothalamic function.

The role of psychological stress factors in the aetiology of amenorrhoea in anorexia nervosa is not fully understood. It has been

proposed that psychological factors acting at the level of the hypothalamic inhibit the regulation of gonadotrophin secretion (Klinefelter et al., 1943; Mecklenburg et al., 1974; Russell, 1972a). Anatomical considerations have led most investigators to propose the hypothalamus as the site at which psychological stress factors exert a disruptive effect on the reproductive cycle (Bajusz, 1967). The results of the present study support the notion that stress exerts an effect at the hypothalamic level. The finding that stress inhibited induction of ovulation by PMS suggests a functional hypothalamic defect since hypothalamic positive feedback mechanisms are presumably involved in PMS-induced ovulation (Ying & Greep, 1971b). That it was a functional disturbance was demonstrated by the resumption of spontaneous ovulatory cycles after the withdrawal of stress.

At present we are not in the position to make confident statements about "how" the aetiological factors exert a disruptive effect on reproductive function. The manner in which malnutrition alters cellular function in the hypothalamus is not yet known and the neural and neuro-endocrine mechanisms by which stimuli emanating from higher centres alter hypothalamic function and gonadotrophin secretion are yet to be elucidated. With the increasing sophistication of investigative procedures it is anticipated that the pathways and mechanisms involved in reproductive disturbance will some day be understood.

APPENDICES

Appendix A

Supplementary studies

Appendix A.1

Supplementary study no.1

The purpose of this study was to obtain the normal growth chart of individually housed rats fed standard pelleted feed and water ad libitum.

Method

Twenty 5 week old Long Evans female rats were housed in individual cages and allowed free access to pelleted rat food and water until they were 15 weeks old. Body weight was recorded at regular intervals.

Results

Body weight, at weekly intervals, and the mean weight gain per week and per day are shown in Table A1.

Table A1

Body weight of female Long Evans rats fed pelleted food and water ad libitum from age 5-15 weeks (n=20)

Age (in weeks)	Mean Body Weight ± SD (g)	Mean Body Weight gain/week (g)	Mean Body Weight gain/day (g)
5	132.6 ± 21.5	-	-
6	160.6 ± 22.5	28.1	4.0
7	187.8 ± 21.0	27.2	3.9
8	215.3 ± 21.8	27.5	3.9
9	231.1 ± 22.1	15.8	2.3
10	246.9 ± 21.4	15.8	2.3
11	258.9 ± 22.1	12.1	1.7
12	265.9 ± 23.5	7.0	1.0
13	274.4 ± 22.8	8.5	1.2
14	281.8 ± 24.4	7.4	1.1
15	288.3 ± 24.2	6.5	.93

Appendix A.2

Supplementary study no.2

The purpose of this study was to determine whether the basal diet was nutritionally adequate to support body growth and to maintain normal oestrous cycling. Stated more specifically the aims of this study were:

(i) to compare the growth rate of sexually mature rats fed the basal diet with the growth rats of rats fed crushed pelleted rat food and rats fed pelleted rat food;

(ii) to compare the consumption rate of rats fed the basal diet with that of rats fed the crushed pelleted food.

An additional aim of this study was to determine the extent of spillage of food from the feeding jars.

Method

Twenty sexually mature female rats with regular 4 day cycles were fed crushed pellets in feeding jars for 8 days (adaptation to eating from feeding jars). Body weight, food consumption and vaginal smears were monitored daily.

On day 1 of the experimental phase the rats were allocated to one of 3 groups (n=10 each) so that the mean body weight of each group was approximately equal. For the following 10 days:

Group 1 received the basal diet and water ad libitum;

Group 2 received the crushed pellets and water ad libitum;

Group 3 feeding jars were removed and the rats were given unlimited access to pelleted food and water.

The daily consumption rates of groups 1 and 2 were measured at 1000h each day. The body weight and vaginal smears of all rats were monitored daily.

During the experimental phase a small tray was mounted under the grill floor, below the feed jars of 5 rats fed the basal diet and 5 rats fed the crushed pellets. The amount of food spilled into these trays was measured daily.

Results

Body weight and food consumption: The mean body weight and consumption rates are shown in Table A2. The body weight values for day 1 are those measured before the introduction of the new diets at 1000h. The consumption rates on day 1 represent the quantity of crushed pellets eaten during the 24 hour period up to 1000h on day 1. The consumption rates of the basal diet and crushed pellets groups on day 2 represent the 24 hr consumption of those diets from 1000h on day 1 to the same time on day 2.

Table A2
Body weight and food consumption rate of rats on 3 different diets over a 10 day period (means ± SD)

	Nature of diet				
	Basal diet		Crushed pellets		Pelleted food
DAY	Mean body weight±SD (g)	Mean consumption±SD (g)	Mean body weight±SD (g)	Mean consumption±SD (g)	Mean body weight±SD (g)
1	249.1±5.8	17.6± .84[a]	248.5±6.2	17.8±1.2[a]	249.9±5.6
2	246.7±6.2	14.1±1.9[b]	250.4±6.0	18.0± .67	251.9±5.6
3	245.5±6.5	16.7± .95[b]	251.5±5.7	18.0±1.2	252.8±5.8
4	249.1±6.2	18.2± .63	252.9±5.5	17.8±1.1	253.7±6.0
5	251.2±6.3	18.4± .70	254.3±5.0	18.1± .99	255.3±6.0
6	252.1±6.3	17.9± .99	256.7±5.6	17.9± .88	256.5±6.1
7	254.9±6.4	18.0± .67	258.9±5.5	17.8± .79	257.6±6.2
8	258.0±6.0	18.1± .99	260.0±5.6	17.8±1.1	259.2±6.3
9	260.9±5.8	18.2± .92	260.8±5.3	18.1± .99	260.3±6.0
10	263.7±5.2	18.2±1.1	262.0±5.4	18.1±1.1	261.6±6.5

a All consumption rates on day 1 represent quantity of crushed pellets eaten in the 24 hr period up to 1000h on day 1.
b Significantly less than crushed pellet diet group ($p < .01$)

Analysis of variance revealed no significant difference in mean body weight of the 3 groups at any stage during the 10 day period ($p > .05$). All groups showed an increase in body weight ($p < .01$).

Analysis of variance revealed that the mean consumption rate of the basal diet group was significantly lower than that of the crushed pellet diet group on days 2 and 3 ($p < .01$). Thereafter there was no significant difference between the two groups.

Vaginal smears: Apart from 2 rats showing a single 5 day cycle, one in the basal group and the other in the pelleted food group, all rats continued to display normal oestrus cycles.

Food spillage: The amount of food spilled from the feed jars varied but, on average, it was found to be less than 2% of the total amount of food consumed.

Discussion

The results indicate that the basal diet is capable of supporting normal growth. Reproductive cycling was not impaired by the introduction of the basal diet. The initial drop in consumption of the basal diet did not have a lasting effect on body weight. However, it did indicate the need to employ an adaptation period to the basal diet to allow consumption rate and body weight to stabilize.

The amount of food spillage was so small that a correction factor was not employed.

Appendix A.3

Supplementary study no.3

The aim of this study was to determine whether the rats displayed a preference for either the basal diet or the crushed pellets when given the choice. The purpose of this study was to eliminate the possiblity that the different consistency and flavour of the basal diet caused the rats distress. It was reasoned that in a setting of free choice the rats would avoid the basal diet if it caused them distress.

Method

Twenty female rats were allocated to one of two groups (n=10 each) so that the mean body weight of each group was approximately the same. One group was fed crushed pellets and the other the basal diet for a period of 5 days. Thereafter both groups received a second feeding jar in their cages containing the alternate diet; the position, in the cage, of the original diet remaining the same. All subjects were then allowed free access to both diets for 7 consecutive days and daily consumption of each diet was recorded.

Results

The quantities of each diet consumed were summed to give the total daily consumption. The percentage of the total consumption for each diet was calculated. The consumption of each diet, over a 7 day period, expressed in terms of percentage of total consumption, is shown in Table A3.

A Student's t test, for independent samples, was performed using the percentage of their original diet eaten by each group during the 7 day choice period as the data for analysis (Columns 2 & 3, Table A3). The results indicate that rats, previously fed either basal diet of crushed pellets ate significantly more basal diet than crushed pellets when given the choice for 7 days ($t(12) = 8.86$, $p < .001$) . It was concluded that the basal diet was a palatable one and that it would provide no source of distress to the rats.

Table A3

Food consumption, expressed as percentage of total daily consumption, of two diets offered simultaneously to groups previously fed basal diet or crushed pellets

	Previously fed			
	Crushed pellets		Basal diet	
	% of total daily consumption			
Days with diet choice	Basal diet	Crushed pellets	Basal diet	Crushed pellets
1	86.8	13.2	51.7	48.3
2	73.7	26.3	53.3	46.7
3	64.3	35.7	62.1	37.9
4	70.5	29.5	70.8	29.2
5	73.5	26.5	69.0	31.0
6	79.8	20.2	74.7	25.3
7	76.1	23.9	74.7	25.3
Column	(1)	(2)	(3)	(4)

Appendix A.4

Supplementary study no.4

In keeping with the tradition adopted by some experimental nutritionists (Leung et al., 1968; Stead & Brock, 1972) an attempt was made to train the rats to adjust to a feeding regime in which access to food was restricted to a single daily 2 hour period.

The rationale for the "interval feeding" is, presumably, to control the time of intake across all experimental animals. After a period of interval feeding training, rats that have gained approximately the same amount of weight and are consuming about the same abount of food are selected for experimentation (Leung, et al., 1968).

The aim of the present study was to train rats to eat their daily requirements of the basal diet during a single daily 2 hour interval. The period of training was extended over a 2 week period.

Method

Twenty female rats with regular 4 day cycles, underwent a period of adaptation to eating the basal diet ad libitum from feeding jars. Subjects were then allocated to one of two groups (n=10 each) so that the mean body weight of each group was approximately equal.

At 1900h on day 1 of the experimental period the feeding jars were removed from the cages of the experimental group and replaced 22 hours later, at 1700h on day 2. After a 2 hr period of access to the basal diet the jars were removed and the 2 hr consumption rate was recorded. Free access to water was maintained throughout the study. The body weight of experimental and control rats were recorded at 1700h on days 1 and 2 and at the same time every second day thereafter. The 24 hr consumption rate of the control group, fed the basal diet and water ad libitum, was measured at 1900h each day. Vaginal smears were taken each day from all rats.

Results

Body weight: The changes in body weight are summarized in Table A4. The mean body weight values for day 1 were those measured at 1700h on day 1, before the basal diet was removed from the experimental group cages for 22 hours. The body weight of both groups were recorded on day 2 and every second day thereafter.

Table A4

Effects of 2 hr interval feeding schedule on body weights

Days on schedule	Mean body weight (g) ±SD	
	Experimental group (n=10)	Control group (n=10)
1	288.8 ± 10.7	288.0 ± 9.8
2	275.1 ± 10.6	289.2 ± 9.6
4	265.9 ± 12.9	291.1 ± 9.4
6	262.0 ± 14.5	292.7 ± 8.6
8	255.3 ± 15.9	294.2 ± 8.1
10	254.8 ± 14.3	295.9 ± 7.9
12	251.3 ± 16.3	297.8 ± 7.7
14	248.6 ± 16.6	299.9 ± 6.8

Analysis of variance revealed that the body weight of the experimental group was significantly lower than that of the control group on day 2 ($p < .05$) and remained significantly lower for the rest of the experimental period ($p < .01$).

Consumption rate: For purposes of statistical analysis the consumption rates were grouped into 3 day period (see Table A5). The consumption rate values for days 2-4 represent:

(i) in the case of the experimental group, the mean quantity of food eaten during a single 2 hr period on days 2, 3 and 4.

(ii) in the case of the control group, the mean 24 hr consumption rate up to 1900h on days 2, 3 and 4.

Table A5

Effects of 2 hr interval feeding schedule on food consumption

Days on schedule	Mean food consumption (g) ±SD	
	Experimental group (n=10)	Control group (n=10)
2-4	6.7±1.0	17.9± .31
5-7	8.7±1.2	18.0± .54
8-10	9.1±1.5	18.2± .55
11-13	9.7±1.5	18.1± .68
14-17	8.7±1.4	18.1± .42

Analysis of variance revealed that the consumption rate of the experimental group was significantly lower than that of the control group at all stages ($p < .01$). The mean consumption rate of the experimental group during the period 2-4 days was significantly lower than that during 5-16 ($p < .01$). There was no significant change in the 24 hr consumption rate of control subjects during the course of the experiment.

Vaginal smears: There was no cessation of cycling in experimental group animals. However, the majority of rats (70%) in the experimental group showed aberrations in the form of lengthened cycles. Although a 5 day cycle was the most common finding some rats had 6 or 7 day cycles with additional days of either leucocytic smear patterns or vaginal cornification. Only one rat in the control group showed evidence of a 5 day cycle.

Discussion

The mean body weight of rats on a 2 hr interval feeding regime remained significantly lower than that of the ad libitum fed control group from the second day onwards. The mean food consumption of the experimental group was significantly lower than that of the control group throughout the experiment. Furthermore, the majority of rats on the 2hr interval feeding regime showed lengthening of their normal 4 day oestrous cycle. It was for these reasons it was concluded that the 2 hr interval feeding procedure was not an appropriate one to employ in the series of nutritional experiments. Normal growth and the maintenance of normal oestrous cycling was not observed with animals on this feeding regime.

These findings appear to be inconsistent with the reports of Dufort (1964) and Dufort et al (1965) who claim that rats adjust to a 23 hr food deprivation schedule within 15-21 days. However, they defined adjustment in terms of stable food intake and weight gain (within group) but not necessarily equal to that of ad libitum fed controls.

The present findings are more consistent with those of Reid & Finger (1955) who found that, after 35 days on a 23 hr food deprivation schedule, the mean body weight and food consumption rate was significantly lower than that of controls.

Appendix A.5

Supplementary study no.5

The aim of this study was to determine whether normal food intake (equivalent to 24 hr intake of ad libitum fed rats) could be achieved by allowing rats two daily 2 hr interval feeding periods.

Method

After a period of adaptation to eating the basal diet from feeding jars 20 female rats were allocated to one of two groups (n=10 each) so that the mean body weight of each group was approximately equal. At 2000h on day 1 the feeding jars of the experimental group were removed for 12 hours until 0800h on day 2. The experimental group were given access to the basal diet during the hours of 0800-1000h (light period) and again during the hours of 2000-2200h (dark period). Consumption rates during both feeding periods were measured and total consumption was computed. The 24 hr consumption rate of the control group, with full access to the basal diet, was also recorded. Double interval feeding was continued for a further 5 days.

Results

The total consumption rate (2x2 hr feeding periods) of the experimental group and the 24 hr consumption rate of the control group are summarized in Table A6.

Analysis of variance revealed that the total consumption rate of the experimental group was significantly lower than that of the control group at all stages ($p < .01$). There was no significant change in the total consumption rate of the experimental group during the experimental phase ($p > .05$).

Table A6

Food consumption of rats on a twice daily 2 hr interval feeding schedule and control rats with ad lib access to food

Days on schedule	Mean food consumption (g) ± SD			
	Experimental group (n=10)			Control group (n=10)
	*L	D	T	
2	4.9±1.1	6.8±1.2	11.7±1.9	18.2±1.3
3	5.0± .84	8.4±1.8	13.4±1.8	18.1± .99
4	5.3± .67	7.1±2.0	12.4±2.0	18.5±1.1
5	5.4± .69	7.4±1.3	12.8±1.7	18.3±1.1
6	5.4± .96	7.8±1.3	13.2±1.5	18.1± .99
7	6.0±1.1	7.0± .94	13.0±1.5	18.3± .94

* L = Light period 0800-1000h

D = Dark period 2000-2200h

T = Total consumption

Discussion

Increasing access to food by a further 2 hour interval did not result in food intake reaching control values. It was thus concluded that this procedure could not be employed in the nutritional experiments of this series.

In a further series of studies, not reported on in detail here, it was found:

(i) that the consumption rate of crushed pellets during a 2 hr feeding interval did not differ significantly from the consumption rate of the basal diet. It would appear that the basal diet did not lead to more rapid satiation than the standard diet.

(ii) the consumption rate of the basal diet during a single 2 hr period during the dark phase was slightly, but not significantly, higher than the consumption rate during a single 2 hr feeding period during the light phase.

(iii) underweight rats, at the end of a period of interval feeding, did not reach the same weight as control animals during a 2 week period of full nutritional rehabilitation.

(iv) the inability of rats to consume their full daily intake of food during a 2 hr interval does not appear to be a function of the age of the animal. The same findings were obtained with young (8-10 weeks) rats and older rats (20-22 weeks).

Appendix A.6

Supplementary study no.6

In an attempt to eliminate the possible stress associated with reduction of bulk intake (Mason, 1968) a study was carried out to determine whether bulk intake could be maintained while controlling the nutritional content. A series of diets containing varying proportions of a non-nutritive bulk filler were presented to different groups of rats.

The basal diet was mixed with five grain vermiculite which is approved by the British Ministery of Agriculture, Fisheries and Food (see their circular DW 142A 28/12/1972) as a binder. That is, a non-nutritional substance which aids the compaction of feeding stuffs. When the basal diet was mixed with small quantities of vermiculite (eg. 15g basal diet + 5g vermiculite) the rats ate readily of this feed. However, when the proportions were reversed, with vermiculite making up the greater volume of the feed, the intake was reduced. For example, rats failed to eat 18g of a feed containing 5g basal diet and 13g vermiculite. Since the intake of such a mixture was both reduced and variable there was no way of controlling the nutritional content of feeds consumed by experimental animals.

It was thus concluded that the procedure of bulk compensation, in instances where nutritional content is exceeded in volume by vermiculite, would introduce a problem of regulation of nutritional content. It is possible that this procedure may be more successful with other species and with other, flavoured, non-nutritive substances.

Appendix A.7

Supplementary study no.7

The aim of this study was to determine the frequency of alterations in vaginal smear patterns of fully fed, non-stressed 4 day cycling rats.

Method

Thirty female rats were housed in individual cages and fed pelleted food and water ad libitum. All rats were transferred from the stock room without an attempt made to determine whether they had regular 4 day cycles. Vaginal smears were taken daily for a period of 12 weeks (spanning 21 anticipated 4 day cycles).

Results

During the first 8 days a 5 day cycle was found in 40% of the rats. This observation could indicate a response to a change in environment since the incidence of 5 day cycles dropped to 16.7% during the following cycle. At the end of 4 weeks 90% of the rats had normal 4 day cycles. The remaining rats had 5 or 6 cycles and continued to display extended cycles throughout the 12 week period.

If rats with persistently extended cycles were excluded then the incidence of irregularities of the oestrous cycle was only 3% during the last 10 weeks. The irregularities were in the form of additional days of either vaginal cornification or of a leucocytic smear pattern.

Discussion

The findings emphasised the need to exclude from experiments rats not showing two normal 4 day cycles during the adaptation phase.

Appendix A.8

Supplementary study no.8

The aim of this study was to obtain, from fully-fed non-stressed rats, control measurement collected at different times of the day on each day of the oestrous cycle. The following control measurements were recorded:

1. Serum LH concentration
2. Pituitary LH content and concentration.
3. Organ weights - pituitary gland, ovaries, uterus and adrenal glands.
4. Number of surface corpora lutea and follicles reaching criterion size.
5. Number of ova present at the ampullary-isthmus junction.

Method

Sixty sexually mature female rats were individualy housed and fed pelleted food and water ad libitum for 10 days. Thereafter 45 rats, with regular 4 day cycles, were allocated to one of 9 groups (n=5 each) so that the mean body weight of each group was approximately the same.

Rats were sacrificed, by means of cervical dislocation, at 1000 and 1600h on each day of the cycle and also at 1900h on pro-oestrus. Trunk blood samples were collected and autopsies were performed.

Results

Serum LH concentration: The serum LH levels at different times of the day on each day of the cycle are summarized in Table A7.

Table A7

Serum LH concentration at different stages of the 4 day oestrous cycle of non-stressed, fully fed rats (each group n=5)

	Mean serum LH (ng/ml) ±SD			
	Stage of cycle			
Time of day (h)	Oestrus	Metoestrus	Dioestrus	Pro-oestrus
1000h	19.4±8.1	46.4±18.8	15.2±13.9	52.6±23.5
1600h	21.9±14.3	45.8±32.1	19.2±11.3	410.0±330.6
1900h	-	-	-	896.0±117.6

In a separate study, blood samples were collected at various intervals from chronically cannulated Long Evans rats. After an appropriate period of post operative recovery blood samples were drawn at 1000, 1500 and 1700h on each day of the cycle. The serum LH values are summarized in Table A8.

Table A8

Serum LH concentration at different stages of the 4 day oestrous cycle of chronically cannulated rats

	Mean serum LH (ng/ml) ±SD			
	Stage of cycle			
Time of day (h)	Oestrus	Metoestrus	Dioestrus	Pro-oestrus
1000h	29.1±14.9(6)	30.8±17.2(3)	16.0± .0(3)	35.1±16.6(7)
1500h	29.8±11.8(5)	26.7±16.7(4)	26.2±10.8(3)	31.8±11.3(5)
1700h	25.9± 6.9(5)	38.6±12.7(5)	22.8± 9.0(4)	406.7±53.9(3)

Note: Figures in parentheses indicate the number of observations.

When this study was carried out the time at which the pro-oestrous LH surge reached its peak in this strain of rat was not known. Additional sampling revealed that the serum LH levels continued to rise after 1700h on pro-oestrus to reach a peak at about 1900h. Consequently blood samples were collected at 1900h, on pro-oestrus, in subsequent studies.

The mean pituitary LH content and concentration are summarized in Table A9.

The mean organ weights are summarized in Table A10.

The mean number of surface corpora lutea and ovarian follicles, the incidence of ovulation and the mean number of ova per ovulating rat is shown in Table A11.

Table A9

Pituitary LH content and concentration at different stages of the 4 day oestrous cycle of non-stressed, fully fed rats (means±SD)

Stage of cycle and time (h)	Mean Pituitary LH content ±SD (µg)	Mean Pituitary LH concentration ±SD (µg/mg wet wt.)
Oestrus		
1000	174.0±24.9	13.6±1.7
1600	160.4±16.6	13.0±1.8
Metoestrus		
1000	197.0±37.4	15.1±2.4
1600	201.8±58.9	15.6±4.6
Dioestrus		
1000	188.6±38.4	15.0±2.5
1600	200.6±17.8	15.2±1.4
Pro-oestrus		
1000	250.1±35.5	18.9±3.2
1600	162.5±12.5	12.5±1.4
1900	195.6±52.4	14.3±3.8

Table A10
ORGAN WEIGHTS OF NON-STRESSED, FULLY FED RATS AT DIFFERENT TIMES OF THE DAY ON EACH DAY OF THE OESTROUS CYCLE

Group				Mean organ weight ±SD							
				Pituitary		Ovaries		Uterus		Adrenals	
Stage of cycle	Time of day (hours)	No. of rats	Mean BW at autopsy ±SD (g)	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW
Oestrus	1000	5	254.0±4.9	12.8± .60	5.0± .15	73.6±3.1	28.9±1.4	432.3±68.7	170.1±25.8	53.4± .93	21.0± .40
	1600	5	253.2±4.9	12.4± .98	4.9± .32	69.7±2.5	27.6± .72	413.8±49.9	163.5±19.8	52.9±2.9	20.9±1.4
Metoestrus	1000	5	252.5±5.9	13.0± .14	5.2± .14	71.7±6.3	28.4±2.1	399.4±50.3	158.4±21.0	52.2±3.2	20.7±1.2
	1600	5	252.6±5.0	12.9± .63	5.1± .31	74.2±4.0	29.3±1.4	409.5±48.6	161.9±19.4	51.0±4.1	20.2±1.4
Dioestrus	1000	5	253.8±5.1	12.6± .85	5.0± .35	73.1±4.8	28.8±2.0	386.5±28.9	152.3± 9.1	50.5±1.7	19.9± .52
	1600	5	254.4±5.6	13.2± .69	5.2± .21	70.0±5.0	27.5±1.1	401.9±39.6	158.0±14.4	52.0±3.4	20.4± .94
Pro-oestrus	1000	5	252.4±4.3	13.2± .74	5.2± .22	77.8±4.3	30.8±1.6	514.9±56.7	203.9±21.1	52.3±3.1	20.7±1.1
	1600	5	254.2±4.2	13.0± .80	5.1± .25	78.2±5.1	30.7±1.7	536.2±64.8	211.1±27.8	51.3±4.5	20.2±1.6
	1900	5	253.8±4.3	13.7± .51	5.4± .46	77.3±2.6	30.5±1.3	609.3±49.5	240.1±19.2	52.2±3.2	20.6±1.3

Table A11

Numbers of corpora lutea and ovarian follicles and incidence of ovulation at different stages of the 4 day oestrous cycle of nonstressed, fully fed rats (Means ±SD)

Stage of cycle and time(h)	No. of rats	Mean no. of corpora lutea±SD	Mean no.of follicles ±SD	Proportion of rats ovulating	Mean no. of ova per ovulating rat
Oestrus					
1000h	5	18.8±3.8	2.6± .89	5/5	12.8± .84
1600h	5	19.8±4.5	2.0±1.6	5/5	10.8±2.4
Metoestrus					
1000h	5	17.8±2.6	1.4±1.1	0/5	-
1600h	5	18.2±3.8	1.2± .84	0/5	-
Dioestrus					
1000h	5	16.0±4.6	5.2±2.6	0/5	-
1600h	5	19.6±2.7	6.4±2.4	0/5	-
Pro-oestrus					
1000h	5	16.4±3.6	11.0±2.2	0/5	-
1600h	5	13.4±2.9	10.6±1.8	0/5	-
1900h	5	15.4±2.7	14.6±2.7	0/5	-

APPENDIX A.9

STATISTICS SUMMARY TABLES: SUPPLEMENTARY STUDIES

TABLE A12

TWO WAY ANALYSIS OF VARIANCE WITH REPEATED MEASURES ON FACTOR B

Supplementary study No 2: Body weight

SOURCE	df	MS	F	p value
Between subjects				
A (Diet)	2	230.90	1.01	ns
Subjects within groups	27	229.00		
Within subjects				
B (Time elapsed)	9	693.37	36.49	<.01
AB	18	26.25	1.35	ns
B x Subjects within groups	243	19.40		

ns = not significant $p > .05$

TABLE A13

TWO WAY ANALYSIS OF VARIANCE WITH REPEATED MEASURES ON FACTOR B

Supplementary study no.2: Food consumption rate

SOURCE	df	MS	F	p value
Between subjects				
A (Diet)	1	9.00	5.23	< .05
Subjects within groups	18	1.72		
Within subjects				
B (Time elapsed)	9	8.38	8.62	< .01
AB	9	8.63	8.88	< .01
B x Subjects within groups	162	.97		

ANALYSIS OF SIMPLE MAIN EFFECTS

Supplementary study no.2: Food consumption rate

SOURCE	df	MS	F	p value
A at B1	1	.20	.19	ns
B2	1	76.05	72.64	< .01
B3	1	8.45	8.07	< .01
B4	1	.80	.76	ns
B5	1	.45	.43	ns
B6	1	.0	.0	ns
B7	1	.20	.19	ns
B8	1	.45	.43	ns
B9	1	.05	.05	ns
B10	1	.05	.05	ns
W cell	180	1.05		
B at A1	9	16.94	17.43	< .01
A2	9	.18	.19	ns
B X Subject within groups	162	.97		

ns = not significant p > .05

TABLE A14

TWO WAY ANALYSIS OF VARIANCE WITH REPEATED MEASURES ON FACTOR B

Supplementary study no.4: Body weight

SOURCE	df	MS	F	p value
Between subjects				
A (Diet)	1	38131.00	37.15	<.01
Subjects within groups	18	1026.28		
Within subjects				
B (Time elapsed)	7	484.00	7.89	<.01
AB	7	1533.71	25.01	<.01
B x Subjects within groups	126	61.32		

ANALYSIS OF SIMPLE MAIN EFFECTS

Supplementary study no.4: Body weight

SOURCE	df	MS	F	p value
A at B1	1	3.20	.02	ns
B2	1	994.00	5.46	<.05
B3	1	3175.20	17.45	<.01
B4	1	4712.40	25.90	.01
B5	1	7566.00	41.59	<.01
B6	1	8446.00	46.42	<.01
B7	1	10811.20	59.42	<.01
B8	1	13158.40	72.32	<.01
W cell	144	181.93		
B at A1	7	1845.14	30.09	<.01
A2	7	172.57	2.81	<.05
B x Subjects within groups	126	61.32		

ns = not significant p > .05

TABLE A15

TWO WAY ANALYSIS OF VARIANCE WITH REPEATED MEASURES ON FACTOR B

Supplementary study no.4: Food consumption rate

SOURCE	df	MS	F	p value
Between subjects				
A (Diet)	1	2246.80	2224.60	< .01
Subjects within groups	18	1.01		
Within subjects				
B (Time elapsed)	4	7.52	18.34	< .01
AB	4	5.38	13.12	< .01
B x Subjects within groups	72	.41		

ANALYSIS OF SIMPLE MAIN EFFECTS

Supplementary study no. 4: Food consumption rate

SOURCE	df	MS	F	p value
A at B1	1	627.20	1183.40	<.01
B2	1	432.45	815.9	<.01
B3	1	414.05	781.20	<.01
B4	1	352.80	665.70	<.01
B5	1	441.80	883.58	<.01
W cell	90	.53		
B at A1	4	12.72	31.02	<.01
A2	4	.13	.32	ns
B x Subjects within groups	72	.41		

ns = not significant p > .05

TABLE A16

TWO WAY ANALYSIS OF VARIANCE WITH REPEATED MEASURES ON FACTOR B

Supplementary study no.5: Food consumption rate

SOURCE	df	MS	F	p value
Between subjects				
A (Diet)	1	907.50	198.14	< .01
Subjects within groups	18	4.58		
Within subjects				
B (Time elapsed)	5	1.66	1.04	ns
AB	5	2.40	1.50	ns
B x Subjects within groups	90	1.60		

ns = not significant $p > .05$

Appendix B

Table B1

Composition of Mineral Mixture

(Dreyer, 1976, pers. comm.)

Ingredients	g/kg
$CaCO_3$	173.3
K_2HPO_4	196.6
$CaHPO_4.2H_2O$	194.3
$Na_2HOP_4.12H_2O$	101.1
$MgSO_4.7H_2O$	208.4
NaCl	66.0
Ca lactate.$5H_2O$	43.5
Fe citrate	16.9
KI	.4519
$MnSO_4.H_2O$	.1582
$CuSO_4.5H_2O$	.1589
$ZnCl_2$	.1476

Table B2

Composition of Vitamin Diet Fortification Mixture (ICN)

Ingredients	g/100 lbs
Vitamin A Concentrate	4.5
Vitamin D Concentrate	.25
Alpha Tocopherol	5.0
Ascorbic acid	45.0
Inositol	5.0
Choline chloride	75.0
Menadione	2.25
p - Amino benzoic acid	5.0
Niacin	4.5
Riboflavin	1.0
Pyridoxine hydrochloride	1.0
Thiamine hydrochloride	1.0
Calcium pantothenate	3.0
Biotin	20.0
Folic Acid	90.0
Vitamin B-12	1.35

STATISTICS SUMMARY TABLES CHAPTER 4-6

TABLE C1

TWO WAY ANALYSIS OF VARIANCE WITH REPEATED MEASURES ON FACTOR B

Experiment 1, Chapter 4:Body weight

SOURCE	df	MS	F	p value
Between subjects				
A (Diet)	3	7838.84	4.18	<.05
Subjects within groups	25	1877.52		
Within subjects				
B (Time elapsed)	7	610.68	29.22	<.01
AB	21	919.67	44.00	<.01
B x Subjects within groups	175	20.90		

ANALYSIS OF SIMPLE MAIN EFFECTS

Experiment 1, Chapter 4: Body weight

SOURCE	df	MS	F	p value
A at B1	3	39.17	.15	ns
B2	3	52.08	.21	ns
B3	3	108.83	.43	ns
B4	3	565.83	2.24	ns
B5	3	1438.08	5.68	<.01
B6	3	2627.75	10.39	<.01
B7	3	3710.08	14.67	<.01
B8	3	4714.33	18.64	<.01
W cell	200	252.98		
B at A1	7	426.93	20.42	<.01
A2	7	41.21	1.97	ns
A3	7	689.79	33.00	<.01
A4	7	1697.86	81.23	<.01
B x Subjects within groups	87	20.90		

ns = not significant p > .05

TABLE C2

TWO WAY ANALYSIS OF VARIANCE WITH REPEATED MEASURES ON FACTOR B

Experiment 2, Chapter 4:Body weight

SOURCE	df	MS	F	p value
Between subjects				
A (Diet)	3	8432.71	71.62	<.01
Subjects within groups	16	117.75		
Within subjects				
B (Time elapsed)	3	38.54	3.52	<.05
AB	9	1338.96	122.42	<.01
B x Subjects within groups	48	10.94		

ANALYSIS OF SIMPLE MAIN EFFECTS

Experiment 2, Chapter 4:Body weight

SOURCE	df	MS	F	p value
A at B1	3	7.67	.20	ns
B2	3	1257.50	33.41	<.01
B3	3	3869.25	102.80	<.01
B4	3	7315.58	194.35	<.01
W cell	64	37.64		
B at A1	3	1058.25	96.75	<.01
A2	3	336.42	30.76	<.01
A3	3	49.92	4.56	<.01
A4	3	2611.00	238.72	<.01
B x Subjects within groups	48	10.94		

ns = not significant $p > .05$

TABLE C3

ONE WAY ANALYSIS OF VARIANCE

Experiment 2, Chapter 4:Serum LH levels

SOURCE	df	MS	F	p value
		Serum LH:After 7 days		
Between groups	3	537.67	1.74	ns
Within groups	16	309.80		
		Serum LH:After 14 days		
Between groups	3	528.32	2.53	ns
Within groups	16	209.13		
		Serum LH:After 21 days		
Between groups	3	1198.20	7.23	<.01
Within groups	16	165.83		

ns = not significant $p > .05$

TABLE TABLE C4

TWO WAY ANALYSIS OF VARIANCE WITH REPEATED MEASURES ON FACTOR B

Experiment 3, Chapter 4: Body weight

SOURCE	df	MS	F	p value
Between subjects				
A (Diet)	1	35166.00	184.06	< .01
Subjects within groups	8	191.05		
Within subjects				
B (Time elapsed)	4	1513.40	7.52	< .01
AB	4	6107.50	30.35	< .01
B x Subjects within groups	32	201.25		

ANALYSIS OF SIMPLE MAIN EFFECTS

Experiment 3, Chapter 4: Body weight

SOURCE	df	MS	F	p value
A at B1	1	12.10	.06	ns
B2	1	476.10	2.39	ns
B3	1	6708.10	33.67	< .01
B4	1	17472.40	87.71	< .01
B5	1	34928.10	175.33	< .01
W cell	40	199.21		
B at A1	4	6827.18	33.92	< .01
A2	4	793.95	3.95	ns
B x Subjects within groups	32	201.25		

ns = not significant $p > .05$

TABLE C5

ONE WAY ANALYSIS OF VARIANCE

Experiment 3, Chapter 4

SOURCE	df	MS	F	p value
		Absolute pituitary weight		
Between groups	3	24.91	38.53	<.01
Within groups	16	.65		
		Relative pituitary weight		
Between groups	3	.17	.88	ns
Within groups	16	.19		
		Absolute ovarian weight		
Between groups	3	1028.90	25.53	<.01
Within groups	16	43.70		
		Relative ovarian weight		
Between groups	3	3.14	.89	ns
Within groups	16	3.52		
		Absolute uterine weight		
Between groups	3	67217.23	26.34	<.01
Within groups	16	2552.53		
		Relative uterine weight		
Between groups	3	3430.42	5.62	<.01
Within groups	16	610.03		
		Absolute adrenal weight		
Between groups	3	31.37	.53	ns
Within groups	16	59.22		
		Relative adrenal weight		
Between groups	3	161.14	9.19	<.01
Within groups	16	17.53		
		Serum LH concentration		
Between groups	3	686.84	9.40	<.01
Within groups	16	73.10		

TABLE C5 continued

ONE WAY ANALYSIS OF VARIANCE

SOURCE	df	MS	F	p value
		Pituitary LH content		
Between groups	3	19146.18	10.67	<.01
Within groups	16	1793.58		
		Pituitary LH concentration		
Between groups	3	52.81	4.42	<.05
Within groups	16	11.94		
		Number of corpora lutea		
Between groups	3	75.87	12.97	<.01
Within groups	16	5.85		

ns = not significant p > .05

TABLE C6

TWO WAY ANALYSIS OF VARIANCE WITH REPEATED MEASURES ON FACTOR B

Experiment 4, Chapter 4: Body weight

SOURCE	df	MS	F	p value
Between subjects				
A (Diet)	1	52334.00	74.67	<.01
Subjects within groups	8	700.88		
Within subjects				
B (Time elapsed)	6	5912.35	369.06	<.01
AB	6	4568.00	285.14	<.01
B x Subjects within groups	48	16.02		

ANALYSIS OF SIMPLE MAIN EFFECTS

Experiment 4, Chapter 4: Body weight

SOURCE	df	MS	F	p value
A at B1	1	.40	.003	ns
B2	1	7236.10	63.53	<.01
B3	1	17139.67	150.48	<.01
B4	1	34290.00	301.05	<.01
B5	1	18960.30	166.46	<.01
B6	1	3764.00	33.04	<.01
B7	1	152.60	1.34	ns
W cell	56	113.86		
B at A1	6	9260.90	578.08	<.01
A2	6	1219.45	76.12	<.01
B x Subjects within groups	48	16.02		

ns = not significant p >.05

TABLE C7

ONE WAY ANALYSIS OF VARIANCE

Experiment 4, Chapter 4

SOURCE	df	MS	F	p value
		Absolute pituitary weight		
Between groups	4	17.63	27.22	< .01
Within groups	20	.65		
		Relative pituitary weight		
Between groups	4	.27	3.42	< .05
Within groups	20	.08		
		Absolute ovarian weight		
Between groups	4	910.03	48.75	< .01
Within groups	20	18.67		
		Relative ovarian weight		
Between groups	4	39.99	10.15	< .01
Within groups	20	3.94		
		Absolute uterine weight		
Between groups	4	34967.11	84.05	< .01
Within groups	20	416.05		
		Relative uterine weight		
Between groups	4	1608.14	56.57	< .01
Within groups	20	28.43		
		Absolute adrenal weight		
Between groups	4	35.30	.79	ns
Within groups	20	44.93		
		Relative adrenal weight		
Between groups	4	38.89	7.45	< .01
Within groups	20	5.22		
		Serum LH concentration		
Between groups	4	239.84	9.53	< .01
Within groups	20	25.18		

TABLE C7 continued

ONE WAY ANALYSIS OF VARIANCE

SOURCE	df	MS	F	p value
		Pituitary LH content		
Between groups	4	21752.30	20.23	< .01
Within groups	20	1075.04		
		Pituitary LH concentration		
Between groups	4	76.47	11.47	< .01
Within groups	20	6.67		
		Number of corpora lutea		
Between groups	4	109.64	16.03	< .01
Within groups	20	6.84		
		Number of ovarian follicles		
Between groups	3	19.33	4.99	< .01
Within groups	16	3.87		

ns = not significant $p > .05$

TABLE C8

ONE WAY ANALYSIS OF VARIANCE

Experiment 6, Chapter 4

SOURCE	df	MS	F	p value
	Initial body weight			
Between groups	2	4.46	.03	ns
Within groups	12	115.06		
	Body weight at first oestrus			
Between groups	2	27.80	.70	ns
Within groups	12	39.36		
	Latency of resumption of oestrous cycles			
Between groups	2	162.46	152.31	<.01
Within groups	12	1.06		

ns = not significant p >.05

TABLE C9

ONE WAY ANALYSIS OF VARIANCE

Experiment 7, Chapter 4

SOURCE	df	MS	F	p value
	Pre body weight			
Between groups	2	1.87	.18	ns
Within groups	12	10.17		
	Body weight at seven days			
Between groups	2	4554.20	192.97	<.01
Within groups	12	23.60		
	Body weight at fourteen days			
Between groups	2	12666.07	640.78	<.01
Within groups	12	19.77		

ns = not significant p > .05

TABLE TABLE C10

TWO WAY ANALYSIS OF VARIANCE WITH REPEATED MEASURES ON FACTOR B

Experiment 7, Chapter 4: Serum LH after LHRH administration

SOURCE	df	MS	F	p value
Between subjects				
A (Treatment)	2	107465.00	36.68	<.01
Subjects within groups	12	2929.90		
Within subjects				
B (Time elapsed)	5	151977.00	100.92	<.01
AB	10	21069.40	13.99	<.01
B x Subjects within groups	60	1505.86		

ANALYSIS OF SIMPLE MAIN EFFECTS

Experiment 7, Chpater 4: Serum LH after LHRH administration

SOURCE	df	MS	F	p value
A at B1	2	1685.40	.97	ns
B2	2	8067.20	4.63	<.05
B3	2	130418.00	74.82	<.01
B4	2	64088.30	36.76	<.01
B5	2	7004.60	4.02	<.05
B6	2	1548.20	.89	ns
W cell	72	1743.20		
B at A1	5	101091.00	67.13	<.01
A2	5	4449.20	2.95	<.05
A3	5	88576.30	58.82	<.01
B x subjects within groups	40	1505.86		

ns = not significant p > .05

TABLE C11

ONE WAY ANALYSIS OF VARIANCE

Experiment 8, Chapter 4

SOURCE	df	MS	F	p value
		Final body weight		
Between groups	6	58.23	.54	ns
Within groups	28	106.84		
		Absolute pituitary weight		
Between groups	6	5.18	6.85	< .01
Within groups	28	.76		
		Relative pituitary weight		
Between groups	6	3.40	8.98	< .01
Within groups	28	.38		
		Absolute ovarian weight		
Between groups	6	75.48	1.32	ns
Within groups	28	56.98		
		Relative ovarian weight		
Between groups	6	26.99	.96	ns
Within groups	28	28.18		
		Absolute uterine weight		
Between groups	6	15690.72	9.61	< .01
Within groups	28	1632.65		
		Relative uterine weight		
Between groups	6	6901.16	7.05	< .01
Within groups	28	979.20		
		Absolute adrenal weight		
Between groups	6	.63	.10	ns
Within groups	28	6.29		
		Relative adrenal weight		
Between groups	6	2.37	.29	ns
Within groups	28	8.12		

TABLE C11 continued

ONE WAY ANALYSIS OF VARIANCE

SOURCE	df	MS	F	p value
		Pituitary LH content		
Between groups	6	367.11	.65	ns
Within groups	28	564.06		
		Pituitary LH concentration		
Between groups	6	4.96	1.62	ns
Within groups	28	3.04		
		Number of corpora lutea		
Between groups	6	17.86	10.60	< .01
Within groups	28	1.69		

ns = not significant $p > .05$

TABLE C12

ONE WAY ANALYSIS OF VARIANCE

Experiment 9, Chapter 4

SOURCE	df	MS	F	p value
		Initial body weight		
Between groups	3	10.27	.19	ns
Within groups	16	54.15		
		Final body weight		
Between groups	3	35.00	3.16	ns
Within groups	16	11.08		
		Absolute pituitary weight		
Between groups	3	7.41	10.00	< .01
Within groups	16	.74		
		Relative pituitary weight		
Between groups	3	2.57	10.50	< .01
Within groups	16	.25		
		Absolute ovarian weight		
Between groups	3	2112.00	273.68	< .01
Within groups	16	7.72		
		Relative ovarian weight		
Between groups	3	782.86	173.16	< .01
Within groups	16	4.52		
		Absolute uterine weight		
Between groups	3	53714.30	84.49	< .01
Within groups	16	635.72		
		Relative uterine weight		
Between groups	3	20164.33	99.17	< .01
Within groups	16	203.34		
		Absolute adrenal weight		
Between groups	3	1.21	.20	ns
Within groups	16	6.09		

TABLE C12 continued

ONE WAY ANALYSIS OF VARIANCE

SOURCE	df	MS	F	p value
		Relative adrenal weight		
Between groups	3	1.46	.80	ns
Within groups	16	1.82		
		Number of corpora lutea		
Between groups	3	4.40	2.07	ns
Within groups	16	2.13		
		Number of ovarian follicles		
Between groups	2	2.60	.30	ns
Within groups	12	8.53		

ns = not significant $p > .05$

TABLE C13

ONE WAY ANALYSIS OF VARIANCE
Experiment 10, Chapter 4.

SOURCE	df	MS	F	p value
		Initial body weight		
Between groups	2	.46	.01	ns
Within groups	12	29.73		
		Final body weight		
Between groups	2	2561.26	75.47	<.01
Within groups	12	33.93		
		Absolute pituitary weight		
Between groups	2	3.33	12.73	<.01
Within groups	12	.26		
		Relative pituitary weight		
Between groups	2	.31	3.30	ns
Within groups	12	.09		
		Absolute ovarian weight		
Between groups	2	77.46	4.16	<.05
Within groups	12	18.61		
		Relative ovarian weight		
Between groups	2	40.83	8.55	<.01
Within groups	12	4.77		
		Absolute uterine weight		
Between groups	2	4814.05	2.13	ns
Within groups	12	2255.30		
		Relative uterine weight		
Between groups	2	437.87	.60	ns
Within groups	12	723.24		
		Absolute adrenal weight		
Between groups	2	5.66	.85	ns
Within groups	12	6.70		

TABLE C13 continued

ONE WAY ANALYSIS OF VARIANCE

SOURCE	df	MS	F	p value
	Relative adrenal weight			
Between groups	2	69.60	22.78	<.01
Within groups	12	3.06		
	Number of corpora lutea			
Between groups	2	8.27	1.45	ns
Within groups	12	5.70		
	Number of ovarian follicles			
Between groups	2	11.67	1.39	ns
Within groups	12	8.37		
	Number of ova per ovulating rat			
Between groups	2	41.52	8.00	<.01
Within groups	8	5.19		

ns = not significant p >.05

TABLE C14

TWO WAY ANALYSIS OF VARIANCE WITH REPEATED MEASURES ON FACTOR B

Experiment 1, Chapter 5: Lick suppression ratios

SOURCE	df	MS	F	p value
Between subjects				
A (Conditioning)	1	11406.30	269.40	< .01
Subjects within groups	18	42.34		
Within subjects				
B (Time elapsed)	4	750.45	40.80	< .01
AB	4	922.93	50.16	< .01
B x Subjects within groups	72	18.40		

ANALYSIS OF SIMPLE MAIN EFFECTS

Experiment 1, Chapter 5: Lick suppression ratios

SOURCE	df	MS	F	p value
A at B1	1	24.20	1.04	ns
B2	1	2420.00	104.36	< .01
B3	1	3781.25	163.06	< .01
B4	1	4867.20	209.87	< .01
B5	1	4004.00	172.66	< .01
W cell	90	23.19		
B at A1	4	1658.03	90.11	< .01
A2	4	15.35	.83	ns
B x subjects within groups	72	18.40		

ns = not significant p >.05

TABLE C15

ONE WAY ANALYSIS OF VARIANCE

Experiment 2, Chapter 5.

SOURCE	df	MS	F	p value
	Serum LH concentration: Dioestrus			
Between groups	4	53.80	.32	ns
Within groups	20	170.24		
Serum LH concentration: Dioestrus incl. normal control values				
Between groups	5	56.37	.32	ns
Within groups	24	175.90		
	Serum LH concentration: Pro-oestrus			
Between groups	2	37053.27	2.02	ns
Within groups	12	18376.40		
Serum LH concentration: Pro-oestrus incl. normal control values				
Between groups	3	44969.40	2.61	ns
Within groups	16	17239.80		
	Final body weight			
Between groups	7	17.77	.69	ns
Within groups	32	25.61		
	Absolute pituitary weight			
Between groups	7	1.17	2.00	ns
Within groups	32	.58		
	Relative pituitary weight			
Between groups	7	.11	2.31	ns
Within groups	32	.46		
	Absolute ovarian weight			
Between groups	7	19.18	.52	ns
Within groups	32	37.06		
	Relative ovarian weight			
Between groups	7	1.91	.50	ns
Within groups	32	3.81		

TABLE C15 continued

ONE WAY ANALYSIS OF VARIANCE

SOURCE	df	MS	F	p value
		Absolute uterine weight		
Between groups	7	31362.65	19.17	< .01
Within groups	32	1636.27		
		Relative uterine weight		
Between groups	7	4258.21	20.33	< .01
Within groups	32	209.43		
		Absolute adrenal weight		
Between groups	7	5.53	1.20	ns
Within groups	32	4.63		
		Relative adrenal weight		
Between groups	7	.53	.77	ns
Within groups	32	.69		

ns = not significant p >.05

TABLE C16

ONE WAY ANALYSIS OF VARIANCE

Experiment 1, Chapter 6

SOURCE	df	MS	F	p value
Mean number of ova per ovulating rat: Full rehabilitation groups				
Between groups	2	24.06	4.83	< .05
Within groups	11	4.98		
Number of corpora lutea: Full rehabilitation groups				
Between groups	2	2.47	.85	ns
Within groups	12	2.90		
Number of follicles: Full rehabilitation groups				
Between groups	2	.87	.87	ns
Within groups	12	1.00		
Serum LH: Full rehabilitation groups including normal control value				
Between groups	3	42.73	.92	ns
Within groups	16	46.50		
Serum LH: Gradual rehabilitation groups including normal control value				
Between groups	2	35.47	.89	ns
Within groups	12	40.00		
Relative adrenal weight: Both rehabilitation groups				
Between groups	4	1.96	2.83	ns
Within groups	20	.69		

ns = not significant p >.05

TABLE C17

ONE WAY ANALYSIS OF VARIANCE
Experiment 2, Chapter 6

Full rehabilitation group

SOURCE	df	MS	F	p value
		Initial body weight		
Between groups	2	2.07	.14	ns
Within groups	12	14.97		
		Final body weight		
Between groups	2	1033.80	31.58	< .01
Within groups	12	32.77		
		Absolute pituitary weight		
Between groups	2	18.66	71.57	< .01
Within groups	12	.26		
		Relative pituitary weight		
Between groups	2	2.14	70.49	< .01
Within groups	12	.03		
		Absolute ovarian weight		
Between groups	2	636.22	32.13	< .01
Within groups	12	19.80		
		Relative ovarian weight		
Between groups	2	74.36	17.61	< .01
Within groups	12	4.22		
		Absolute uterine weight		
Between groups	2	9470.75	51.46	< .01
Within groups	12	184.04		
		Relative uterine weight		
Between groups	2	1106.25	62.31	< .01
Within groups	12	17.75		
		Absolute adrenal weight		
Between groups	2	12.17	3.03	ns
Within groups	12	4.02		

TABLE C17 continued

ONE WAY ANALYSIS OF VARIANCE

SOURCE	df	MS	F	p value
		Relative adrenal weight		
Between groups	2	1.95	2.55	ns
Within groups	12	.76		
		Number of corpora lutea		
Between groups	2	7.27	1.80	ns
Within groups	12	4.03		
		Number of ovarian follicles		
Between groups	2	88.07	32.22	< .01
Within groups	12	2.73		

ns = not significant p >.05

TABLE C18

ONE WAY ANALYSIS OF VARIANCE

Experiment 2, Chapter 6
Gradual rehabilitation groups

SOURCE	df	MS	F	p value
		Initial body weight		
Between groups	2	1.27	.10	ns
Within groups	12	13.03		
		Final body weight		
Between groups	2	45.00	.92	ns
Within groups	12	48.97		
		Absolute pituitary weight		
Between groups	2	12.36	103.89	< .01
Within groups	12	.12		
		Relative pituitary weight		
Between groups	2	2.37	91.36	< .01
Within groups	12	.03		

TABLE C18 continued

ONE WAY ANALYSIS OF VARIANCE

SOURCE	df	MS	F	p value
		Absolute ovarian weight		
Between groups	2	1811.17	118.07	< .01
Within groups	12	15.34		
		Relative ovarian weight		
Between groups	2	373.74	235.17	< .01
Within groups	12	1.59		
		Absolute uterine weight		
Between groups	2	38466.99	123.40	< .01
Within groups	12	311.72		
		Relative uterine weight		
Between groups	2	7955.95	322.51	< .01
Within groups	12	24.67		
		Absolute adrenal weight		
Between groups	2	18.01	3.04	ns
Within groups	12	5.92		
		Relative adrenal weight		
Between groups	2	3.35	1.84	ns
Within groups	12	1.82		
		Number of corpora lutea		
Between groups	2	1.87	.76	ns
Within groups	12	2.47		

ns = not significant p > .05

TABLE C19

ONE WAY ANALYSIS OF VARIANCE
Experiment 2, Chapter 6
Low weight maintenance groups

SOURCE	df	MS	F	p value
		Initial body weight		
Between groups	2	.07	.003	ns
Within groups	12	20.13		
		Final body weight		
Between groups	2	55.27	2.08	ns
Within groups	12	26.60		
		Absolute pituitary weight		
Between groups	2	9.21	45.56	< .01
Within groups	12	.20		
		Relative pituitary weight		
Between groups	2	3.05	67.02	< .01
Within groups	12	.05		
		Absolute ovarian weight		
Between groups	2	2176.74	337.28	< .01
Within groups	12	6.45		
		Relative ovarian weight		
Between groups	2	703.50	771.10	< .01
Within groups	12	.91		
		Absolute uterine weight		
Between groups	2	75147.79	340.17	< .01
Within groups	12	220.91		
		Relative uterine weight		
Between groups	2	24547.47	2164.54	< .01
Within groups	12	11.34		
		Absolute adrenal weight		
Between groups	2	4.57	.88	ns
Within groups	12	5.21		

TABLE C19 continued

ONE WAY ANALYSIS OF VARIANCE

SOURCE	df	MS	F	p value
		Relative adrenal weight		
Between groups	2	.02	.009	ns
Within groups	12	2.13		
		Number of corpora lutea		
Between groups	2	29.40	8.56	< .01
Within groups	12	3.43		

ns = not significant p > .05

APPENDIX D

Table D1
EFFECTS OF SHOCK ADMINISTRATION ON BODY AND ORGAN WEIGHTS AT FIRST OESTRUS DURING REHABILITATION AFTER 21 DAYS FEEDING ON DFF DIET: EXPERIMENT 1,CHAPTER 6

Group	No. of rats	Mean initial BW±SD (g)	Mean final BW±SD (g)	BW Change (%)	Mean organ weight ±SD							
					Pituitary		Ovaries		Uterus		Adrenals	
					Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW
Full rehab												
1	5	251.8±5.4	244.2±3.5	-3.0	9.6± .59	3.9± .21	63.2±2.6	25.9±1.6	309.0±16.0	126.5±5.3	51.1±2.4	20.9±1.2
2	5	250.8±5.8	246.6±5.5	-1.7	9.4± .60	3.8± .23	68.9±3.4	28.0±2.0	322.7±18.4	130.9±6.2	49.3±2.7	20.0± .97
3	5	252.0±5.9	273.8±4.4	+8.6	12.8± .45	4.7± .15	78.6±4.8	28.7±2.7	372.8±13.2	136.2±4.4	57.9±2.0	21.1± .52
Gradual rehab												
4	5	252.0±6.4	275.7±3.1	+9.4	13.6± .89	4.9± .36	74.3±6.2	27.0±3.1	364.5±14.9	132.2±4.9	59.2±2.3	21.5± .76
5	5	251.6±5.9	247.0±4.7	-1.8	10.7± .48	4.3± .33	67.4±3.8	27.3±1.7	338.4±11.4	137.0±3.5	50.0±2.1	20.3± .54

Table D2
EFFECTS OF SHOCK ADMINISTRATION AND 20IU PMS ON ORGAN WEIGHTS DURING FEEDING ON 3 DIETARY REGIMEN FOLLOWING 21 DAYS CALORIC DEPRIVATION: EXPERIMENT 2,CHAPTER 6

Group	No. of rats	Mean initial BW±SD (g)	Mean final BW±SD (g)	BW Change (%)	Mean organ weight ±SD							
					Pituitary		Ovaries		Uterus		Adrenals	
					Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW	Absolute (mg)	/100gBW
Full rehab												
1	5	251.4±3.4	244.2±3.8	-2.9	13.3± .38	5.5± .21	86.1±4.7	35.3±2.4	385.2±16.6	157.7±5.9	51.5±2.5	21.1±1.3
2	5	252.6±4.5	268.8±8.1[a]	+6.4	13.8± .47	5.1± .06	87.9±3.6	32.8±1.9	389.5±9.6	144.9±2.3[b]	53.5±1.7	19.9± .18
3	5	251.6±6.7	243.6±4.4	-3.2	10.2± .64[c]	4.2± .21[c]	67.6±5.0[c]	27.7±1.8[c]	312.0±13.5[c]	128.1±3.7[c]	50.4±1.7	20.7± .72
Gradual rehab												
4	5	252.0±4.4	210.4±6.9	-16.5	11.7± .43	5.9± .05	82.9±2.7	39.4±1.5	376.3±20.8	178.8±6.1	53.2±2.9	25.3±1.8
5	5	252.4±3.4	213.4±9.2	-15.4	12.0± .29	5.6± .27	87.3±5.4	40.9±1.0	394.7±18.4	185.0±3.6	51.1±2.4	24.0±1.1
6	5	251.4±2.9	207.4±4.0	-17.5	9.1± .30[d]	4.4± .07[d]	52.4±3.2[d]	25.2±1.2[d]	234.4±12.9[d]	113.0±4.8[d]	49.4±1.9	23.8± .96
Low weight main.												
7	5	252.4±4.0	172.0±5.2	-31.9	9.8± .48	5.7± .15	72.6±2.5[f]	42.2±1.4[f]	362.4±19.0	210.6±4.7	50.8±1.9	29.6±1.8
8	5	252.6±4.5	178.6±6.2	-29.3	10.1± .22	5.7± .17	78.5±3.3	44.0± .76	372.5±16.1	208.5±2.7	52.7±2.8	29.5±1.3
9	5	252.6±4.9	176.0±3.8	-30.3	7.6± .57[e]	4.3± .29[e]	39.8±1.5[e]	22.6± .50[e]	155.3±6.5[e]	88.2±2.2[e]	52.1±2.0	29.6±1.2

significantly greater than groups 1 and 3 ($p < .01$)
significantly lower than group 1 ($p < .01$)
significantly lower than groups 1 and 3 ($p < .01$)
significantly lower than groups 4 and 5 ($p < .01$)
significantly lower than groups 7 and 8 ($p < .01$)
significantly lower than group 8 ($p < .05$)

REFERENCES

Aiyer, M.S., Chiappa, S.A. & Fink, G. A priming effect of luteinizing hormone releasing factor on the anterior pituitary gland in the female rat.
Journal of Endocrinology, 1974, 62, 573-588. (a)

Aiyer, M.S., Fink, G. & Greig, F. Changes in the sensitivity of the pituitary gland to luteinizing hormone releasing factor during the oestrous cycle of the rat.
Journal of Endocrinology, 1974, 60, 47-64. (b)

Aiyer, M.S., Greig, F. & Fink, G. Changes in sensitivity of the anterior pituitary gland to synthetic luteinizing hormone releasing factor during the oestrous cycle of the rat.
Journal of Endocrinology, 1973, 57, xxxvi.

Ajika, K., Kalra, S.P., Fawcett, C.P., Krulich, L. & McCann, S.M. The effect of stress and nembutal on plasma levels of gonadotrophins and prolactin in ovariectomized rats.
Endocrinology, 1972, 90, 707-715.

Amatayakul, K., Ryan, R., Uozumi, T. & Albert, A. A reinvestigation of testicular-anterior pituitary relationship in the rat: 1. Effects of castration and crypto orchidism.
Endocrinology, 1971, 88, 872-880.

Anorexia nervosa: Editorial; British Medical Journal. 1971, No. 5781, 183-184.

Antonov, A.N. Children born during the seige of Leningrad in 1942.
Journal of Pediatrics, 1947, 30, 250-259.

Aono, T., Kinugasa, T., Yamamoto, T. Miyake, A., & Kurachi, K. Assessment of gonadotrophin secretion in women with anorexia-nervosa.
Acta Endocrinologica, and supplementum, 1975, 80, 530-641.

Ariumura, A. & Schally, A.V. Augmentation of pituitary responsiveness to LH-releasing hormone (LH-RH) by estrogen.
Proceedings of the Society for Experimental Biology and Medicine, 1971, 136, 290-293.

Arimura, A., Matsuo, H., Baba, Y., Debeljuk, L., Sandow, J., & Shally,A.V. Stimulation of release of LH by synthetic LHRH in vivo. I. Comparative study of natural and synthetic hormone.
Endocrinology, 1972, 90, 163-168.

Arimura, A. & Schally, A.V. Physiological and clinical studies with natural and synthetic luteinizing hormone-releasing hormone (LHRH).
Medical Journal of Osaka University. 1972, 23, 77-100.

Bainbridge, J.G. & Labhsetwar, A.P. The role of oestrogens in spontaneous ovulation: location of site of action of positive feedback of oestrogen by intracranial implantation of the anti-oestrogen I.C.I. 46474.
Journal of Endocrinology, 1971, 50, 321-327.

Baird, D.T., Corker, C.S.,Fraser, I.S., Hunter, W.M., Michie, E.A. & Vanlook, P.F.A. Pituitary-ovarian relationships in polycystic ovarian disease.
Journal of Endocrinology, 1975, 64, 53-54.

Bajusz, E. (Ed)
An introduction to clinical neuroendocrinology. Baltimore: Williams & Wilkins, 1967.

Balint, M.A. A contribution to the psychology of menstruation.
Psychoanalytic Quarterly, 1937, 6, 346.

Ball, Z.B., Barnes, R.H. & Visscher, M.B. The effects of dietary caloric restriction on maturity and senescence, with particular reference to fertility and longevity.
American Journal of Physiology, 1947, 150, 511-519.

Banks, J.A. & Freeman, M.E. The temporal requirement of progesterone on proestrus for extinction of the estrogen-induced daily signal controlling luteinizing hormone release in the rat.
Endocrinology, 1978, 102, 426-432.

Barraclough, C.A. Sex steroid regulation of reproductive neuroendocrine processes. In R.O. Greep (Ed), Handbook of physiology. Section 7: Endocrinology Vol 11 Female reproductive system, Part 1. Washington, D.C.: American Physiological Society, 1973.

Barraclough, C.A., Collu, R., Massa, R. & Martini, L. Temporal inter-relationships which exist amongst plasma LH, ovarian secretion rates, and peripheral plasma progesten concentrations in the rat: Effects of Nembutal and exogenous gonadotrophins.
Endocrinology, 1971, 88, 1437-1447.

Barraclough, C.A. & Gorski, R.A. Evidence that the hypothalamus is responsible for androgen-induced sterility in the female rat.
Endocrinology, 1961, 68, 68-79.

Bass, F. L'amenorrhee au camp de concentration de Terezin (Theresienstadt).
Gynaecologia, 1947, 123, 211.

Beardwood, C.J.
The relationship between psychogenic feeding disorders and the output of gonadotrophic hormones in the human.
Unpublished doctoral dissertation, University of London, 1969.

Beardwood, C.J. Relationship between body weight and gonadotrophin excretion in anorexia nervosa and obesity.
South African Medical Journal, 1974, 48, 53-58.

Bell, E.T., Harkness, R.A., Loraine, J.A. & Russell,G.F.M. Hormone assay studies in patients with anorexia nervosa.
Acta Endocrinologica, 1966, 51, 140-148.

Bentley, P.J.
Comparative vertebrate endocrinology. Cambridge: Cambridge Univ. Press, 1976.

Berson, S.A. & Yalow, R.S. (Eds),
Methods in investigative and diagnotic endocrinology: Peptide hormones Vol 2A, Part 1 and 11. North Holland Publishing Co.: Amsterdam, 1973.

Beumont, P.J.V. Anorexia nervosa in male subjects.
Psychotherapy and Psychosomatics, 1970, 18, 365-371.

Beumont, P.J.V., Carr, P.J. & Gelder, M.G. Plasma levels of luteinizing hormone and of immunoreactive oestrogens (oestradiol) in anorexia nervosa: response to clomiphene citrate.
Psychological Medicine, 1973, 3, 495-501.

Beumont, P.J.V., Friesen, H.G., Gelder, M.G. & Kolakowska, T. Plasma prolactin and luteinizing hormone levels in anorexia nervosa.
Psychological Medicine, 1974, 4, 219-221.

Beumont, P.J.V., George, G.C.W., Pimstone, B.L. & Vinik, A.I. Body weight and the pituitary response to hypothalamic releasing hormones in patients with anorexia nervosa.
Journal of Clinical Endocrinology and Metabolism, 1976, 43, 487-496.

Bhattacharya, A.N., Dierschke, D.J., Yamaji, T. & Knobil, E. The pharmacologic blockade of the circhoral mode of LH secretion in the ovariectomized Rhesus monkey.
Endocrinology, 1972, 90, 778-786.

Blake, C.A. A detailed characterization of the proestrous luteinizing hormone surge.
Endocrinology, 1976, 98, 445-450. (a)

Blake, C.A. Simulation of the proestrous luteinizing hormone (LH) surge after infusion of LH-releasing hormone in phenobarbital-blocked rats.
Endocrinology, 1976, 98, 451-460. (b)

Blake, C.A. Changes in plasma luteinizing hormone-releasing hormone and gonadotrophin concentration during constant rate intravenous infusion of luteinizing hormone-releasing hormone in cyclic rats.
Endocrinology, 1978, 102, 1043-1052.

Blitzer, J.R., Rollins, N. & Blackwell, A. Children who starve themselves: anorexia nervosa.
Psychosomatic Medicine, 1961, 23, 369-383.

Bogdadove, E.M. Direct gonad-pituitary feedback: An analysis of effects of intracranial estrogenic depots on gonadotropin secretion.
Endocrinology, 1963, 73, 696-712.

Bogdadove, E.M. The role of the brain in the regulation of pituitary gonadotropin secretion.
Vitamins and Hormones, 1964, 22, 206-254.

Bohanan, E.H. Effects of environmental factors on the length of the oestrous cycle in the rat.
American Journal of Hygiene, 1939, 29, 1-10.

Boyar, R.M., Finkelstein, J.W., Roffwarg, H., Kapen, S., Weitzman,E., & Hellman, L. Synchronization of augmented luteinizing hormone secretion with sleep during puberty.
The New England Journal of Medicine, 1972, 287, 582-586.

Boyar, R.M., Finkelstein, J.W., & David, R. Twenty four patterns of plasma luteinizing hormone and follicle-stimulating hormone in sexual precocity.
The New England Journal of Medicine, 1973, 289, 282-286.

Boyar, R.M., Katz, J., Finkelstein, J.W., Kapen, S., Weiner, H., Weitzman, E.D. & Hellman, L. Anorexia nervosa: Immaturity of the 24-hour luteinizing hormone secretory pattern.
The New England Journal of Medicine, 1974, 291, 861-865.

Boyar, R.M. & Katz, J. Twenty-four hour gonadotrophin-secretory patterns in anorexia nervosa. In R.A. Vigersky (Ed.), Anorexia nervosa. New York: Raven Press, 1977.

Brady, J.V. & Nauta, W.J.H. Subcortical mechanisms in emotional behavior : Affective changes following septal forebrain lesions in the albino rat.
Journal of Comparative and Physiological Psychology, 1953, 46, 339-346.

Brimer, C.J. & Dockrill, F.J. Partial reinforcement and the CER.
Psychonomic Science, 1966, 5, 185-186.

Brown, G.M., Garfinkel, P.E., Jeuniewic, N., Moldofsky, H. & Stancer, H.C. Endocrine profiles in anorexia nervosa. In R.A. Vigersky (Ed.), Anorexia nervosa. New York: Raven Press, 1977.

Brown-Grant, K. The induction of ovulation by ovarian steroids in the adult rat.
Journal of Endocrinology, 1969, 43, 553-562.

Brown-Grant, K. Physiological aspects of the steroid hormone-gonadotropin interrelationship. In R.O. Greep (Ed.), International Review of Physiology. Reproductive Physiology 11, Vol 13. Baltimore: University Park Press, 1977.

Brown-Grant, K., Corker, C.S. & Naftolin, F. Plasma and pituitary luteinizing hormone concentrations and peripheral plasma oestradiol concentration during early pregnancy and after the administration of progestational steroids in the rat.
Journal of Endocrinology, 1972, 53, 31-35.

Brown-Grant, K., Exley, D. & Naftolin, F. Peripheral plasma oestradiol and luteinizing hormone concentrations during the oestrous cycle of the rat.
Journal of Endocrinology, 1970, 48, 295-296.

Brown-Grant, K. & Greig, F. A comparison of changes in the peripheral plasma concentrations of luteinizing hormone and follicle-stimulating hormone in the rat.
Journal of Endocrinology, 1975, 65, 389-397.

Brown-Grant, K. & Naftolin, F. Facilitation of luteinizing hormone secretion in the female rat by progesterone. Journal of Endocrinology, 1972, 53, 37-46.

Bruch, H. Anorexia nervosa and its differential diagnosis. Journal of Nervous and Mental Disease, 1965, 141, 555-566.

Bullock, D.W. & Kappauf, B.H. Dissociation of gonadotropin-induced ovulation and steroidogenesis in immature rats. Endocrinology, 1973, 92, 1625-1628.

Burger, H.G., Catt, K.J. & Brown, J.B. Relationship between plasma luteinizing hormone and urinary estrogen excretion during the menstrual cycle. Journal of Clinical Endocrinology and Metabolism, 1968, 28, 1508-12.

Bush, I.E. General discussion. In G.E.W. Wolstenholme & C.M. O'Connor (Eds.), Metabolic effects of adrenal hormones. Boston: Little, 1960.

Butcher, R.L., Collins, W.E. & Fugo, N.W. Plasma concentration of LH, FSH, prolactin, progesterone and estradiol-17β throughout the 4 day estrous cycle of the rat. Endocrinology, 1974, 94, 1704-1708.

Caligaris, L., Astrada, J.J. & Taleisnik, S. Release of luteinizing hormone induced by estrogen injection into ovariectomized rats. Endocrinology, 1971, 88, 810-815. (a)

Caligaris, L., Astrada, J.J. & Taleisnik, S. Biphasic effect of progesterone on the release of gonadotropin in rats. Endocrinology, 1971, 89, 331-337. (b)

Campbell, G.A., Kurcz, M., Marshall, S. & Meites, J. Effects of starvation in rats on serum levels of follicle stimulating hormone, luteinizing hormone, thyrotropin, growth hormone and prolactin; response to LH-releasing hormone and thyrotropin-releasing hormone. Endocrinology, 1977, 100, 580-587.

Campbell, H.J., Feuer, G., Garcia, J. & Harris, G.W. The infusion of brain extracts into the anterior pituitary gland and secretion of gonadotrophic hormone. Journal of Physiology, 1961, 157, 30 P.

Cargille, C.M., Ross, G.T. & Yoshimi, T. Daily variations in plasma follicle stimulating hormone, luteinizing hormone and progesterone in the normal menstrual cycle. Journal of Clinical Endocrinology, 1969, 29, 12-19.

Casper, R.C., Davis, J.M. & Pandey, G.N. The effect of the nutritional status and weight changes on hypothalamic function tests in anorexia nervosa. In R.A. Vigersky (Ed.), Anorexia nervosa. New York: Raven Press, 1977.

Charters, A.C., Odell, W.D. & Thompson, J.C. Anterior pituitary function during surgical stress and convalescence. Radioimmunoassay measurement of blood TSH, LH, FSH and growth hormone. Journal of Clinical Endocrinology and Metabolism, 1969, 29, 63-71.

Chedd, G. Nobel prizes 1977: Medicine.
New Scientist, 1977, 76, 144-145.

Chowers, I., Einat, R. & Feldman, S. Effects of starvation on levels of corticotropin-releasing factor, corticotropin and plasma corticosterone in rats.
Acta Endocrinologica, 1969, 61, 687-694.

Chowers, I. & McCann, S.M. Content of luteinizing hormone-releasing factor and luteinizing hormone during the estrous cycle and after changes in gonadal steroid titers.
Endocrinology, 1965, 76, 700-708.

Corker, C.S., Naftolin, F. & Exley, D. Interrelationship between plasma luteinizing hormone and oestradiol in the human menstrual cycle.
Nature, 1969, 222, 1063.

Crisp, A.H. The possible significance of some behavioural correlates of weight and carbohydrate intake.
Journal of Psychosomatic Research, 1967, 11, 117-131.

Crisp, A.H., Chen, C., MacKinnon, P.C.B. & Carter, C.S. Observations on gonadotrophic and ovarian hormone activity during recovery from anorexia nervosa.
Postgraduate Medical Journal, 1973, 49, 584-590.

Crisp, A.H. & Stonehill, E. Relation between aspects of nutritional disturbance and menstrual activity in primary anorexia nervosa.
British Medical Journal, 1971, 3, 149-151.

Critchlow, B.V. & Bar-sela, M.E. Control of the onset of puberty. In L. Martini & W.F. Ganong (Ed.),
Neuroendocrinology, Vol 11, New York: Academic Press, 1967.

Crosignani, P.G., Reschini, E., D'Alberton, A., Troisi, L., Cantalamessa,L. & Guistina, G. Variability of gonadrotropin response to luteinizing hormone-releasing hormone in amenorrheic women.
American Journal of Obstetrics and Gynecology, 1976, 120, 376-384.

Crowell, C.R. & Brown, J.S. Electrifiable restraining cage for small animals.
American Journal of Psychology, 1973, 86, 851-854.

Daane, T.A. & Parlow, A.F. Periovulatory patterns of rat serum follicle stimulating hormone and luteinizing hormone during the normal estrous cycle: effects of pentobarbital.
Endrocrinology, 1971, 88, 653-663.

Dally, P.J.
Anorexia nervosa. London: Heinemarin, 1969.

Dally, P.J. & Gomez, J.
Anorexia nervosa. London: Heinemann Medical Books, 1979.

Dally, P.J. & Sargant, W. Treatment and outcome of anorexia nervosa. British Medical Journal, 1966, 2, 793.

Daniel, P.M. & Prichard, M.M.L. Studies of the hypothalamus and the pituitary gland: with special reference to the effects of transection of the pituitary stalk. Acta Endocrinologica, Supplementum 201, 1975, 80, 1-216.

Danowski, T.S., Livstone, E., Gonzales, A.R., Jung, Y. & Khurana, R.C. Fractional and partial hypopituitarism in anorexia nervosa. Hormone Research, 1972, 3, 105-118.

David, M.A., Fraschini, F. & Martini, L. Control of LH secretion: role of "short" feedback mechanism. Endocrinology, 1966, 78, 55-60.

Davidson, J.M. Feedback control of gonadotropin secretion. In W.F. Ganong & L. Martini (Eds.), Frontiers in neuro-endocrinology. New York: Oxford University Press, 1969, p.p. 343-388.

Debeljuk, L., Rettori, V. & Rozados, R. Pituitary levels and response to LH-RH in female rats pretreated with oestradiol. Journal of Reproduction and Fertility, 1977, 51, 127-129.

Debons, A.F., Krimsky, I., Likarski, H.J., From, A. & Cloutier, R.J. Gold thioglucose damage to the satiety center: Inhibition in diabetes. American Journal of Physiology, 1968, 214, 652-658.

De Neef, J.C. Clinical endocrine cytology. New York: Hoeber, 1965.

Dickerman, E., Negro-Vilar, A. & Meites, J. Effects of starvation on plasma GH activity, pituitary GH and GH-RF levels in the rat. Endocrinology, 1969, 84, 814-819.

Dierschke, D.J., Bhattacharya, A.N., Atkinson, L.E. & Knobil, E. Circhoral oscillations of plasma LH levels in the ovariectomized Rhesus monkey. Endocrinology, 1970, 87, 850-853.

Döcke, F. & Dörner, G. The mechanism of the induction of ovulation by oestrogens. Journal of Endocrinology, 1965, 33, 491-499.

Döhler, K. & Wuttke, W. Serum LH, FSH, prolactin and progesterone from birth to puberty in female and male rats. Endocrinology, 1974, 94, 1003-1007.

Donovan, B.T. Ovarian hormones and the regulation of pituitary function in the female. In R.P. Michael (Ed.), Endocrinology and human behaviour. London: Oxford University Press, 1968.

Donovan, B.T. Mammalian neuroendocrinology. London, McGraw-Hill, 1970.

Donovan, B.T. The control of ovarian function.
Acta Endocrinologica, 1971, 66, 1-15.

Donovan, B.T. The hypothalamic control of the menstrual cycle.
Postgraduate Medical Journal, 1972, 48, 3-4.

Drew, F.L. & Stifel, E.N. Secondary amenorrhoea among young women entering religious life.
Obstetrics and Gynaecology, 1968, 32, 47-51.

Dreyer, J.J. Personal communication, Jan. 14, 1976.

Drillien, C.M.A. A study of normal and abnormal menstrual function in Auxillary Territorial Service.
Journal of Obstetrics and Gynaecology of the British Empire, 1946, 53, 228.

Dufort, R.H. The rats adjustment to 23-, 47- and 71-hr food-deprivation schedules.
Psychological Reports, 1964, 14, 663-669.

Dufort, R.H., Rollins, H.A. & Funderburk, A.J. Adjustment of the rat to the 23-hr food-deprivation schedule under two conditions of water availability.
Psychological Reports, 1965, 17, 869-870.

Dunn, J.D., Arimura, A. & Scheving, L.E. Effects of stress on circadian periodicity in serum L.H. and prolactin concentration.
Endocrinology, 1972, 90, 29-33.

Ershoff, B.H. Nutrition and the anterior pituitary with special reference to the general adaptation syndrome.
Vitamins and Hormones, 1952, 10, 79-140.

Eshkol, A., Lunimeld, B. & Insler, V. The effect of sex steroids on pituitary responsiveness to gonadotropin releasing hormone.
Journal of Steroid Biochemistry, 1975, 6, 1061-1066.

Eskay, R.L., Oliver, C., Ben-Jonathan, N.& Porter, J.C. Hypothalamic hormones in portal and systemic blood. In M. Motta, P.G. Crosignani, & L. Martini (Eds.),
Hypothalamic hormones. London: Academic Press, 1975.

Estep, H.L. Neuroendocrine aspects of surgical stress. In E. Bajusz (Ed.), An Introduction to clinical neuroendocrinology, Basel: S. Karger, 1967.

Estes, W.K. & Skinner, B.F. Some quantitative properties of anxiety.
Journal of Experimental Psychology, 1941, 29, 390-400.

Euker, J., Meites, J. & Riegle, G. Serum LH and prolactin following restraint stress in the rat.
Physiologist, 1973, 16, 307.

Euker, J.S., Meites, J. & Riegle, G.D. Effects of acute stress on serum LH and prolactin in intact, castrate and dexamethasone-treated male rats.
Endocrinology, 1975, 96, 85-92.

Evans, H.M. & Bishop, K.S. The relations between fertility and nutrition. 11. The ovulation rhythm in the rat on inadequate nutritional regimes.
Journal of Metabolic Research, 1922, 1, 335-355.

Everett, J.W. Progesterone and estrogen in the experimental control of ovulation time and other features of the estrous cycle in the rat.
Endocrinology, 1948, 43, 389-405.

Everett, J.W. The time of release of ovulating hormone from the rat hypophysis.
Endocrinology, 1956, 59, 580-585.

Everett, J.W. Prepoptic stimulative lesions and ovulation in the rat: "Thresholds" and LH-release time in late diestrus and proestrus. In E. Bajusz & G. Jasmin (Eds.),
Major problems in neuroendocrinology. Basel: Karger, 1964.

Everett, J.W. Neuroendocrine aspects of mammalian reproduction.
Annual Review of Physiology, 1969, 31, 383-415.

Everett, J.W. & Sawyer, C.H. Estimated duration of the spontaneous activation which causes release of ovulating hormone from the rat hypophysis.
Endocrinology, 1953, 52, 83-92.

Exley, D. & Dutton, A. Peripheral plasma 17β-oestradiol concentrations during the oestrous cycle of a 4-day cyclic rat.
Excerpta Medica 1970, 210, 498.

Ferin, M., Tempone, A., Zimmering, P.E. & Van de Wiele, R.L. Effect of antibodies to 17β-estradiol and progesterone on the estrous cycle of the rat.
Endocrinology, 1969, 85, 1070-1078.

Fink, G., Aiyer, M.S., Jamieson, M.G. & Chiappa, S.A. Factors modulating the responsiveness of the anterior pituitary gland in the rat with special reference to gonadotrophin releasing hormone (GnRH). In M. Motta, P.G. Crosignani, & L. Martini (Eds.),
Hypothalamic hormones. London: Academic Press, 1975.

Fink, G. & Jamieson, M.G. Immunoreactive luteinizing hormone releasing factor in rat pituitary stalk blood: effects of electrical stimulation of the medial preoptic area.
Journal of Endocinology, 1976, 68, 71-87.

Flerko, B. & Bardos, V. Pituitary hypertrophy after anterior hypothalamic lesions.
Acta Endocrinologica, 1960, 35, 375-380.

Follis, R.H.
Deficiency disease. Springfield, Ill.: Charles C. Thomas, 1958.

Franchimont, P. The regulation of follicle stimulating hormone and luteinizing hormone secretion in humans. In L. Martini & W.F. Ganong (Eds.), Frontiers in neuroendocrinology. London: Oxford University Press, 1971.

Franchimont, P., Becker, H., Ernould, C., Thys, C., Demoulin, A., Bourguignon, J.P., Legros, J.J. & Valcke, J.C. The effects of hypothalamic luteinizing hormone releasing hormone (LH-RH) on plasma gonadotrophin levels in normal subjects. Clinical Endocrinology, 1974, 3, 27-39.

Frankel, R.J. & Jenkins, J.S. Hypothalamic-pituitary function in anorexia nervosa. Acta Endocrinologica, 1975, 78, 209-221.

Freeman, M.E., Dupke, K.C. & Croteau, C.M. Extinction of the estrogen induced daily signal for LH release in the rat: a role for the proestrous surge of progesterone. Endocrinology, 1976, 99, 223-229.

Fries, H. Secondary amenorrhoea, self induced weight reduction and anorexia nervosa. Acta Psychiatrica Scandinavica Supplementum 248, 1974, p.p.5-45.

Frisch, R.E. Weight at menarche: similarity for well-nourished and under-nourished girls at differing ages, and evidence for historical constancy. Pediatrics, 1972, 50, 445-450.

Frisch, R.E. & McArthur, J.W. Menstrual cycles: fatness as a determinant of minimum weight for height necessary for their maintenance or onset. Science, 1974, 185, 949-951.

Gay, V.L., Midgley, A.R. & Niswender, G.D. Patterns of gonadotrophin secretion associated with ovulation. Federation Proceedings; 1970, 29, 1880-1887.

Gillman, J. & Gillman, T. Perspectives in human malnutrition. New York: Grune & Stratton, 1951.

Gombe, S., Apgar, I. & Hansel, W. Effect of zinc deficiency and restricted food intake on plasma and pituitary LH and hypothalamic LRF in female rats. Biology of Reproduction, 1973, 9, 415-419.

Gomez-Mont, F. Endocrine changes in chronic human undernutrition. In Reproductive physiology and protein nutrition. New Brunswick, N.J.: Rutgers Univ. Press, 1959.

Goodman, R.L. A quantitative analysis of the physiological role of estradiol and progesterone in the control of tonic and surge secretion of luteinizing hormone in the rat. Endocrinology, 1978, 102, 142-151. (a)

Goodman, R.L. The site of the positive feedback action of estradiol in the rat. Endocrinology, 1978, 102, 151-159. (b)

Gordon, J.H. & Reichlin, S. Changes in pituitary responsiveness to luteinizing hormone-releasing factor during the rat estrous cycle. Endocrinology, 1974, 94, 974-978.

Grayburn, J.A. & Brown-Grant, K. The role of oestrogen in the induction of ovulation in the gonadotrophin-treated immature rat. Journal of Endocrinology, 1968, 42, 409-416.

Greeley, G.H.Jr.,Allen, M.B.Jr. & Mahesh, V.B. Potentiation of luteinizing hormone release by estradiol at the level of the pituitary. Neuroendocrinology, 1975, 18, 233-234.

Greep, R.O. Physiology of the anterior hypophysis in relation to reproduction. In W.C. Young (Ed.), Sex and internal secretions, Vol. 1, Baltimore: Williams & Wilkins, 1961.

Gregory, B.A.J.C. The menstrual cycle and its disorders in psychiatric patients 11. Journal of Psychosomatic Research, 1957, 2, 61-79.

Greig, F. & Weisz, J. Preovulatory levels of luteinizing hormone, the critical period and ovulation in rats. Journal of Endocrinology, 1973, 57, 235-245.

Grewal, T., Mickelsen, D. & Hafs, H.D. Androgen secretion and spermatogenesis in rats following semistarvation. Proceedings of the Society of Experimental Biology and Medicine, 1971, 138, 723-727.

Guevara, A., Luria, M.H. & Wieland, R.G. Serum gonadotropin levels during medical stress (myocardial infarction). Metabolism, 1970, 19, 79-83.

Guilbert, H.R. & Goss, H. Some effects of restricted protein intake on the estrous cycle and gestation in the rat. Journal of Nutrition, 1932, 5, 251-265.

Guillemin, R. The adenohypophysis and its hypothalamic control. Annual Review of Physiology, 1967, 29, 313-348.

Halasz, B. & Gorski, R.A. Gonadotrophic hormone secretion in female rats after partial or total interruption of neural afferents to the medial basal hypothalamus. Endocrinology, 1967, 80, 608-622.

Harper, A.E. Sequence in which the amino acids of casein become limiting for the growth of the rat. Journal of Nutrition, 1959, 67, 109. (a)

Harper, A.E. Amino acid balance and imbalance. I. Dietary level of protein and aminor acid imbalance. Journal of Nutrition, 1959, 68, 405-418. (b)

Harris, G.W. Neural control of the pituitary gland. London: Edward Arnold, 1955.

Henderson, K.M. & McNatty, K.P. A possible interrelationship between gonadotrophin stimulation and prostaglandin $F_{2\alpha}$ inhibition of steroidogenesis by granulosa-luteal cells in vitro. Journal of Endocrinology, 1977, 73, 71-78.

Herrera, E. & Freinkel, N. Interrelationships between liver composition, plasma glucose and ketones, and hepatic acetyl-COA and citric acid during prolonged starvation in the male rat.
Brochimica et Biophysica Acta, 1968, 170, 244-253.

Hohlweg, W. & Junkmann, K. Die hormonal-nervösal Regulierung der Funktion des Hypophysen borderlappens.
Klinische Wochenschrift, 1932, 15, 321-323.

Hori, T., Ide, M. & Miyake, T. Ovarian estrogen secretion during the estrous cycle and under the influence of exogenous gonadotropins in rats.
Endocrinologia Japonica, 1968, 15, 215-222.

Howland, B.E. Gonadrotrophin levels in female rats subjected to restricted feed intake.
Journal of Reproduction and Fertility, 1971, 27, 467-470.

Howland, B.E. Effect of restricted feed intake on LH levels in female rats.
Journal of Animal Science, 1972, 34, 445-447.

Howland, B.E. The influence of feed restriction and subsequent re-feeding on gonadotrophin secretion and serum testosterone levels in male rats.
Journal of Reproduction and Fertility, 1975, 44, 429-436.

Howland, B.E., Beaton, D.B. & Jack, M.I. Changes in serum levels of gonadotropins and testosterone in the male rat in response to fasting, surgery and ether.
Experientia, 1974, 30, 1223-1225.

Howland, B.E. & Skinner, K.R. Effect of starvation on gonadotropin secretion in intact and castrated male rats.
Canadian Journal of Physiology and Pharmacology, 1973, 51, 759-762.

Ibrahim, E.A. & Howland, B.E. Effect of starvation on pituitary and serum follicle stimulating hormone and luteinizing hormone following ovariectomy in the rat.
Canadian Journal of Physiology and Pharmacology, 1972, 50, 768-773.

Igarashi, M., Tohma, K., Ozawa, M., Hosaka, H. & Matsumoto, S. Pathogenesis of psychogenic amenorrhea and anovulation.
International Journal of Fertility, 1965, 10, 311-319.

Ihalainen, O. Psychosomatic aspects of amenorrhoea.
Acta Psychiatrica Scandinavica Supplementum 262, 1975, pp 9-109.

Imada, H. & Soga, M. The CER and BEL as a function of predictability and escapability of an electric shock.
Japanese Psychological Research, 1971, 13, 115-122.

Jackson, G.L. Blockage of progesterone-induced release of luteinizing hormone by actinomycin-D or reserpine and comparison of effects of progesterone and estrogen on release of luteinizing hormone in rats.
Endocrinology, 1972, 90, 874-879.

Jacobson, A., Marshall, J.R., Ross, S.T. & Cargille, C.M. Plasma gonadotrophins during clomiphene induced ovulatory cycles.
American Journal of Obstetrics and Gynecology, 1968, 102, 284-290.

Jaffe, R.B. & Keye, W.R. Estradiol augmentation of pituitary responsiveness to gonadotropin-releasing hormone in women. Journal of Clinical Endocrinology and Metabolism, 1974, 39, 850-855.

Jeffcoate, T.N.A. Amenorrhoea. British Medical Journal, 1965, 2, 383-388.

Kagan, H. Anorexia and severe inanition associated with a tumour involving the hypothalamus. Archives of Diseases of Childhood, 1958, 33, 257-260.

Kalish, H.I. Strength of fear as a function of the number of acquisition and extinction trials. Journal of Experimental Psychology, 1954, 47, 1-9.

Kalra, S.P. & Kalra, P.S. Effects of circulating estradiol during rat estrous cycle on LH release following electrochemical stimulation of preoptic brain or administration of synthetic LRF. Endocrinology, 1974, 94, 845-851.

Kalra, P.S., Kalra, S.P., Krulich, L., Fawcett, C.P. & McCann, S.M. Involvement of norepinephrine transmission of the stimulatory influence of progesterone on gonadotropin release. Endocrinology, 1972, 90, 1168-1176.

Kalra, P.S. Krulich, L., Quijada, M., Kalra, S.P., Fawcett, C.P. & McCann, S.M. Feedback effects of gonadal steroid on gonadotropins and prolactin in the rat. Excerpta Medica, 1970, 210, 53.

Kalra, S.P. & McCann, S.M. The stimulatory effect of gonadotropin release of implants of estradiol or progesterone in certain sites in the central nervous system. Neuroendocrinology, 1975, 19, 289-302.

Kanis, J.A., Brown, P., Fitzpatrick, K., Hibbert, D.J., Horn, D.B., Nairn, I.M., Shirling, D., Strong, J.A. & Walton, H.J. Anorexia nervosa: A clinical psychiatric and laboratory study. Quarterly Journal of Medicine, 1974, 43, 321-338.

Kapen, S., Boyar, R.M. & Hellman, L. Episodic release of luteinizing hormone at mid-menstrual cycle in normal adult women. Journal of Clinical Endocrinology and Metabolism, 1973, 36, 724-729.

Kastin, A.J., Gual, C. & Schally, A.V. Clinical experience with hypothalamic releasing hormones. Part 2: Luteinizing and other hypophysiotropic releasing hormones. Recent Progress in Hormone Research, 1972, 27, 201-217.

Katz, M. Endocrine investigation of the hypothalamic-pituitary-ovarian axis. South African Journal of Hospital Medicine, 1977, 3, 100-107.

Kay, D.W. & Leigh, D. The natural history, treatment and prognosis of anorexia nervosa, based on a study of 38 patients. The Journal of Mental Science, 1954, 100, 411-431.

Kennedy, G.C. & Mitra, J. Hypothalamic control of energy balance and the reproductive cycle in the rat.
Journal of Physiology, 1963, 166, 395-407. (a)

Kennedy, G.C. & Mitra, J. Body weight and food intake as initiating factors for puberty in the rat.
Journal of Physiology, 1963, 166, 408-418. (b)

Keye, W.R. & Jaffe, R.B. Modulation of pituitary gonadotropin response to gonadotropin-releasing hormone by estradiol.
Journal of Clinical Endocrinology and Metabolism, 1974, 38, 805-810.

Keyes, P.L. Luteinizing hormone: action on the Graafian follicle in vitro.
Science, 1969, 164, 846-847.

Keys, A., Brozek, J., Henschel, A., Micklensen, O. & Taylor, H.L. The biology of human starvation. Minneapolis: Univ. of Minnesota Press, 1950.

King, A. Primary and secondary anorexia nervosa syndromes.
British Journal of Psychiatry, 1963, 109, 470-479.

Kirk, R.E.
Experimental design: Procedures for the behavioural sciences.
Belmont: Brooks/Cole, 1968.

Kirsch, R.E.
The kinetics of albumin metabolism in experimental protein-calorie malnutrition.
Unpublished M.D. Thesis, University of Cape Town, 1972.

Klinefelter, H.F., Albright, F., & Griswold, G.C. Experience with quantitative test for normal or decreased amounts of follicle stimulating hormone in urine in endocrinological diagnosis.
Journal of Clinical Endocrinology, 1943, 3, 529-544.

Knobil, E., Dierschke, D.J., Yamaji, T., Karsh, F.J., Hotchkiss, J., & Weick, R.F. Role of estrogen in the positive and negative feedback control of LH secretion during the menstrual cycle of the Rhesus monkey. In B.B. Sayena (Ed.),
Gonadrotrophins.
New York: Wiley-Interscience, 1972.

Kollar, E.J. Psychological stress: a re-evaluation.
Journal of Nervous and Mental Diseases, 1961, 132, 382-396.

Kreezer, G.L. Technics for the investigation of behavioural phenomena in the rat. In E.J. Farris & J.Q. Griffith (Eds.),
The rat in laboratory investigation.
Philadelphia: Lippincott, 1949.

Krey, L.C., Butler, W.R., Weiss, G., Weick, R.F., Dierschke, D.J. & Knobil, E. Influence of endogenous and exogenous gonadal steroids on the actions of synthetic LRF in the Rhesus monkey. In C. Gual & E. Rosenberg (Eds.),
Hypothalamic hypophysiotropic hormones. Amsterdam: Excerpta Medica, 1973.

Lamming, G.E. & Krause, J.B. The effect of a low plane of nutrition on the endocrine glands of the female rat.
Proceedings of the Sixth International Congress on Nutrition, Edinburgh. Edinburgh:Livingstone, 1963.

Lazarus, R.S.
Psychological stress and the coping process.
New York: McGraw-Hill, 1966.

Leaf, R.C. & Muller, S.A. Simple method for CER conditioning and measurement.
Psychological Reports, 1965, 17, 211-215.

Leathem, J,H, Hormones and protein nutrition.
Recent Progress in Hormone Research, 1958, 14, 141-176.

Leathem, J.H. Nutritional effects on hormone production.
Journal of Animal Science, 1966, 25, 68-82.

Leaton, R.N. Long-term retention of the habituation of lick suppression in rats.
Journal of Comparative and Physiological Psychology, 1974, 87, 1157-1164.

Legan, S.J. & Karsch, F.J. Modulation of pituitary responsiveness to luteinizing hormone-releasing factor during the estrous cycle of the rat.
Endocrinology, 1975, 96, 571-575.

Leung, P.M., Rogers, Q.R. & Harper, A.E. Effect of amino acid imbalance in rats fed adlibitum, interval fed or force fed.
Journal of Nutrition, 1968, 95, 474-482.

Lewin, K., Mattingly, D. & Millis, R.R. Anorexia nervosa associated with hypothalamic tumour.
British Medical Journal, 1972, 2, 629-630.

Loeser, A.A. Effects of emotional shock on hormone release and endometrial development.
Lancet, 1943, i , 518.

Longenecker, D.E. & Gallo, D.G. The inhibition of PMSG-induced ovulation in immature rats by melatonin (35634).
Proceedings of the Society for Experimental Biology and Medicine, 1971, 137, 623-625.

Lundberg, P.O., Walinder, J., Werner, I. & Wide, I. Effects of thyrotrophin-releasing hormone on plasma levels of TSH, FSH, LH and GH in anorexia nervosa.
European Journal of Clinical Investigation, 1972, 2, 150-153.

Lutwak-Mann, C. The dependence of gonadal function upon vitamins and other nutritional factors.
Vitamins and Hormones, 1958, 16, 35-71.

Mahesh, V.B., Muldoon, T.G., Eldridge, J.C. & Korach, K.S. The role of steroid hormones in the regulation of gonadotropin secretion. Journal of Steroid Biochemistry, 1975, 6, 1025-1036.

Malaisse, W.J., Malaisse-Lagae, F. & Wright, P.H. Effect of fasting upon insulin secretion in the rat. American Journal of Physiology, 1967, 213, 843-848.

Malmo, R.B. & Shagass, C. Standardized pain stimulation as controlled stress in physiological studies of psychoneurosis. Science, 1948, 108, 509-511.

Mann, D.R. & Barraclough, C.A. Role of estrogen and progesterone in facilitating LH release in 4-day cyclic rats. Endocrinology, 1973, 93, 694-699.

Mann, D.R., Korowitz, C.D. & Barraclough, C.A. Adrenal-gland involvement in synchronizing preovulatory release of LH in rats. Proceedings of the Society for Experimental Biology, 1975, 150, 115-120.

Marrian, G.F. & Parkes, A.S. Effects of anterior pituitary preparations administered during dietary anestrus. Proceedings of the Royal Society (London), 1929, 105, 248-258.

Marshall, J.C. & Fraser, T.R. Amenorrhoea in anorexia nervosa: Assessment and treatment with clomiphene citrate. British Medical Journal, 1971, 4, 590-592.

Marshall, J.C., Morris, R., Court, J. & Butt, W.R. Absent positive feedback of oestrogens: a cause of amenorrhoea in hypothalamic-pituitary disease. Journal of Endocrinology, 1973, 59, xxiv.

Martini, L., Fraschini, F. & Motta, M. Comments on "long" and "short" feedback loops. In R.P. Michael (Ed.), Endocrinology and human behaviour. London: Oxford Univ. Press, 1968.

Mason, J.W. "Overall" hormone balance as a key to endocrine organization. Psychosomatic Medicine, 1968, 30, 791-808.

Maurer, R. & Wooley, D. Distribution of ^{3}H-estradiol in clomiphene-treated and neonatally androgenised rats. Endocrinology, 1971, 88, 1281-1287.

Mazer, C. & Israel, L. Diagnosis and treatment of menstrual disorders and sterility. New York: Hoeber, 1959.

McCance, R.A. & Widdowson, E.M. Protein deficiencies and calorie deficiencies. Lancet, 1966, ii, 158-159.

McCann, S.M. A hypothalamic luteinizing hormone-releasing factor. American Journal of Physiology, 1962, 202, 396-400.

McCann, S.M., Dhariwal, A.P.S. & Porter, J.C. Regulation of the adenohypophysis. Annual Review of Physiology, 1968, 30, 589-640.

McCann, S.M. & Ramirez, V.D. The neuroendocrine regulation of hypophysial luteinizing hormone secretion.
Recent Progress in Hormone Research, 1964, 20, 131-181.

McCann, S.M. & Taleisnik, S. The effect of estrogen on plasma luteinizing hormone (LH) activity in the rat.
Endocrinology, 1961, 69, 909-914.

McCann, S.M., Taleisnik, S. & Friedman, H.M. Luteinizing hormone-releasing factor in hypothalamic extracts.
Proceedings of the Society for Experimental Biology and Medicine, 1960, 104, 432-434.

McCormick, W.O. Amenorrhoea and other menstrual symptoms in student nurses.
Journal of Psychosomatic Research, 1975, 19, 131-137.

McKay, D.W., Wood, S.M. & Reinke, D.A. Stress effects on ovulation and serum hormone levels in the rat.
Federation Proceedings, 1975, 34, 288.

Mecklenburg, R.S., Loriaux, D.L., Thompson, R.H. Hypothalamic dysfunction in patients with anorexia nervosa.
Medicine (Baltimore) 1974, 53, 147-159.

Meites, J. & Reed, J.O. Effects of restricted feed intake in intact and ovariectomized rats on pituitary lactogen and gonadotrophin.
Proceedings of the Society for Experimental Biology and Medicine, 1949, 70, 513-516.

Memon, G.N., Antoniewicz, R.J., Benevenga, N.J., Pope, A.L. & Casida,L.E. Some effects of differences in dietary energy and protein levels on the ovary and anterior pituitary gland of the ewe.
Journal of Animal Science, 1969, 28, 57.

Midgley, A.R. & Jaffe, R.B. Regulation of human gonadotropins X. Episodic fluctuation of LH during the menstrual cycle.
Journal of Clinical Endocrinology and Metabolism, 1971, 33, 962-969.

Midgley, A.R., Niswender, G.D. & Rebar, R.W. Principles for the assessment of the reliability of radio immunoassay methods (precision, accuracy, sensitivity and specificity). In E. Diczfalusy & A. Diczfalusy (Eds.),
Karolinska symposium on research methods in reproductive endocrinology. Transactions of the first symposium. Stockholm, 1969.

Miyashita, T. Proper control procedure in the "on-the-base-line" type of CER experiment.
Japanese Psychological Research, 1971, 13, 176-182.

Monroe, S.E., Rebar, R.W., Gay, V.L. & Midgley, A.R. Radioimmunoassay determination of luteinizing hormone during the estrous cycle of the rat.
Endocrinology, 1969, 85, 720-724.

Moore, C.R. & Price D., Gonad hormone functions and the reciprocal influence between gonads and hypophysis with its bearing on the problem of sex-hormone antagonisms.
American Journal of Anatomy, 1932, 50, 13-71.

Morishige, W.K. & Rothchild, I. A paradoxical inhibiting effect of ether on prolactin release in the rat: comparison with effect of ether on LH and FSH.
Neuroendocrinology, 1974, 16, 95-107.

Mortimer, C.H., Besser, G.M, McNeilly, A.S., Marshall, J.C., Harsoulis, P., Tunbridge, W.M.G., Gomez-Pan, A. & Hall, R. Luteinizing hormone and follicle stimulating hormone-releasing hormone test in patients with hypothalamic-pituitary-gonadal dysfunction.
British Medical Journal, 1973, 4, 73-77.

Mulinos, M. & Pomerantz, L. Pseudo-hypophysectomy-A condition resembling hypophysectomy produced by malnutrition.
Journal of Nutrition, 1940, 19, 493.

Naftolin, F., Brown-Grant, K. & Corker, C.S. Plasma and pituitary luteinizing hormone and peripheral plasma oestradiol concentrations in the normal oestrous cycle of the rat and after experimental manipulation of the cycle.
Journal of Endocrinology, 1972, 53, 17-30.

Naftolin, F. & Corker, C.S. An ultramicro method for the measurement of luteinizing hormone by radioimmunoassay. In K.E. Kirkham & W.M. Hunter (Eds.),
Radioimmunoassay methods. Edinburgh:Churchill-Livingstone, 1971.

Nageishi, Y. & Imada, H. Suppression of licking behaviour in rats as a function of predictability of shock and probability of conditioned-stimulus-shock pairings.
Journal of Comparative and Physiological Psychology, 1974, 87, 1165-1173.

Negro-Vilar, A., Dickerman, E. & Meites, J. Effects of starvation on hypothalamic FSH-RF and pituitary FSH in male rats.
Endocrinology, 1971, 88, 1246-1249.

Negro-Vilar, A., Orias, R., McCann, S.M. Evidence for a pituitary site of action for the acute inhibition of LH release by estrogen in the rat.
Endocrinology, 1973, 92, 1680-1684.

Neill, J.D. Effect of "stress" on serum prolactin and luteinizing hormone during the estrous cycle of the rat.
Endocrinology, 1970, 87, 1192-1197.

Neill, J.D. Sexual differences in the hypothalamic regulation of prolactin secretion.
Endocrinology, 1972, 90, 1154-1159.

Neill, J.D. & Reichert, L.E. Development of a radioimmunoassay for rat prolactin and evaluation of the NIAMD rat prolactin radioimmunoassay,
Endocrinology, 1971, 88, 548 - 555.

Newton, J. Hormone excretion patterns in anovulatory infertility.
Postgraduate Medical Journal, 1972, 48, 5-9.

Newton, J. & Dixon, P. Site of action of clomiphene and its use as a test of pituitary function.
Journal of Obstetrics and Gynaecology of the British Commonwealth, 1971, 78, 812-821.

Nillius, S.J., Fries, H. & Wide, L. Successful induction of follicular maturation and ovulation by prolonged treatment with LH-releasing hormone in women with anorexia nervosa.
American Journal of Obstetrics and Gynecology, 1975, 122, 921-928.

Nillius, S.J. & Wide, L. The LH-releasing hormone test in 31 women with secondary amenorrhoea.
Journal of Obstetrics and Gynaecology of the British Commonwealth, 1972, 79, 874-882.

Nillius, S.J. & Wide, L. The pituitary responsiveness to acute and chronic administration of gonadotrophin-releasing hormone in acute and recovery stages in anorexia nervosa. In R.A. Vigersky (Ed.), Anorexia Nervosa. New York: Raven Press, 1977.

Osofsky, H.J. & Fisher, S. Psychological correlates of the development of amenorrhoea in a stress situation.
Psychosomatic Medicine, 1967, 29, 15-23.

Palka, Y.S., Ramirez, V.D. & Sawyer, C.H. Distribution and biological effects of tritiated estradiol implanted in the hypothalamo-hypophysial region of female rats.
Endocrinology, 1966, 78, 487-499.

Palmer, R.L., Crisp, A.H., MacKinnon, P.C.B., Franklin, M., Bonnar, J. & Wheeler, M. Pituitary sensitivity to 50 ng LH/FSH-RH in subjects with anorexia nervosa in acute and recovery stages.
British Medical Journal, 1975, 1, 179-182.

Piacsek, B.E. & Hautzinger, G.M. Effects of duration, intensity and spectrum of light exposure on sexual maturation time of female rats.
Biology of Reproduction, 1974, 10, 380-387.

Piacsek, B.E. & Meites, S.M. Effects of castration and gonadal hormones on hypothalamic content of luteinizing hormone releasing factor.
Endocrinology, 1966, 79, 432-439.

Piacsek, B.E. & Meites, J. Reinitiation of gonadotropin release in underfed rats by constant light or epinephrine.
Endocrinology, 1967, 81, 535-541.

Piacsek, B.E., Schneider, T.C. & Gay, V.L. Sequential study of luteinizing hormone (LH) and "Progestin" secretion on the afternoon of proestrus in the rat.
Endocrinology, 1971, 89, 39-45.

Platt, B.S. Protein-calorie deficiency.
Lancet, 1966, ii, 283.

Platt, B.S. Experimental protein-calorie deficiency. In R.A. McCance & E.M. Widdowson (Eds.),
Calorie deficiencies and protein deficiencies. London:Churchill, 1968.

Querido, D.
Applications of a radioimmunoassay technique to the study of luteinizing hormone secretion in the rat. Unpublished M.Sc. thesis, University of Cape Town, 1975.

Querido, D. & Beardwood, C.J. A micro-radioimmunoassay for measurement of rat luteinizing hormone.
South African Journal of Animal Science, 1977, 7, 161-166.

Rakoff, A.E. Endocrine mechanisms in psychogenic amenorrhoea. In R.P. Michael (Ed.)
Endocrinology and human behaviour. London: Oxford University Press, 1968.

Ramirez, V.D., Abrams, R.M. & McCann, S.M. Effect of estradiol implants in the hypothalamo-hypophysial region of the rat on the secretion of luteinizing hormone.
Endocrinology, 1964, 75, 243-248.

Reeves, J.J., Arimura, A. & Schally, A.V. Changes in pituitary responsiveness to luteinizing hormone-releasing hormone (LH-RH) in anestrous ewes pretreated with estradiol benzoate.
Biology of Reproduction, 1971, 4, 88-92.

Reid, L.S. & Finger, F.W. The rat's adjustment to 23-hour food deprivation cycles.
Journal of Comparative and Physiological Psychology, 1955, 48, 110-113.

Reifenstein, E.C. Psychogenic or "hypothalamic" amenorrhea.
Medical Clinics of North America, 1946, 30, 1103-1114.

Reiter, R.J., Monaco, L., Donofrio, R.J. The influence of the pineal gland and its constituents on PMS-induced ovulation in immature rats.
American Zoologist, 1969, 9, 1087.

Riegle, G.D. & Meites, J. The effect of stress on serum prolactin in the female rat.
Proceedings of the Society for Experimental Biology and Medicine, 1976, 152, 441-448.

Rinaldini, L.M. Effect of chronic inanitition on the gonadotrophic content of the pituitary gland.
Journal of Endocrinology, 1949, 6, 54-62.

Root, A.W. & Duckett, G.E. In vivo and in vitro effects of synthetic luteinizing hormone-releasing hormone (LH-RH) upon the secretion of luteinizing hormone (LH) and follicle stimulating hormone (FSH) in intact and castrated, fed and starved adult male rats.
Proceedings of the Society for Experimental Biology and Medicine, 1973, 144, 30-33.

Root, S.W., Reiter, E.O., Duckett, G.E. & Sweetland, M.L. Effect of short term castration and starvation upon hypothalamic content of luteinizing hormone releasing hormone in adult male rats.
Proceedings of the Society for Experimental Biology and Medicine, 1975, 150, 602-605.

Root, A.W. & Russ, R.D. Short-term effects of castration and starvation upon pituitary and serum levels of luteinizing hormone and follicle stimulating hormone in male rats.
Acta Endocrinologica, 1972, 70, 665-675.

Rose, S. & Nelson, J.F. Direct effect of estradiol on the pars distalis.
Australian Journal of Experimental Biology and Medical Science, 1957, 35, 605-610.

Ross, G.T., Cargille, C.M., Lipsett, M.B., Rayford, P.L., Marshall, J.R., Scott, C.A. & Rodbard, D. Pituitary and gonadal hormones in women during spontaneous and induced ovulatory cycles.
Recent Progress in Hormone Research, 1970, 26, 1-62.

Roth, J.C., Kelch, R.P., Kaplan, S.L. & Grumbach, M.M. FSH and LH response to luteinizing hormone-releasing factor in prepubertal and pubertal children, adult males and patients with hypogonadotropic and hypergonadotropic hypogonadism.
Journal of Clinical Endocrinology and Metabolism , 1972, 35, 926-930.

Rubin, R.T., Kales, A., Adler, R., Fagan, T. & Odell, W. Gonadotropin secretion during sleep in normal adult men.
Science, 1971, 175, 196-198.

Russell, G.F.M. Metabolic aspects of anorexia nervosa.
Proceedings of the Royal Society of Medicine, 1965, 58, 811-814.

Russell, G.F.M. The nutritional disorder in anorexia nervosa.
Journal of Psychosomatic Research, 1967, 11, 141-149.

Russell, G.F.M.
The neuroendocrinology of human under nutrition, with special reference to anorexia nervosa. Paper presented at 3rd International Meeting of Endocrinology, Marseilles, 1968.

Russell, G.F.M.
Metabolic, endocrine and psychiatric aspects of anorexia nervosa. In The Scientific Basis of Medicine Annual Reviews.
London: Athlone Press, 1969.

Russell, G.F.M. Premenstrual tension and "psychogenic" amenorrhoea: psychophysical interactions.
Journal of Psychosomatic Research, 1972, 16, 279-287. (a)

Russell, G.F.M. Psychological and nutritional factors in disturbances of menstrual function and ovulation.
Postgraduate Medical Journal, 1972, 48, 10-13. (b)

Russell, G.F.M. & Beardwood, C.J. The feeding disorders with particular reference to anorexia nervosa and its associated gonadotrophin changes. In R.P. Michael (Ed.)
Endocrinology and human behaviour. London: Oxford Univ. Press, 1968.

Russell, G.F.M., Loraine, J.A., Bell, E.T. & Harkness, R.A. Gonadotrophin and oestrogen excretion in patients with anorexia nervosa.
Journal of Psychosomatic Research, 1965, 9, 79-85.

Russell, G.F.M. & Wakeling, A.
The endocrine and menstrual response to clomiphene citrate in patients with anorexia nervosa. Paper presented at 10th European Conference on Psychosomatic Research, Edinburgh, Scotland, September, 1974.

Sakar, D.K., Chiappa, S.A., Fink, G. & Sherwood, N.M. Gonadotropin-releasing hormone surge in pro-oestrous rats.
Nature, 1976, 264, 461-463.

Samuels, L.T.
Nutrition and hormones. Springfield, Ill.:Charles C. Thomas, 1948.

Santen, R.J. & Bardin, C.W. Episodic luteinizing hormone secretion in man. Pulse analysis, clinical interpretation, physiologic mechanisms.
Journal of Clinical Investigation, 1973, 52, 2617-2628.

Schaeffer, R.W. & Premack, D. Licking rates in infant albino rats.
Science, 1961, 134, 1980-1981.

Schally, A.V., Arimura, A., Baba, Y., Nair, R.M.G., Matsuo, H., Redding,T.W., Debeljuk, L. & White, W.F. Isolation and properties of the FSH and LH-releasing hormone.
Biochemical and Biophysical Research Communications, 1971, 43, 393-399.

Schally, A.V., Arimura, A., Bowers, C.Y., Kastin, A.J., Sawano, S., & Redding, T.W. Hypothalamic neurohormones regulating anterior pituitary function.
Recent Progress in Hormone Research, 1968, 24, 497-588.

Schally, A.V., Ariumura, A., Kastin, A.J., Matsuo, H., Baba, Y., Redding,T.W., Nair, R.M.G., Debeljuk, L. & White, W.F. Gonadotropin-releasing hormone: One polypeptide regulates secretion of luteinizing and follicle stimulating hormones.
Science, 1971, 173, 1036-1038.

Schally, A.V., Kastin, A.J. & Arimura, A. FSH-releasing hormone and LH-releasing hormone.
Vitamins and Hormones, 1972, 30, 83-164.

Schneider, H.P.G. & McCann, S.M. Mono-and indolamines and control of LH secretion.
Endocrinology, 1970, 86, 1127-1133.

Schneider, T., Piacsek, B.E. & Gay, V. Simultaneous measurements of progesterone and 20-α-OH-pregn-4-en-3-one in systemic blood on the afternoon of proestrus in the rat.
Federation Proceedings, 1970, 29, 440.

Schwartz, N.B. A model for the regulation of ovulation in the rat.
Recent Progress in Hormone Research, 1969, 25, 1-53.

Schwartz, N.B. & Bartosik, D. Changes in pituitary LH content during the rat estrous cycle.
Endocrinology, 1969, 71, 756-762.

Seki, K., Seki, M., Yoshihari, T. & Maeda, H. Radioimmunoassay for rat follicle stimulating and luteinizing hormones. Endocrinologica Japonica, 1971, 18, 477-485.

Seligman, M.E.P. Chronic fear produced by unpredictable electric shock. Journal of Comparative and Physiological Psychology, 1968, 66, 402-411.

Seligman, M.E.P. Control group and conditioning: A comment on operationism. Psychological Review, 1969, 76, 484-491.

Seligman, M.E.P., Maier, S.F. & Solomon, R.L. Unpredictable and uncontrollable aversive events. In F.R. Brush (Ed.), Aversive conditioning and learning. New York: Academic Press, 1971.

Selye, H. The general adaption syndrome and the disease of adaptation. Journal of Clinical Endocrinology, 1946, 6, 117-230.

Selye, H. Stress. Montreal: Acta Medical Publishers, 1950.

Seyler, L.E. & Reichlin, S. Luteinizing hormone release in the rat induced by blood volume depletion. Endocrinology, 1973, 92, 295-302.

Shearman, R.P. Progress in the investigation and treatment of anovulation. American Journal of Obstetrics and Gynecology, 1969, 103, 444-463.

Sherman, B.M., Halmi, K.A. & Zamudio, R. LH and FSH response to gonadotropin-releasing hormone in anorexia nervosa: Effect of nutritional rehabilitation. Journal of Clinical Endocrinology and Metabolism,1975, 41, 135-142.

Shipley, R. Extinction of conditioned fear in rats as a function of several parameters of CS exposure. Journal of Comparative and Physiological Psychology, 1974, 87, 699-707.

Siegal, S. Non-parametric statistics for the behavioral sciences. New York: McGraw-Hill, 1956.

Smith, C.A. Effects of maternal under nutrition upon the new born infant in Holland (1944-45). Journal of Pediatrics, 1947, 30, 229-243.

Smith, E.R., Bower, C.Y. & Davidson, J.M. Circulating levels of plasma gonadotropins in 4 and 5-day cycling rats. Endocrinology, 1973, 93, 756-758.

Smith E.R. & Davidson, J.M. Role of estrogen in the cerebral control of pubery in female rats. Endocrinology, 1968, 82, 100-108.

Smith, M.S., Freeman, M.E. & Neil, J.D. The control of progesterone secretion during the estrous cycle and early pseudopregnancy in the rat: prolactin, gonadotropin and steroid levels associated with rescue of the corpus luteum of pseudopregnancy. Endocrinology, 1975, 96, 219-226.

Society of Actuaries. Build and blood pressure study. Vol. 1. Chicago: Society of Actuaries, 1959.

Speroff, L. & Vande Wiele, R.L. Regulation of the human menstrual cycle. American Journal of Obstetrics and Gynecology, 1971, 109, 234-247.

Srebnik, H.H. & Nelson, M.M. The influence of diet on the gonadotrophins of the anterior pituitary gland. Proceedings of the 6th International Congress of Nutrition.pp 375-386 Edinburgh: Livingstone, 1963.

Stead, R.H. & Brock, J.F. Experimental protein-calorie malnutrition: Rapid induction of protein depletion signs in early-weaned rats. Journal of Nutrition, 1972, 102, 1357-1366.

Steele, R.E. & Weisz, J. Changes in sensitivity of the estradiol-LH feedback system with puberty in the female rat. Endocrinology, 1974, 96, 513-520.

Steffens, A.B. A method for frequent sampling of blood and continuous infusion of fluids in the rat without disturbing the animal. Physiology and Behaviour, 1969, 4, 833-836.

Stumpf, W.E. Estrogen neurons and estrogen-neuron systems in the periventricular brain. American Journal of Anatomy, 1970, 129, 207-217.

Svendsrod, R. & Ursin, H. A factor-analytic study of the acqusition of a conditioned emotional response in rats. Journal of Comparative and Physiological Psychology, 1974, 87, 1174-1179.

Swerdloff, R.S. & Odell, W.D. Serum luteinizing and follicle stimulating hormone levels during sequential and nonsequential contraceptive treatment of eugonadal women. Journal of Clinical Endocrinology, 1969, 29, 157-163.

Sydenham, A. Amenorrhoea at Stanley Camp, Hong Kong, during internment. British Medical Journal, 1946, 2, 159.

Szepesi, B. & Berdanier, C.D. Time course of starve-refeed response in rats; the possible role of insulin. Journal of Nutrition, 1971, 101, 1563-1574.

Tepperman, J. Metabolic and endocrine physiology. (3rd ed.), Chicago: Year Book Medical Publications, 1973.

Travaglini, P. Some aspects of hypothalamic-pituitary function in patients with anorexia nervosa. Acta Endocrinoligica, 1976, 81, 252-262.

Tsai, C.C. & Yen, S.S.C. The effects of ethinyl estradiol administration during early follicular phase of the cycle on the gonadotropin levels and ovarian function.
Journal of Clinical Endocrinology and Metabolism, 1971, 33, 917-923.

Underwood, E.J.
Trace elements in human and animal nutrition. (3rd ed.) New York: Academic Press, 1971.

Vande Wiele, R.L., Bogumil, J., Dryenfurth, I., Ferin, M., Jewelewicz, R., Warren, M., Rizkallah, T. & Mikhail, G. Mechanisms regulating the menstrual cycle in women.
Recent Progress in Hormone Research, 1970, 26, 63-95.

Verplanck, W.S. & Hayes, J.R. Eating and drinking as a function of maintenance schedule.
Journal of Comparative and Physiological Psychology, 1953, 46, 327-333.

Vigersky, R.A. & Loriaux, D.L. Anorexia nervosa as a model of hypothalamic dysfunction. In R.A. Vigersky (Ed.),
Anorexia nervosa, New York: Raven Press, 1977.

Wakeling, A., De Souza, V. & Beardwood, C.J. Effects of administered estrogen on luteinizing hormone release in subjects with anorexia nervosa in acute and recovery stages. In R.A. Vigersky (Ed.),
Anorexia nervosa. New York:Raven Press, 1977.

Wakeling, A., Marshall, J.C., Beardwood, C.J., de Souza, V.F.A. & Russell, G.F.M. The effects of clomiphene citrate on the hypothalamic-pituitary-gonadal axis in anorexia nervosa.
Psychological Medicine, 1976, 6, 371-380.

Wang, C.F. & Yen, S.S.C. Direct evidence of estrogen modulation of pituitary sensitivity to luteinizing hormone-releasing factor during the menstrual cycle.
Journal of Clinical Investigation, 1975, 55, 201-204.

Warren, M.P. Weight loss and responsiveness to LHRH. In R.A. Vigersky (Ed.),
Anorexia nervosa. New York: Raven Press, 1977.

Warren, M.P., Jewelewicz, R., Dyrenfurth, I., Ans, R., Khalaf, S., & Van de Wiele, R.L. The significance of weight loss in the evaluation of pituitary response to LH-RH in women with secondary amenorrhea.
Journal of Clinical Endocrinology and Metabolism, 1975, 40, 601-611.

Warren, M.P. & Van de Wiele, R.L. Clinical and metabolic features of anorexia nervosa.
American Journal of Obstetrics and Gynecology, 1973, 117, 435-449.

Weick, R.F. & Davidson, J.M. Localization of the stimulatory feedback effect of estrogen on ovulation in the rat.
Endocrinology, 1970, 87, 693-700.

Weiss, J.M. Somatic effects of predictable and unpredictable shock.
Psychosomatic Medicine, 1970, 33, 397-408.

Weiss, J.M. Psychological factors in stress and disease.
Scientific American, 1972, 226, 104-113.

Werner, S. Failure of gonadotrophic function of the rat hypophysis during chronic inanition.
Proceedings of the Society for Experimental Biology and Medicine, 1939, 41, 101-105.

Whiteacre, F.E. & Barrera, B. War amenorrhoea.
Journal of the American Medical Association, 1944, 124, 399.

Widdowson, E.M. The place of experimental animals in the study of human malnutrition. In R.A. McCance & E.M. Widdowson (Eds.), Calorie deficiencies and protein deficiencies. London:Churchill, 1968.

Widdowson, E.M. The response of the sexes to nutritional stress.
Proceedings of the Nutrition Society, 1976, 35, 175-180.

Widdowson, E.M., Mavor, W.O. & McCance, R.A. The effect of undernutrition and rehabilitation on the development of the reproductive organs:rats.
Journal of Endocrinology, 1964, 29, 119-126.

Wiegelmann, W. & Solbach, H.G. Effects of LH-RH on plasma levels of LH and FSH in anorexia nervosa.
Hormone and Metabolic Research, 1972, 4, 404.

Winer, B.J.
Statistical principles in experimental design (2nd ed). New York: McGraw-Hill, 1971.

Winter, N. Menorrheal problems in college women.
American Journal of Obstetrics and Gynecology, 1946, 52, 803.

Wolpe, J. Experimental neuroses as learned behaviour.
British Journal of Psychology, 1952, 43, 243-268.

Wuttke, W. & Meites, J. Effects of ether and pentobarbital on serum prolactin and LH levels in proestrous rats.
Proceedings of the Society for Experimental Biology and Medicine, 1970, 135, 648-652.

Yen, S.S.C., Vandenberg, G. & Siler, T.M. Modulation of pituitary responsiveness to LRF by estrogen.
Journal of Clinical Endocrinology and Metabolism, 1974, 39, 170-177.

Ying, S-Y, Fang, V.S. & Greep, R.O. Estradiol benzoate (EB)-induced changes in serum-luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in immature female rats.
Fertility and Sterility, 1971, 22, 799-801.

Ying, S-Y. & Greep, R.O. Effects of age of rat and dose of a single injection of estradiol benzoate (EB) on ovulation and the facilitation of ovulation by progesterone (P).
Endocrinology, 1971, 89, 785. (a)

Ying, S-Y, & Greep, R.O. Effect of a single low dose of estrogen on ovulation, pregnancy and lactation in immature rats.
Fertility and Sterility, 1971, 22, 165-169. (b)

Ying, S-Y. & Meyer, R.K. Dose dependent PMS-induced ovulation in immature rats.
Proceedings of the Society for Experimental Biology and Medicine, 1969, 130, 40-43.

Yoshimoto, Y., Moridera, K. & Imura, H. Restoration of normal pituitary gonadotropin reserve by administration of luteinizing-hormone-releasing hormone in patients with hypogonadotropic hypogonadism.
The New England Journal of Medicine, 1975, 292, 242-245.

Yoshinaga, K., Hawkins, R.A. & Stocker, J.F. Estrogen secretion by the rat ovary in vivo during the estrous cycle and pregnancy.
Endocrinology, 1969, 85, 103-112.

Zarrow, M.X. & Brown-Grant, K. Inhibition of ovulation in the gonadotrophin treated immature rat by chlorpromazine.
Journal of Endocrinology, 1964, 30, 87-95.

Zarrow, M.X. & Quinn, D.L. Superovulation in the immature rat following treatment with PMS alone and inhibition of PMS-induced ovulation.
Journal of Endocrinology, 1963, 26, 181-188.

Zeballos, G. & McCann, S.M. Alterations during the estrous cycle in the responsiveness of the pituitary to subcutaneous administration of synthetic LH-releasing hormone (LHRH).
Endocrinology, 1975, 96, 1377-1384.

Zimmerman, D.R., Spies, H.G., Self, H.G. & Casida, L.E. Ovulation rate in swine as affected by increased energy intake just prior to ovulation.
Journal of Animal Science, 1960, 19, 295.

Zondek, B. & Tamari, I. Effect of audiogenic stimulation on genital function and reproduction.
American Journal of Obstetrics and Gynecology, 1960, 80, 1041-1048.

Zubiran, S. & Gomez-Mont, F. Endocrine disturbance in chronic human malnutrition.
Vitamins and Hormones, 1953, 11, 97-132.

12 JUL 1982