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PRENATAL MATERNAL STRESS IN *MUS MUSCULUS*: EFFECTS ON THE OFFSPRING, AND THE ROLE OF THE MOTHER

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Abstract

The aim of this project was to examine certain aspects of prenatal stress, by providing a more comprehensive study of one strain of animal in one laboratory than hitherto presented. The stressor employed was that of crowding during the last third of gestation, and special attention was given to the problem of distinguishing between prenatally and postnatally-caused alterations to the offspring. In order to assess the mother's role in postnatal mediation of these changes, the behaviour towards the young of both stressed and non-stressed dams raising either type of litter was monitored throughout the lactation period. Differences were observed in the ways in which frequencies and diurnal rhythms of behaviour changed as the young matured, and efforts have been made to relate these to observed differences in offspring.

As to the effects on the offspring themselves, their "emotionality" was examined employing three common methods - the open-field test, the holeboard test and a passive avoidance test. The results indicated that prenatal stress may differentially affect male and female offspring, and that the importance of prenatal versus postnatal influences on emotionality is likewise sex-dependent. The sexuality of female offspring was investigated, with measures being taken of puberty onset, adult ovarian cyclicity, and receptivity. While the results of this section were not clear-cut, they did indicate that prenatal stress may act to reduce responsiveness (to e.g., pheromonal stimuli) in female offspring. An investigation of adrenocortical function was also carried out, examining the daily corticosterone rhythm and its onset, and also the adrenocortical response to mild stress. Rhythm onset was advanced in prenatally-stressed animals, and while no differences were observed

in the adult corticosterone rhythm of males, that of females suggested that prenatal stress may alter pituitary-gonadal function. A theoretical model for the mediation of prenatal stress effects, based upon the results obtained in this work and elsewhere, is presented.

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Chapter 1: Introduction

It is generally accepted today that stress has an important role to play in the life and general health of mankind. The effects of stress are most frequently thought of as deleterious, acting as contributory factors to, for example, coronary disease, peptic ulcers and some psychiatric disorders. At the same time, it is recognised that a moderate degree of stress can be beneficial, better adapting an individual to cope with more stressful situations in life. A major problem in stress research lies in its definition. To the layman, stress is a form of psychological pressure which when brought to bear on an individual makes it hard for him to lead a normal existence. Scientists have tried to define stress more objectively, with the result that a wide variety of definitions now exist, ranging from "any aversive stimulus for an animal" (Coffey, 1971), to "the prolonged inability to remove a source of potential danger, leading to activation of systems for coping with danger beyond their range of maximal efficiency" (Archer, 1979). Some workers have defined stress in terms of the factors which elicit changes in the animal rather than in terms of changes themselves. Here, these factors will be termed stressors, for clarity's sake. It should be noted that stressors can be either physical stressors, e.g. electric shock, or psychological e.g. fear. It may be important to distinguish between these, as I shall explain later.

Many definitions of stress (e.g. Brain, 1975) include activation of the pituitary-adrenocortical system as a consequence of stress, deriving from Selye's original hypothesis of a "General Adaptation Syndrome" in 1936. This hypothesis stated that an animal would respond in two ways to noxious stimuli - specifically and generally. The general response can be seen in terms of three stages, which can

be assessed by changes in adrenal weight. During the first, "alarm", phase, the adrenal glands are activated, and increase in weight. Provided the aversive stimulus is not removed, the response will attain a "resistance" phase in which the adrenals maintain their enlarged state. Eventually a state of exhaustion is reached, at which point adrenal size is reduced as its secretory capacity declines, and death finally ensues. How much of this G.A.S. response is shown will depend on the duration of the stress. Today it is this pituitary-adrenal activation in response to aversive stimuli which is most often taken as an indicator of stress. Other measurements are sometimes taken - for example adrenalin release, heartbeat rate and skin conductance.

The effects of stress are commonly thought of as acting in adult life, whereas in fact stress during infancy or even before birth can have a profound effect as most advanced animals are particularly sensitive to changes in the environment during early development. A large body of evidence exists for effects of early postnatal stimulation (e.g. Ader, 1968; Haltmeyer et al., 1967) , and the late 1950's saw the development of an interest in prenatal stress. A number of investigations and/or observations of human beings suggested that prenatal psychological stress influences the behaviour and development of the offspring. The stressors in these studies were ill-defined, but these studies showed effects on aspects such as neonatal activity and crying (Ottinger & Simmons, 1963, 1964), reading ability (Kawi & Pasamanick, 1959), temperamental impairment (Stott, 1959), and childhood behaviour disorders (Pasamanick & Lilienfield, 1955; Pasamanick, Rogers & Lilienfield, 1956). Because of the many problems inherent with working on human beings - either in ethical terms, in terms of finding enough subjects, or in terms of the problems associated with controlling for external variables - a

number of workers have taken up the problem with rats or mice. Indeed, it was because of the effects of prenatal stress seen in humans that W.R. Thompson carried out the first animal experiment on this subject in 1957. Since then a great body of work has been carried out in this area, and the interest in prenatal psychological stress has been extended to examine the effects of other prenatal treatments, such as electric shock treatment, malnutrition, drugs and radiation.

It is in examining the effects of prenatal stress that one must be careful to distinguish between those stressors that may directly impinge on the foetus, such as electric shock, and those whose effects are mediated indirectly via alterations in the mother's internal *milieu*, such as fear or anxiety. It is also worth mentioning here that the term "prenatal stress" could be interpreted to mean "stress before birth", and this in turn implies a stress response on the part of the foetus. What is really implied is a stress response by the mother, since the foetus may well as yet be incapable of such a response. However, for brevity's sake, I will refer here to "prenatal maternal stress" as "prenatal stress", and to the offspring of stressed mothers as "prenatally stressed".

In assessing the effects of prenatal stress, several aspects of both behaviour and physiology have been studied. Known effects on offspring include reduced birth weights and retarded reflex development (e.g. Chevins, 1981), delayed puberty in females (Harvey, in press), altered sexual behaviour (e.g. Dahlöf et al., 1977) and altered emotional behaviour (e.g. Thompson, 1957). There is, however, an element of confusion here, in the variation of the age and sex of the animal studied, their postnatal experience, and in the characteristics of the tests and testing procedures themselves. This is particularly true of the behavioural studies.

Inasmuch as behavioural tests are concerned, a good deal of interest has centred on the concept of "emotionality", and many tests are specifically designed to investigate this trait. This interest probably stems from Thompson's (1957) observation that prenatal influences act on an organism when it cannot perceive discrete cues and has no (or little) neural memory store, and so such treatments act as sensitizing agents, affecting the general responsivness of the organism - characteristics such as "emotionality", "fearfulness", temperament etc. - rather than more specific responses. A more detailed discussion of emotionality is given in Chapter 4, but here it is sufficient to say that, in general, tests of emotionality examine an animal's response to novelty. The most common of these is the open-field test, in which an animal's activity and defecation (both considered indices of the emotional response) are measured. However, there are variations on this test; the open-field arena may be circular or square, and the dimensions vary enormously from one study to another; the number of times an animal is exposed to the arena can vary from once to twelve times on consecutive days; and the duration of each exposure can vary from one minute (e.g. Ader & Belfer, 1962) to ten minutes (e.g. Bruell, 1969). The results of these tests vary: most authors report decreased ambulation as a result of prenatal stress (e.g. Ader & Belfer 1962; De Fries & Weir, 1964; Thompson 1957). Others report no significant change (e.g. Ader & Conklin, 1963) or increased ambulation (e.g. Porter & Wehmer, 1969) in the prenatally treated animals. Similarly varying results have been obtained for ambulation latencies and defecation, but on the whole there is a trend towards the behavioural consequences of prenatal stress being decreased ambulation and increased defecation in a novel area. This is thought to represent increased timidity or fearfulness on the part of these animals.

Other tests involving an animal's behavioural response to novel situations include the response to handling (e.g. Ader & Plaut 1968), and home-cage emergence tests (e.g. Thompson, 1957), which indicate decreased emotionality in prenatally-stressed animals. Other behavioural tests assess the animal's reaction to learning situations, principally maze-learning (e.g. Thompson & Quinby, 1964) and conditional avoidance tests (e.g. Joffe, 1965_b) tests. The results of the maze learning tests generally indicate that prenatally stressed animals take longer to learn mazes and make more errors than do controls, while the results of conditioned avoidance tests indicate that experimental animals show more avoidance responses, and more readily, than do controls. The former seem to indicate increased fearfulness, while the latter might suggest the opposite. However both could be interpreted in either way, and physiological measures of the response are needed to clarify the situation. Overall, though, the trend is again in favour of prenatal stress causing increased emotionality, although this depends on the age and sex of the offspring, the characteristics of the test used, and especially by the strain of rats or mice used.

The results of tests for physiological consequences of prenatal stress are on the whole more clear cut than those of behavioural tests. As mentioned above, stress during pregnancy has been shown to reduce birth weights of the offspring (e.g. Chevins, 1981; Politch & Herrenkohl, 1979) and to retard early development (Chevins, 1981; Harvey, Doctoral thesis, in preparation). Reduced adrenal weights have been shown to result from injections of hydrocortisone and adrenalin (Gunberg, 1957, and Thompson & Goldenberg, 1962, respectively). Male animals have been shown to be 'demasculinised', displaying less intermale aggression and male sexual behaviour, by Ward (1972) and

Dahlöf et al. (1977). Puberty may be delayed in female mice (Harvey, in press). An increased susceptibility to gastric ulcers has also been shown to be a consequence of prenatal stress, both by Ader and Plaut (1968) using prenatal handling, and by Bell et al (1965,1967) using prenatal adrenalin injections and conditional avoidance. Ader and Plaut (1968) also studied the effect of prenatal handling on the offspring's hormonal response to mild and more severe stress. Basal levels of plasma corticosterone were unaffected by the prenatal treatment, as were the levels following electric shock. Differences were seen in plasma corticosterone levels following handling, but only in females, where experimental animals had lower levels than controls. Development of the diurnal corticosterone rhythm itself has been demonstrated to be affected by prenatal maternal treatment (Ader & Dietchman 1970).

Workers in this field have used a number of different procedures to induce prenatal stress, for example conditioned avoidance tests (Thompson, 1957; Ader & Belfer, 1962), handling (Ader & Plaut, 1968), daily exposure to swimming, tilting and noise (DeFries, Weir & Hegmann, 1967), and crowding (Keeley, 1962). Others have employed injections of hormones in an attempt to mimic the effects of stress, and these include epinephrine (DeFries, Weir & Hegmann,1967), norepinephrine (Young, 1963), and hydrocortisone (Lieberman, 1963). With the exception of the hormone injections, the direction of the effects would seem not to depend on the type of stressor used. However, even when these researchers employ the same stressor, there may still be differences in the timing or intensity of the stress procedure. Intensity of stress can be important: Thompson and Quinby (1964) induced high and low levels of stress in pregnant rats, and found that the higher the degree of maternal stress the less the offspring activity in an open-field. Likewise with timing: Thompson

et al. (1963) found that offspring of mothers injected with adrenalin or saline during the second trimester of pregnancy showed decreased ambulation in an open field when compared with offspring of mothers treated during either of the other two trimesters. It is sometimes the case too that different controls are employed: Ader and Belfer (1962) and Hockman (1961) both looked at the effects of prenatal conditioned avoidance, but Hockman's control animals were handled daily while Ader & Belfer's controls were left untreated. These inconsistencies between experiments mean that care must be taken before generalising from one study to another. Even the species of animal employed, and particularly the strain within each species, can determine the direction of the effect of maternal stress on offspring behaviour. DeFries (1964) stressed females of two strains of mice (BALB/Crgl and C57BL/Crgl) and tested the offspring in an open-field. The activity of the BALB mice was decreased, while that of the less active C57 strain was increased. Joffe (1965_a) also showed this effect on two strains of rats, Maudsley reactives (MR) and nonreactives (MNR): using a different stress procedure, the activity of the MR offspring in the open-field was decreased, while that of the MNR offspring was increased. Thus the genetic composition of the animal is important in the outcome of such experiments.

Due to problems such as those outlined above, it is hard to draw any definite conclusions from experiments investigating the effects of prenatal stress. In many cases the direction of the effect would appear to be independent of the stressor used, which then implies a common effect of these stressors on the mother's internal environment, probably mediated by the pituitary-adrenocortical and sympathetico-adrenal medullary systems.

The question of how the effects of prenatal stress are mediated has been considered from the outset by workers in this area of

research. Since there is no neural connection between mother and foetus, it is likely that some blood-borne substance is involved. Hormones are the most likely substances if this is the case, especially given that the word "stress" implies activation of the pituitary-adrenocortical system. There is now a general hypothesis of hormonal mediation of the effects of prenatal stress, founded on the basis that stress activates the maternal pituitary-adrenal system. The consequent increase in foetal glucocorticoids (especially corticosterone) was thought to produce long-term changes in the functioning of some areas of the foetal central nervous system, and the behavioural alterations were seen to be due to neural or neuroendocrine alterations in areas governing that behaviour. The changes in the central nervous system were also hypothesised to be in the mechanisms controlling pituitary-adrenal function which in turn altered behaviour, but since behavioural effects of prenatal stress have been demonstrated in experiments in which no detectable effects on pituitary-adrenocortical function were found (e.g. Joffe, 1977) this latter hypothesis somewhat lacks support. This hypothesis is based on evidence from two sources: firstly, on the evidence for the relationship between maternal and foetal pituitary-adrenocortical systems, and secondly, from hypotheses on their effects of neonatal manipulations on the behaviour and pituitary-adrenocortical functioning of the offspring.

The relationship between maternal and foetal pituitary-adrenocortical systems has been extensively studied. It has been established (Zarrow, Philpott and Denenberg, 1970) that corticosteroids cross the placenta, while ACTH does not (Milković et al., 1966). Adrenalectomy of pregnant rats results in hypertrophy of foetal and neonatal adrenals (e.g. Angervall, 1962), and in increased levels of plasma corticosterone at birth (Thoman, Sproul & Levine,

(1970). Here, adrenalectomy leads to a decrease in adrenal steroids in foetal circulation, and when the foetal pituitary-adrenocortical system is functional (day 18 of gestation) this promotes foetal ACTH output. The increase in foetal ACTH leads to foetal adrenal hypertrophy. The consequent increase in foetal corticosterone production maintains the maternal plasma corticosterone at normal levels via placental transfer from the foetus to the mother. Administration of cortisone (e.g. Knobil & Briggs, 1955) or of dexamethasone or corticosterone (e.g. Paul & d'Angelo, 1972) to the pregnant rat results in foetal adrenal atrophy. Here both maternal and foetal ACTH output is suppressed, leading to reduced adrenal development and reduced adrenocortical output. Administering ACTH to pregnant females (e.g. Jones, Lloyd & Wyatt, 1953) also reduces adrenal development. In this case the ACTH promotes an increase in maternal steroid levels, and hence in foetal steroid levels. This in turn suppresses ACTH output by the foetal pituitary, and leads to reduced adrenal development in the foetus.

Various postnatal manipulations (e.g. handling, electric shock) of immature rodents which have been shown to affect development and behaviour also produce pituitary-adrenocortical changes, such as accelerated development of the diurnal corticosterone rhythm (Ader, 1969), altered development of the pituitary-adrenocortical response to stress (Levine, 1968), and altered adult functioning of the pituitary-adrenocortical system (e.g. Ader & Grota, 1969). Adult behaviour may also be modified by direct manipulation of the neonatal pituitary-adrenocortical system: for for example, Howard and Granoff (1968) found the neonatal corticosterone implantation in the mouse increased activity and decreased motor coordination. These effects have all contributed to the general hypothesis for hormonal mediation

of prenatal stress.

While this hypothesis is still accepted by many workers, the evidence in favour of it is somewhat patchy (Joffe, 1978). Studies of the effects of prenatal hormone treatments on behaviour have produced inconsistent results, and one can only conclude that since certain pituitary-adrenocortical hormone manipulations can produce effects on behaviour, then prenatal stress effects may be mediated by the pituitary-adrenocortical system. While there is plenty of evidence that manipulation of the maternal pituitary-adrenocortical system can affect foetal and neonatal hormone levels and adrenal size, there has been only limited success in studies trying to obtain the same results from prenatal stress. Maternal starvation (Picon, 1957), exposure to low temperatures or chronic pain (Milković et al., 1963), or formalin injection (Schnürer, 1963) produce adrenal atrophy similar to that seen as a consequence of maternal corticosterone or ACTH injections. However, there is very little evidence for prenatal stress effects on hormonal levels in the foetus. The only real support for the hypothesis that changes in pituitary-adrenocortical function occur in prenatally stressed animals comes from a study by Ader and Plaut (1968), who found that female offspring of handled mothers showed a lower plasma corticosterone response to handling than did controls, although the response to a more severe stimulation (electric shock) did not differ between the groups. Another line of evidence which seems to belie the general hypothesis for the hormonal mediation of prenatal stress effects is based on the reasoning that if activation of the maternal pituitary-adrenocortical system is necessary to produce the behavioural effects of stress, then attenuating or abolishing the stress response should attenuate the effects. Smith et al. (1970) investigated this theory, as did Joffe (1977). While they used different prenatal stress procedures, both

found that the effects of stress on birth weights (and, in Smith et al.'s case, weaning weights) were attenuated by inactivation of the pituitary-adrenocortical system using adrenalectomy, chlorpromazine or dexamethasone administration. However, in no case where prenatal stress affects control animals' behaviour were consistently attenuated effects obtained by pituitary-adrenocortical manipulation. On the whole then, it seems unlikely that the effects of prenatal stress are due solely to altered pituitary-adrenocortical hormone levels during development. It is likely that there is some other factor, or group of factors, operating, which respond(s) to stress and to ACTH.

Having accepted that any manipulation of the mother during the early development of the offspring may affect them in some way, it must be pointed out that the period during which the stress is administered is not the only time during which the effects of the stress can be mediated. The mother plays an important role in the development of an animal, especially among mammals, where she can influence her offspring's development at two distinct phases. The first of these phases is during pregnancy, when the mother can affect the biochemical environment of the foetus by the hormones and antibodies she produces, and by placental transfer of waste matter, gas and nutrients. The second phase is after parturition, when the mother is the primary source of food, and provides warmth and sensory stimulation. In other words, prenatal stress effects could be due either to interference with normal *in utero* development, or else to altered mothering by the previously stressed mother, or else again to some combination of the two. Most researchers concern themselves with the first of these options, largely ignoring the second. As a consequence there is yet another inconsistency among studies on the effects of prenatal stress, some workers fostering and

cross-fostering the offspring (e.g. Thompson, 1957; Ader & Conklin, 1963), and others not (e.g. Lieberman, 1963; Young 1963). The area of mother-offspring relationships and their implications is discussed more fully in Chapter 3. However, it should be mentioned here that the initiation of maternal behaviour in virgin mice can be delayed by oestrogen or progesterone, whether of ovarian or adrenal origin (Leon et al., 1975). Progesterone treatment of pregnant rats during the last days of pregnancy with doses too large to allow normal delivery has no observable effect on multiparous rats but causes primiparous rats to either cannibalise or ignore their young (Moltz, Levin & Leon, 1969). Given that activation of the pituitary-adrenal system is a direct consequence of stress, and that ACTH induces progesterone secretion from the adrenals (Feder & Ruff, 1969; Resko, 1969), the fact that postnatal maternal behaviour may be affected by prenatal maternal stress should be considered. It is also possible that prenatally-stressed offspring, which are usually born underdeveloped - at least in terms of birth weight, are less stimulating to the mother, or provide less good cues for the initiation, maintainence and further development of maternal behavior.

The stressor to be used in this particular study is based on that of Keeley (1962), who crowded 15 animals in a 6" x 12" x 5" cage during pregnancy. Crowding is a treatment intended to be more relevant to stress in natural situations where there are high population densities. In such situations it is necessary that the population size is regulated, and in 1950 J.J. Christian formulated a hypothesis whereby population regulation was achieved by "social stress". In an increasing population, he argued, social encounters would increase and intensify, leading to this social stress, activation of the adrenal cortex and other organs of adaptation, increased mortality, dispersion and decreased reproduction. Evidence

for this hypothesis derives from three types of population studies: studies of natural populations, studies of semi-natural populations (in which small populations are allowed to grow freely in enclosed area), and laboratory studies of animals grouped together in cages. In each case physiological measures - of adrenal weight, reproductive condition etc. - are taken, and then compared with the population density. Studies of natural populations of small mammals derive most information from rats, mice and voles. In high population densities all show a decrease in number of pregnancies (e.g. Davis, 1953; Southwick, 1958), and other measures of reproductive efficiency may also be affected: for example, inhibition of maturation and reproduction occurs in voles and mice (Kalela, 1957; Chitty, 1952; Christian 1971_b), ovulatory rate is affected in voles (Hoffmann, 1958) and the number of foetal resorptions is increased in mice (Evans, 1959). There is also a corresponding increase seen in adrenal weight (e.g. Christian, 1959; Christian, 1971_b). In confined, "semi-natural", populations of mice there are many effects of increasing density on reproduction: female fertility is reduced (Southwick 1955), there is a reduction in litter size as a result of both abortion/resorption of embryos and a reduction in the number of ova (Christian, 1959), and infant mortality is increased, due to nest disturbance (Southwick, 1955) and suppressed lactation (Christian, 1959). Christian (1956, 1961, 1963_a) has also examined a number of endocrine parameters, and aside from the general increase in adrenal weight, has found an increase in zone widths of the adrenal cortex, decreased ovary, testis and seminal vesicle weight, and a decrease in thymus weight. Christian and Lemunyan (1958) have demonstrated persistent stunting in offspring of crowded mothers, presumed to be due to deficient lactation.

Thus the consequences of high population densities on the

behaviour and reproductive efficiency of the animals involved can be profound. Grouping mothers during the last trimester of pregnancy has also been shown to affect offspring sexual behaviour. In male mice there is an increased tendency to show lordotic (female) behaviour (Dahlöf et al., 1977; Herrenkohl & Whitney, 1976), and an increased latency to mounting and intromission, as well as a decrease in the number of mounts and intromissions (Harvey & Chevins, 1984). The results of group-housing mothers during pregnancy have been less extensively studied in female than in male offspring. However, Allen and Haggett (1977) have shown less sexual receptivity following ovariectomy and hormone replacement therapy in females of group-housed mothers than in offspring of control females. Albeit using a different stress procedure, Herrenkohl and Politch (1978) have demonstrated that the oestrous cycle may also be affected by prenatal stress, the effect being an overall lengthening of the cycle, primarily by lengthening the oestrous-metoestrous stage. Although the two situations of long-term high population densities and the (comparatively) short-term high population density for a fixed period during gestation are not directly comparable, it nevertheless seems that some parallels may exist in terms of consequences on reproductive efficiency.

The overall aim of this study is to examine some of the effects of prenatal stress, with particular reference to the importance of the mother's role in the mediation of these effects. The means employed to induce stress is that of crowding during the last trimester of gestation (for details see Chapter 2). The last trimester of gestation is chosen in preference to earlier stages mainly because it reduces the chances of foetal abortion or resorption. No really detailed study exists of the effects of minute differences in the timing of the stress procedure, but many studies

of prenatal crowding use the last week of pregnancy as the crowding phase. Another reason for using this phase is that it is in this point during foetal development that neurogenesis is at its highest - little occurs before day 9 of gestation (taking as day 0 the day a vaginal plug is seen). A major part of neurogenesis occurs over gestational days 11-18, although production levels are low as parturition approaches. After birth there is a return to high levels of proliferation in some areas of the hippocampus, the olfactory bulb and the cerebellum, but by the end of the third week of postnatal life neurogenesis is virtually complete (Rodier, 1980). Foetal development should therefore be particularly susceptible to environmental alterations during this last prenatal week.

The particular aim of this work was to try and give a more complete picture of the effects of prenatal stress by investigating several aspects of behaviour and physiology while at the same time using only one type of stress and one strain of mouse. Given the confusing results of emotionality tests, and the lack of consistency in controlling for the treatment of the mother, the main concern was to try and clarify the importance of the mother's role in the mediation of prenatal stress effects, with particular respect to her behaviour towards the offspring after birth. To this aim, all litters were either fostered or cross-fostered, and the maternal behaviour of both control and prenatally stressed females was examined over the 21-day lactation period. The intention was to determine in this way whether the treatment of the litter affected the mother's maternal care, or again whether the mother's treatment affected her behaviour towards the litter.

The area of emotionality and emotionality-related effects was investigated, and examined in the light of both maternal and foetal treatment. Standard tests of emotionality were used, for example the

open-field test and holeboard apparatus. In addition, pain thresholds and avoidance behaviour were examined.

Because of the comparative lack of information on female sexuality as affected by prenatal stress, an attempt has been made here to expand the data, and the area of pheromonal control of puberty and oestrous cycles has been investigated, as well as aspects of female sexual behaviour. Effects on male sexuality have not been studied here, primarily because another worker in this research group has already done this (Harvey, 1984).

Aspects of the adrenal rhythm have also been studied here. Ader and Dietchman (1970) have demonstrated that development of the circadian rhythm of corticosterone secretion may be affected by prenatal treatment, and the study of maternal behaviour in this work indicated that rhythmicity in maternal behaviour was altered by prenatal crowding. Hence the onset of the diurnal corticosterone rhythm was examined here, again as a function of both maternal and foetal treatment. The adult rhythm was also studied, as was the corticosterone response to stress.

In summary, this project aimed to examine known effects of prenatal maternal stress, to attempt to correlate results of different tests, and to clarify the results in terms of maternal vs. offspring treatment.

Chapter 2: Materials and Methods: General

Animals

The animals used throughout this work were outbred "TO" albino mice, obtained from A. Tuck & Son Ltd., Battlesbridge, Essex. In one experiment another strain was used: this was a "wild" strain, derived from a "TO" x "wild" mouse cross, and since then maintained by the occasional addition of a wild mouse to the breeding colony in our laboratory. All "TO" females used for breeding in the experiments were either purchased 2-3 weeks prior to the start of the experiment and given time to become accustomed to laboratory conditions, or else had been bred in our laboratories (1st or 2nd generation).

All animals were housed in one of two animal houses, under similar lighting and temperature regimes. A reversed lighting schedule was employed, of 14:10 hours light:dark, with lights on at 22.00 hours. Temperature was maintained at 18°C-22°C, food (Labsure Animal Diet, Christopher Hill Ltd., Dorset) and water were available *ad libitum*, and wood shavings were provided as bedding material. In all but one experiment, both males and females were present in each animal room, though housed in single-sex cages. All cages were cleaned out once every seven days, except for those of lactating females which were left undisturbed (except to replace bedding inadvertently soaked, etc.) until weaning.

Prior to mating, females were housed in groups of 8-10 in large plastic cages with stainless steel tops measuring 42 x 25 x 11 cm (Source: North Kent Plastics Ltd.). At 10-12 weeks of age they were placed in small plastic cages (30 x 13 x 11 cm) with a sexually mature male and examined daily for vaginal plugs: when a plug was observed (designated Day 0 of gestation) the male was removed. On day 12 of gestation the females were assigned to one of two groups.

Control animals were transferred individually to large cages where they remained until parturition, while experimental animals were transferred to "crowding cages". "Crowding cages" were large cages containing 25-28 adult males, and enough experimental females were added to bring the total number of animals in the cage up to 30. Late on day 16 or day 17 of gestation (depending on the experiment - see Chapter 3) the experimental females were removed from the crowding cages and placed individually in large cages to give birth. At parturition, litters were randomly culled to eight pups and then fostered.

The particular form of the fostering again depended on the experiment in question, but could be of four different forms: control mothers gaining control pups (abbreviated in later discussion to CC), control mothers gaining experimental pups (CE), experimental mothers gaining experimental pups (EE), and experimental mothers gaining control pups (EC). Foster mothers had all given birth to their own litters 0-24 hours prior to fostering. In each of the designated abbreviations, the first letter refers to the treatment of the mother and the second to that of the litter, where C = control, and E = experimental. Hence CE mothers are control mothers raising experimental offspring, and CE offspring are experimental offspring raised by control mothers.

Blood Sampling:

All blood-sampling was carried out using retro-orbital puncture (after Riley, 1960), a method that allows repeated blood-sampling from one animal, although no animal was sampled more than once in this work. Plasma corticosterone levels obtained by this method do not differ from those obtained by decapitation, the other common method (Nichols, 1980). Using this method, the animal is first

anaesthetised, and a small glass tube is then inserted into the orbital sinus. Blood flows out from the sinus through the glass tube and is collected in 400µl plastic vials, where it is heparinised by means of repeated uptake and ejection using heparinised capillary tubes. The samples are then centrifuged for 3 minutes at approximately 3,500 rpm, and following this are frozen and stored at -20°C until assaying. In order to obtain true resting values of plasma corticosterone, only as many animals were taken from each cage at any given sampling time as could be sampled within three minutes of cage disturbance (after Levine & Treiman, 1969).

Plasma corticosterone levels:

Total plasma corticosterone levels were determined using a radioimmunoassay developed by Nichols (1980). Samples were given an initial wash with 2,2,4 trimethyl pentane and then extracted into ethyl acetate. The antiserum used in the assay was rabbit anti-corticosterone-21-thyroglobulin serum, supplied by Miles-Yeda Ltd. This antiserum interacts significantly (>10%) only with progesterone and deoxy-corticosterone, and not with cortisol, testosterone, or any other important steroid. Since progesterone is removed by washing, and since deoxycorticosterone does not occur in significant quantities in mouse plasma (Gross et al., 1972), the assay has good specificity for corticosterone in this species. Recovery of corticosterone added to mouse plasma averaged 86.7%. The relationship between the amount of corticosterone added to a plasma pool to the amount estimated was linear over the range 10-400 ng/ml and the mean interassay variation was 3.74%. Sensitivity was 60pg, and the least detectable concentration was less than 5 ng/ml. A Packard Tri-Carb 300 scintillation counter was used to count the samples, and the counts were converted to concentrations (ng/ml) using a log transformation incorporated into a computer programme.

Ovarian function:

Ovarian function was assessed by the lavage method of taking vaginal smears. These were taken 2-3 hours prior to the changeover from light to dark. The smears were dried, stained with diluted (1:20) Giemsa stain for 20-30 minutes, washed in water and then dried again. They were then staged according to the description given by Bingel and Schwartz (1969).

Where vaginal smears and blood samples were taken from the same animal on any one day, the vaginal smears were taken four hours prior to the midday ("peak") blood-sampling time, so as to minimise disturbance effects on resting corticosterone levels.

Statistical Procedures

Physiological parameters such as body weight and plasma corticosterone levels, which may be assumed to have a normal distribution, were analysed using parametric tests: results are expressed in terms of means and standard errors. Unless otherwise stated, behavioural measures, which cannot be assumed to be normally distributed, were analysed using non-parametric tests: these results are expressed in terms of medians and 95% confidence limits.

To control for litter effects, no litter was represented more than twice in any one group of animals, on any of the tests outlined in this work. The only possible exception to this case is in the case of the maternal behaviour studies where adult females were "bought in" prior to the experiment: in these it was not possible to assess the relatedness of the animals.

Chapter 3: Maternal Behaviour

It has already been suggested here (Chapter 1) that the effects of prenatal treatment may be mediated not only by the prenatal action of the treatment on the pups but also indirectly through altered maternal behaviour. This hypothesis must also be considered in the light of evidence that the development and maintenance of normal maternal behaviour patterns depends, to some extent, on stimulation from the litter itself (Rosenblatt and Lehrman, 1963). Maternal behaviour was one of the earliest natural patterns of behaviour to be studied in the laboratory, in the early 1900's, and for a while was an active area of research. Interest was revived in the late 1940's and early 1950's with the "nature vs. nature" controversy. Concurrently there was the growing interest of psychologists and ethologists in social behaviour, and in mother-infant interactions as the first social relationship of the developing young. Since then it has been established that maternal behaviour is regulated by both hormonal and non-hormonal factors, and that different factors initiate pre- and postpartal behaviour.

Before proceding further a clarification of the term "maternal behaviour" is required. Few authors seek to define maternal behaviour. This is perhaps wise, as a number of different aspects of maternal care have been studied and to postulate a common causation for these different activities is not necessarily justified. The term does however remain a convenient collective description, and as such will be included here. Aspects of maternal behaviour usually studied include nest-building, behaviour during parturition, nursing and cleaning the young, retrieving offspring outside the nest, and aggression towards intruders. Many of these behaviours can be observed in naive rodents, (those that are not mothers and have not

previously encountered pups other than their own littermates): these animals require a habituation or "sensitisation" period of exposure to pups before maternal behaviour is displayed, whereas newly-parturient females, naive or experienced, show an immediate maternal response to pups. Mice require a shorter sensitisation period than rats, and unlike rats, all adult mice will show maternal reponses. There are genetic differences within species in maternal behaviour too, and there may be differences between colonies (Noirot, 1971). Nevertheless, the ability of naive animals to show maternal behaviour and the existance of these sensitisation periods have provided valuable clues to the mechanisms controlling maternal responsiveness to the young.

The onset of maternal behaviour occurs before parturition. Nest-building can be initiated in late-pregnant rats around 34 hours prepartum, following regular or continous exposure to pups (Slotnick et al., 1973). Retrieving can be initiated in the same manner in some animals as early as 28 hours prepartum, with 75-80% retrieving pups by 4 hours prepartum (Rosenblatt & Siegel, 1975). Again however there may be genetic differences: only 45% of Slotnick et al. (1973)'s Wistar rats showed prepartum retrieving (not beginning till 14 hours prepartum) as opposed to the 75% of Rosenblatt and Siegel (1975)'s Sprague-Dawley animals. Maternal aggression may also begin before parturition, although it is hard to demonstrate other than in the subordinate posture adopted by an intruder male. This occurs more rapidly and for longer periods of time with late pregnant females than with non-pregnant females (Erskine, 1978).

Although maternal caretaking behaviour can be virtually indistinguishable in sensitised and lactating rats, they can be distinguished by retrieval in a T-maze extension of the home cage (Stern & Mackinnon, 1976). Only a small percentage of sensitised

females retrieve pups in a T-maze whereas thelectomised postparturient females, lactating females and hormonally-induced maternal females all showed retrieval, and retrieval of similar quality. This indicated that hormonal factors associated with pregnancy and/or parturition but not suckling stimulation may facilitate T-maze retrieval of pups. However pup-induced maternal virgins resemble lactating females more than they do non-maternal virgins with respect to frequency and duration of retrieval and related behaviours, and also in the analysis of behavioural sequences in relation to choices of pups over toys for retrieval.

Sensitisation latencies can be reduced by injecting maternal blood into intact, nonpregnant females: this will stimulate nest-building, crouching over the young, retrieving and pup-licking with an average latency of 2.25 days compared with the 4-6 days required when plasma from a nonpregnant female or saline is injected (Terkel, 1972). Cross-transfusion was even more effective in this study, and 88% of the females receiving this treatment exhibited most components of maternal behaviour (except nest-building) with an average latency of 14.5 hours.

Maternal behaviour in nonpregnant animals can also be induced by hormone treatment. Several workers have tried to simulate hormonal changes during pregnancy, concentrating principally on progesterone, oestradiol and prolactin. As pregnancy progresses circulating progesterone levels increase dramatically, dropping sharply just prior to parturition. Oestradiol also increases, slowly at first, but latterly faster. Prolactin remains at a low level throughout pregnancy, rising sharply immediately prior to parturition. Hence Moltz et al. (1970) took ovariectomised nulliparous females and administered oestradiol benzoate for eleven days together with progesterone on days 6-9, and two injections of prolactin, one late

on day 9, the other early on day 10. On the evening of day 10 pups were presented. Females were maternally responsive to pups, slowing retrieval, nest-building, and crouching behaviour with latencies of 35-40 hours. Zarrow et al., (1971) using different doses and different timing of hormone injections, reported latencies of 36-72 hours.

It should be noted that despite the success by various workers in reducing the latencies to maternal behaviour by several hours, or even days, no-one has yet managed to reduce latencies so far that non-parturient females are indistinguishable from parturient females.

Thus prepartal hormonal factors are determinants of the expression (or non-expression) of maternal behaviour. Postpartal regulation of maternal behaviour also depends on other, non-hormonal factors, chief among which are stimuli from the pups. Maternal behaviour has been shown to decline rapidly if pups are removed at parturition (Rosenblatt & Lehrman, 1963): if rat pups were removed and then returned on the third day postpartum, about 30% of mothers still retrieved the pups and showed other components of maternal behaviour, but if pups were not returned till the fifth day postpartum none of the mothers showed any components of maternal behaviour other than nest-building (which rose rapidly on the return of pups). Hence postpartal maintainance of maternal behaviour would seem to rely on the young. There has been some debate whether rat and mouse pups maintain maternal behaviour by evoking hormonal secretion, or solely through the stimuli they provide. Following parturition the female rat will undergo a single oestrous cycle during which she may be mated and become pregnant. Following this postpartum oestrus she will become acyclic, and suckling by the pups will prevent oestrous cycling by inhibiting the release of follicle-stimulating hormone and luteinising hormone (eg. Rothchild, 1960; Gala, 1970). If pups are

removed immediately after parturition (in unmated females), cycling is resumed within 4-5 days in nearly half the females, and by 10-15 days in the remainder (Rothchild, 1960). Although circulating prolactin rises prepartum and is maintained at high levels throughout lactation, it does not appear to play a major role in the maintainance of maternal behaviour postpartum. Blocking the release of prolactin with ergocornine has no effect on maternal behaviour, although lactation fails and the pups receive inadequate milk (Numan et al., 1972; Zarrow et al., 1971). Similarly prevention of oxytocin release does not prevent the appearance and maintainance of maternal behaviour (Terkel, 1970). Cross-transfusion of blood from females 24 hours after parturition is ineffective in stimulating maternal behaviour in nonpregnant females, in contrast to the positive effect from blood of newly-parturient females (Terkel & Rosenblatt, 1972). This provides a general indication that humoral factors are no longer influencing maternal behaviour. There is in fact little evidence that maternal behaviour, once initiated under hormonal influences prepartally, requires hormones to maintain it. Hormones such as prolactin and oxytocin may play a role in modulating the mother's potential, but she does not rely on them for maintainance of maternal behaviour. Evidence points to a change from a principally hormonal regulation of the outset of maternal behaviour prepartally to a nonhormonal regulation postpartally. Pup stimulation is required immediately after parturition to maintain this hormonally-established behaviour - in their absence maternal behaviour, especially maternal aggression (Erskine, 1978), declines rapidly.

A transition period between pre- and postpartum maternal behaviour is clearly implied. This time is presumed to commence with parturition and involve a certain minimum exposure to pups, before the nonhormonal regulation can be established. Tests of retention of

maternal behaviour have been used to define the amount of pup exposure necessary. Bridges (1975, 1977) has shown that sensitisation latencies (25 days postpartum) are markedly reduced in females that have had 48 hours of postpartum contact, and even in females only given 6-8 hours contact with pups. Fleming and Cummings (cited in Rosenblatt et al., 1979) further demonstrated that only direct contact, as opposed to contact with sight and/or smell and sound of the pups, was successful in inducing immediate responsiveness to pups presented on day 7 postpartum. During the transition phase there is a period of overlap between the effects of hormonal and pup stimulation, and evidence suggests that hormonal effects may still be present while nonhormonal stimulation is becoming more effective - hormonal effects may be potentiated by early pup stimulation (Rosenblatt et al., 1979).

After parturition the interaction between mother and young determines the nature of the mother's behaviour towards her offspring. Her responsiveness depends on cues from the pups, principally auditory and olfactory cues, as well as tactile and visual factors. Olfaction is a primary element of the early mother-infant bond: many newborn altricial mammals, including mice, use olfactory cues to locate the nipple prior to attachment. These odour cues derive from the mother's saliva and amniotic fluid and are spread over the nipples when the mother grooms. Teicher and Blass (1977) have shown that newborn rats fail to attach to nipples that have been washed or covered with foreign odours. Infant rats only a few hours old are also able to discriminate between the saliva of their own mother (or any lactating female) and that of a virgin female. Human infants at 2-7 days of age can distinguish between the odour of their mother's breast pad and a clean breast pad (Macfarlane, 1975) and one day-old *Acomys* offspring show a preference

for their own home cage bedding and bedding from other litters of the same age over clean bedding and bedding from nulliparous females. Bilateral olfactory bulbectomy and anosmia caused by intranasal zinc sulphate treatement both lead to deficiencies in nipple attachment and suckling behaviour in rats as young as 2 days of age (Singh & Tobach, 1975; Singh et al., 1976). The tendency to huddle, which serves as a means of thermoregulation, is also disrupted by olfactory bulbectomy and zinc sulphate anosmia (Singh & Tobach, 1975; Alberts, 1978). Huddling in rats is under thermal, tactile and olfactory control, with temperature being the most important in early life, and olfaction showing increased importance between 5-10 days of age, (Alberts, 1978; Freeman & Rosenblatt, 1978_a). At this stage the young animal bgins to use olfactory cues to discriminate between objects and to orientate itself towards specific objects (prior to eye-opening) - before this stage locomotor responses do not appear to be a reliable indicator of olfactory discrimination (Leon & Moltz, 1971).

Rosenblatt has suggested that the maternal odour has two components, one being a general lactation odour and the other an individual odour specific to each female. Young animals can initially only differentiate between lactatory and non-lactatory odours, and later learn to discriminate between the odour of their mother and those of other lactating females, although the precise age at which this occurs is not known. The maternal odour originates in a secretion called caecotrophe, which is excreted via the anus with the faeces. Its production is dependent on prolactin secretion: ergocornine (which inhibits prolactin) treatment prevents caecotrophe production whereas adrenalectomy and ovariectomy have no effect (Leon & Moltz, 1973). Emission is preceded by a sequence of events starting with increased prolactin in the blood of the lactating female,

followed by an increase of prolactin at the liver, where it acts to increase cholic acid levels in the bile. Selected bacteria in the caecum then transform the cholic acid, the resulting cholic acid derivative being what is referred to as "the maternal pheromone" (Schumacher & Moltz, 1983). Schumacher and Moltz (1983) have argued that pup attraction to the maternal pheromone is innate, although since then Rosenblatt (1984) has pointed out that there is a preference for general maternal odours which develops before that for maternal faeces (and hence caecotrophe). Diet can affect the caecotrophe odour (Leon, 1975) - animals fed on different diets will produce different caecotrophe odours. This is probably more relevant - and adaptive - in the wild, where animals may to some extent eat different foodstuffs. In the laboratory, where animal feed is usually of a standard (often pelleted) form, it is less likely that odours will be affected in this way: it is possible that this fact may facilitate mother/offspring acceptance in fostering studies. Caecotrophe odours serve to attract the pups not only to the mother, but also to the nest and to littermates. Caecotrophe in the mother's faeces may be deposited in the nest or on the pups, and pups, by eating these faeces, may produce their own caecotrophe odour (Leon, 1974). Sixteen day-old rat pups are more attracted to the odour of littermates than an empty goal box. They are also attracted to the odour of unfamiliar 16 day-old pups, but not to the odour of similarly-aged pups whose mothers have been fed on sucrose and produce no caecotrophe (Leon, 1974).

Odours also serve to attract the mother to her offspring, and recognition of the offspring by odour develops early in lactation. In humans, by six days postpartum a mother can recognise her own child's garments by odour alone (Porter et al., 1983). Ostermeyer and Elwood (1982) have demonstrated that both male and female mice differentiate

between their own and alien young, and that this discrimination is probably based on olfactory, and possibly gustatory, cues rather than auditory ones. Beach and Jaynes (1956) also demonstrated that while alien pups are retrieved more slowly than own pups, and may be rejected once or twice before being retrieved when own pups are never rejected, maternal olfactory bulbectomy eliminates these differences, and both sets of pups are accepted with equal readiness. Chantrey and Jenkins (1981) have confirmed that mother mice discriminate between own and alien pups using olfactory cues.

Licking the young may serve to "imprint" them to the mother and create some kind of maternal bond. It has been shown (Klopfer & Gamble, 1966) that this is important in goats: a mother will push her kid away as it attempts to suckle if it was removed from her at birth for 1 hour, whereas if she is allowed to lick the kid for as little as 2-5 minutes at birth separation for up to 3 hours does not affect her maternal behaviour.

Another important factor in the maintainance and/or establishment of the mother-infant bond is ultrasonic vocalisations by the offspring. Rodent pups in distress emit high frequency sounds to which adults respond. Noirot (1972) and Bell (1974) have demonstrated that these calls can be of two types, one in response to cold and the other in response to unusual tactile stimuli. These may have different effects on the mother, either attracting the female and initiating searching, retrieval and nest-building, or else inducing her withdrawal and hence leading to the cessation of rough handling or aggression towards them. Sounds audible to man (ie. below ca. 20kHz) tend to fall into the latter category.

The rate of ultrasonic calling can depend to a large extent on environmental temperature, Okon (1970_a, 1970_b) showed that mouse pups give extremely few calls in response to cold during the very early

poikilothermic phase, and rapidly go into a cold coma, ceasing to call and even breathe. Calling rate increases as the pups grow older, but later in life (days 5-6 in Okon's studies), as the ability to regulate their own body temperature develops, the pups' ultrasonic response to cold again drops off. By the time thermoregulation is fully developed (day 17-18) the frequency of calling in response to cold has again become very rare.

Sales and Skinner (1979) have also related developmental changes in thermoregulation to ultrasonic calling, examining litters of mice exposed to different temperature over a range of ages. The animals were able to maintain their own body temperature (at 35-37^{o}C) by day 8 when exposed to 22^{o}C, at which temperature very little calling occured at any age. At 12^{o}C the animals managed to maintain body temperature by day 19, where peaks of calling occured at 4 and 6 days. At 3^{o}C body temperature was not maintained till day 21, and here two calling peaks were observed, at days 6 and 16. Hence an overall relationship between calling frequency and thermoregulatory ability can be seen, with calling frequency increasing with age until a peak is reached just prior to the point at which the animal starts to be able to maintain its body temperature. As the animal becomes increasingly able to "cope" so calling rate drops off.

Okon found that the highest rate of calling was during the first few days after birth, although Elwood and Keeling (1982) found a peak at day 9. Elwood and Keeling also found a change with age in the number of calls given in bouts as opposed to single calls: only 2% of the calls of very young animals (day 3) occurred singly, whereas in day 13 animals this figure rose to 38%. The same study showed that the mean bout length was maximal around day 3 and minimal at day 13, and the age at this maximal bout length would seem to relate closely to Okon's "highest-rate-of-calling" age, (although it should be

remembered that ambient temperature is also important in this respect).

Ultrasonic calling interacts with pup odours as means of directing the mother to pups outside the nest (Smotherman et al., 1978). Although ultrasounds are effective directional cues, odours are not, but odours increase search speed, and will also increase search response when the cues are presented together. This work suggested that stimuli from the pups not only initiate and direct location of pups, but also maintain the female's predisposition to retrieve. Hennessy et al., (1980) also demonstrated that retrieval may be stimulated by ultrasonic signals, pups of a strain emitting a high number of ultrasounds following handling being retrieved faster than pups of a less reponsive strain. Although pups emitting more ultrasounds were retrieved faster they were licked less than were the other pups suggesting that ultrasonic signals do not stimulate maternal licking. The pups that were retrieved faster were those that were slower to develop thermoregulation, although strain difference in ultrasonic signalling were not related to differences in degree of hypothermia.

The amount of time a female spends on the nest is to some extent regulated by pup vocalisations. Jans and Leon (1982) have shown that in rats the duration of intervals between bouts of contact with the young in the nest is influenced by the animal's ablity to dissipate body heat, but while thermal factors can initiate these bouts, pup vocalisations may terminate them. However if the pups do not attach to the nipples soon after the contact bout is initiated the mother may terminate it, especially if the pups are warm. If the pups are cool she is more likely to maintain contact. Jans and Leon also showed that the mothers did not attempt to either maximise or minimise the duration of contact bouts with the offspring: when given

a choice of temperatures in which to rest these females spent approximately half the time on the nest compared to those in ambient laboratory temperatures. Neither did these females seek out the coolest areas of "cool off" in during off-nest intervals. Leon et al., (1984) have demonstrated that mother rats had longer contact bouts with their offspring during the day than during the night, and that this could be related to maternal brain temperature, which peaked during the night. When the daily temperature cycle was suppressed by removal of adrenal and ovarian hormones (via adrenalectomy and ovariectomy) this daily cycle in contact bouts was also suppressed.

Maternal behaviour will change over the lactation period as the pups become increasingly more able to fend for themselves. The amount of time a mother spends on the nest and the quality of the nest decline over the lactation period (Hughes et al., 1978). The developing pups will over this phase be developing the ability to regulate their own body temperature and hence will be less in need of the warmth provided by the mother's presence and a good quality nest. Also as the pups get older and their eyes open they are more likely to leave the nest for short periods of time and again the need for a good nest is less. Retrieval will likewise decline with the onset of thermoregulation and decline in ultrasonic calling - and indeed with the increasing size of pups. Expression of maternal aggression also follows a time scale over lactation. Postpartum aggression is highest (in rats) over days 3-8 of lactation, declines over days 9-14, and over days 15-21 is present towards males but not females (Svare & Gandelman, 1973).

Strain differences may also affect the expression of maternal behaviour. Strain differences with regard to ultrasonic calling have already been mentioned. Ward (1980) examined the frequency of a

variety of activities in inbred female mice of three strains, in the presence of either isogenic (own or fostered) or nonisogenic offspring. He found that differences existed in the frequency of grooming, nursing and handling the offspring and in the time spent off the nest, which could be related to differences in the rate of early postnatal development in two of the strains. In general, pups receiving less maternal attention developed more rapidly. Ward suggested that the cause of this more rapid development lay in the sequential ordering of the foster mother's manipulation of her young, rather than in the total quantity of care received. Mann et al., (1983) have demonstrated genetic differences in infanticide, both in the age of onset and in the proportions of animals that exhibit infanticide. Hennessy et al., (1980_a) looked at maternal behaviour of C57BL/6 and A/J mice after daily infantile handling and found that maternal behaviour differed in a number of ways irrespective of the pup's strain, although no differences had been observed prior to handling. Mothers with the same strain pups treated them differently from the way they did other strain pups. Recovery of body temperature following handling was slow for both strains of pups and depended to some degree on the strain of the foster mothers. St. John and Corning (1973) found large strain differences in maternal aggression among five strains of mice. There are also strain differences in pup repsonsivesness: Hennessy et al. (1980_b) found that C57BL/6J pups spend more time near a same-strain or A/J mother than they do with a virgin female, whereas A/J pups show no preference. In a study by Carlier et al. (1983), reflex development was affected in 16/34 cases by pup strain and in 10/34 cases by maternal strain. Survival was only affected by pup strain, but weight was affected by maternal, pup and interactive factors. There is other evidence that rearing by a nonisogenic foster mother can affect the development of the

offspring. For example, Southwick (1968) reported that A/J mice became more aggressive if raised by CFW dams, but that fostering CFW mice to A/J dams had no effect on the aggression of CFW mice. Lagerspetz and Wuorinen (1965) reported similar results for cross-fostering of mice selectively bred for high or low incidence of aggressive behaviour.

Meier and Schutzman (1968) examined and re-interpreted behavioural effects of early experimental manipulation of the offspring in the light of altered interaction between mother and infant. They proposed that the mother responds differently to certain behaviours of her offspring or predominantly to those offspring showing such behaviours. This theory was supported by the work of Bell and Little (1978), among others, who observed parental behaviour following the return to the nest of pups subjected to differing degrees of "stress". Mothers attended more to pups subjected to intermediate levels of stress, the differences persisting across the age span (3,6,9 and 12 days) without change. Fathers exhibited marked changes in responsiveness to the young across the ages studied, their attention to the young correlating with the rate of ultrasonic calling by the young. Sherrod et al., (1974) also showed that early handling led to increases in maternal behaviour that was stimulating to the pups, such as licking, carrying them, retrieving them and cleaning the nest. Perinatal treatments can affect both the mother's behaviour and offspring development. Johanson (1980_a, 1980_b; Johanson et al., 1980) has studied the effects of hyperthyroidism and hypothyroidism. Hypothyroid offspring showed delays in the development of home orientation and in the development of olfactory reponsiveness relative to control animals, whereas hyperthyroid offspring developed home orientation at the normal age (peak percentage homing between days 12-16, as opposed to day 20 in

hypothyroid young) and showed an accelerated development of olfactory responsiveness. Vis-a-vis the mothers of these offspring, the normal decrease in nesting and nursing with pup age was delayed in hypothyroid litters but accelerated in hyerthyroid litters. Retrieval was not affected, but grooming was seen less in hypothyroid groups. Here, physiologically abnormal pups were being treated differentially by their mother: this had implications for prenatal treatment and prenatal stress studies, where the offspring may be born underdeveloped and provide altered stimuli for the mother. Levine (1959, 1960, 1962) maintains that handling and other forms of neonatal stimulation accelerate the development of such characteristics as eye-opening, fur growth, locomotion and body size, although several other workers have failed to confirm this. Perhaps because of this confusion, attempts to relate early development to maternal and to neonatal treatments have been disappointing - Herrenkohl and Whitney (1976) examined the effects of prepartal stress (heat, restraint and bright lights) on postpartal nursing behaviour, litter development and adult sexual behaviour. They found that stress affected litter weights at birth and at 21 days, and altered adult sexual behaviour, but found no differences in maternal behaviour towards these offspring. Cross and Labarba (1978) also found no evidence to suggest that neonatal handling leads to accelerated development. However, in Herrenkohl and Whitney's study a very limited study of maternal behaviour was made, and in Cross and Labarba's work small samples sizes were used. Other researchers have had more success. Maternal adrenalectomy has been shown to affect litter weight gain by Hennessy et al., (1978). They managed to show that cues from adrenalectomised females' pups are less arousing to lactating females. There was also a retarded development of ultrasonic signalling in these pups, which Hennessy et al. presumed

was due to the malnutrition that results from maternal adrenalectomy: malnourished pups had similar body weight to pups of adrenalectomised mothers at 15 days, and Hunt et al. (1976) have shown that pups reared by malnourished mothers also produce fewer ultrasounds. Weiner et al. (1977) have likewise demonstrated that mothers of malnourished offspring (this time achieved through a low-protein maternal diet) show deficits in retrieval and the rate of nest-building although adrenalectomised mothers did not. Both Weiner et al. (1976) and Thoman and Levine (1970) have shown that adrenalectomised mothers spend more time on the nest than controls - as do mothers of undernourished offspring. Fleischer and Turkewitz (1981) have demonstrated that undernutrition can also affect maternal care in rat pups rotated between lactating and non-lactating foster mothers. There was no difference in maternal care until the third week of lactation, when females caring for pups fed only 8 hours per day nursed more and built better nests than females caring for pups fed for 16 and 24 hours per day.

In the work described in this chapter the aim was to see whether or not prenatal crowding affected maternal behaviour in the "TO" strain of mouse. No data is apparently available on this to date. Knowing the evidence for prenatal and postnatal influences on maternal responsiveness, it was hoped that differences in maternal behaviour might be seen, and if so, related to either the prenatal treatment of the mother herself, or to the prenatal treatment of her pups. The exposure of mothers to crowding with males and to harrassment by them in the days immediately before birth is known to elevate maternal corticosterone secretion, at least acutely (Harvey, Doctoral thesis in preparation), but it is not know what other repercussions may arise in the complex endocrine changes which accompany both. As the above review has demonstrated the importance

of hormones in the initiation of maternal behaviour, it seems likely that some disturbance to the mother's behaviour may be observable. Prenatally stressed offspring are known to be lower in weight at birth, and if other aspects of their development (eg. thermoregulation) are equally retarded, this too may result in alterations to maternal responsiveness. All four combinations of control and experimental mothers and pups were studied in order to separate causal factors of purely maternal origin from those due to the offspring.

Materials and Methods

Animals used were virgin females aged 10-12 weeks, and were treated during pregnancy according to the procedure given in Chapter 2.

The lactation period was studied from two angles: firstly, in terms of maternal behaviour, and secondly in terms of litter development.

Maternal observations (see below) were made in four studies (Expts. 1a-d). The first of these (Experiment 1a) examined maternal behaviour in CC and EE females of two different strains, "TO" and "Wild" (see Chapter 2). Two strains were examined, as an original aim of the project was to compare the two strains not only on maternal behaviour but also in behavioural and physiological tests: this idea was abandoned as behaviour tests were carried out at 30-35 days, and the "Wild" offspring proved nearly impossible to handle at this age. The next study (Experiment 1b) was carried out to confirm a difference observed between control and experimental "TO" females rearing pups of like treatement: Wild females were not included here. The third study (Experiment 1c) extended the observation made in the

prior studies to CC, CE, EE and EC females. The crowding period used in Expts. 1a-c was days 12-16 of gestation. In this research group the normal practice is to crowd animals over days 12-17, and the change to a day 12-16 crowding period had arisen as a result of a large number of premature births (in crowding cages) in a pilot study employing the standard crowding procedure. It is possible that day 17 may be important in determining the extent of the effect of prenatal stress and also that the temporal proximity of stress to parturition may determine whether there are effects on the initiation of maternal behaviour: hence a further study (Experiment 1d) was carried out to see whether an extra day's crowding had any obvious effect on maternal behaviour not seen when the crowding period only extended to day 16. This study again compared all four groups of animals.

Experiment 2a consisted of retrieval tests, carried out on CC and EE mothers (crowding over days 12-16): details are given below. A separate attempt had been made to study nest-building and maternal aggression, but in the course of this study the offspring contracted an infantile disease which was manifested by diarrhoea, stunted body growth, and eventually death. This disease persisted in the animal house to some extent over a year, and was eventually only eliminated by killing all animals in the room, and by thoroughly sterilising the room using ultra-violet light and fumigation. The results of the nest-building and aggression tests were considered subject to misinterpretation, and have not been reported here. Nest quality was assessed (Experiment 2b) in all four groups daily over the lactation period (crowding days 12-17), according to the method given below.

Pup development was assessed from three points of view. Firstly (Experiment 3a) a record was made of the age of eye-opening, for CC and EE pups only (crowding days 12-16). Secondly (Experiment 3b) an assessment was made of body weight (crowding days 12-17): although

birth weights were examined in every batch of mice bred throughout this work to assess whether or not the prenatal treatment had had an effect, this was extended here to compare birth and weaning weights of all four groups of pups, as well as an intermediary weight at eleven days of age. The mother was removed prior to weighing, and the pups weighed individually on a Metler P1100 electronic top-pan balance (accurate to 0.01g) as speedily as possible. The mother was returned to the cage. Weights were not measured at other ages so as to minimise handling, and hence possible alterations in development (e.g. Ader, 1969). Thirdly, the frequency of ultrasonic vocalisation was assessed (Experiment 3c), again in CC and EE animals only (crowding days 12-17).

Test Methods:

Maternal Observations:

Observations were made of maternal behaviour twice daily over the 21-day lactation period. The two observation periods took place at one hour after "lights-off" (13.00 hrs) and at nine hours after "lights-on". These times were deliberately chosen to coincide respectively with the active and inactive phases of the animals' daily activity cycles. Observations were made on a "spot-check" basis (as in Hennessy et al., 1979): each cage was examined in turn, the behaviour in progress being noted before passing on to the next one. This was repeated at 5-minute intervals over a one-hour period. Thus twelve observations were made for each cage on two occasions each day. Behaviour in progress was sorted into the following categories:

a) "On nest" - mother is on the nest.

b) "Other pup-directed activity" - includes nursing, nest-building and pup-licking.

c) "Feed" - mother is feeding at food hopper.

d) "Other" - includes grooming of self, inactivity, and general activity (climbing on the bars of the cage etc.).

The components of the categories "Other pup-directed-activity" and "Other" had been treated separately in a pilot study. However, many of them appeared too infrequently for sensible analysis, and so here are grouped into two categories. Pup directed activity was seen to be representative of the mother's involvement with her young and as such a useful category. "Other" was a category used to include non-pup-directed-activity. Feeding was kept apart from this category, in part to determine whether possible differences between control and experimental females in access to food during the gestation period were reflected in feeding patterns, and in part to see whether differences in feeding existed that might reflect pup development (and hence demand).

The daily frequencies of each behaviour category in white light (a.m.) and red light (p.m.) phases of the cycle were summed to give total scores. "On nest" was further analysed in terms of differences between the amount of time spent on the nest in the two phases, as development of any diurnal rhythm in pups may well be dependent on maternal rhythmicity. Because of the sheer volume of the data collected in these studies, data was analysed using a parametric two-way analysis of variance with repeated measures. This analysis is robust enough to tolerate some violation of the assumptions underlying parametric analysis (Meyers and Grossen, 1974), and so this test was used in preference to a non-parametric test as computing facilities did not provide for non-parametric analysis on this scale.

In Expt. 1c nursing was also assessed separately, in addition to its inclusion in the category "PDA".

c) "Feed" - mother is feeding at food hopper.

d) "Other" - includes grooming of self, inactivity, and general activity (climbing on the bars of the cage etc.).

The components of the categories "Other pup-directed-activity" and "Other" had been treated separately in a pilot study. However, many of them appeared too infrequently for sensible analysis, and so here are grouped into two categories. Pup directed activity was seen to be representative of the mother's involvement with her young and as such a useful category. "Other" was a category used to include non-pup-directed-activity. Feeding was kept apart from this category, in part to determine whether possible differences between control and experimental females in access to food during the gestation period were reflected in feeding patterns, and in part to see whether differences in feeding existed that might reflect pup development (and hence demand).

The daily frequencies of each behaviour category in white light (a.m.) and red light (p.m.) phases of the cycle were summed to give total scores. "On nest" was further analysed in terms of differences between the amount of time spent on the nest in the two phases, as development of any diurnal rhythm in pups may well be dependent on maternal rhythmicity. Because of the sheer volume of the data collected in these studies, data was analysed using a parametric two-way analysis of variance with repeated measures. This analysis is robust enough to tolerate some violation of the assumptions underlying parametric analysis (Meyers and Grossen, 1974), and so this test was used in preference to a non-parametric test as computing facilities did not provide for non-parametric analysis on this scale.

In Expt. 1c nursing was also assessed separately, in addition to its inclusion in the category "PDA".

Maternal tests:

Retrieval tests were carried out over days 1-14 postpartum inclusive, after which date virtually no females were any longer retrieving pups: at this age many pups have their eyes open, and if retrieved to the nest are quite likely to climb out again. The mother to be tested was removed from the cage, and her litter was taken from the nest and placed as far from it as possible. The mother was then returned to the nest and three latencies were measured - the latency to emerge from the nest, the latency to retrieve the first pup, and the latency to retrieve the entire litter. A pup was said to have been "retrieved" only if it was returned to the nest: pups carried to another part of the cage were "not retrieved". To minimise disturbance of the litters no test period lasted longer than 5 minutes. If retrieval was not completed within this time the the pups were returned to the nest and the cage replaced in the rack. CC and EE groups only were tested (crowding over days 12-16).

Nest ratings:

Nests of CC, CE, EE and EC mothers were observed daily and scored according to quality. The scoring was as follows:

0 = no nest - no obvious sign of nest.

1 = poor - slight hollow in bedding, or bare patch on floor of cage.

3 = average - a small mound of bedding.

6 = good - a large mound of bedding, well structured.

9 = "volcano" - incorporating all bedding, well structured.

Scores in between those given above were occasionally awarded.

Pup vocalisations:

Frequencies of ultrasonic calling were recorded in CC and EE pups aged 3, 7, 9, 11 and 13 days of age. The pup to be tested was removed from the nest and placed in a glass dish. After being left for two minutes (to reduce calls in response to tactile stimuli and to enable the change of temperature to be felt) the number of ultrasonic calls were manually counted using a QMC mini bat detector (supplied by QMC Instruments Ltd., London). This detector could be set to pick up specific frequencies in the range 10-160 kHz (in this study calling at 60kHz was measured), and had an earphone to enable recording while minimally disturbing the animals. Recording was carried out over 2 minutes, after which the pup was returned to the nest. To control for possible litter effects, no more than two pups from each litter were used at each age tested. Testing was carried out between 2-6hrs after "lights-off". These litters were not manipulated in any other way, or used for other studies.

Summary of Experiments

Expt. 1: Maternal Observations:

a) CC, EE - TO and Wild strains	Stressed days 12-16
b) CC, EE - TO	Stressed days 12-16
c) CC, CE, EE, EE - TO	Stressed days 12-16
d) CC, CE, EE, EC - TO	Stressed days 12-17

Expt. 2: Maternal Tests:

a) Retrieval - CC, EE	Stressed days 12-16
b) Nest Quality - CC, CE, EE, EC	Stressed days 12-17

Expt. 3: Pup Development:

a) Eye-opening - CC, EE	Stressed days 12-16
b) Body weight at day 0, day 11, and day 21 - CC, CE, EE, EC	Stressed days 12-17
c) Ultrasonic vocalisation - CC, EE	Stressed days 12-17

Results

Experiment 1.

a) Observations of maternal behaviour of CC and EE animals, in both TO and Wild Strains:

This data (see Tables 3.1-3.3) was analysed using a two-way ANOVA, looking for differences in total quantities of each category of behaviour between CC and EE groups within each strain. No such differences were obtained. "On nest" was further analysed in terms of the difference between a.m. and p.m. levels of each behaviour: here there was a difference seen ($p < 0.05$) between CC and EE TO females in that the daily rhythm shown by CC females is not seen in EE groups. This difference appeared to exist to some extent in Wild females as well (see Figs. 3.1 and 3.2) but was not statistically significant in this case.

b) Observations of maternal behaviour of CC and EE animals (TO strain):

This experiment sought only to confirm the rhythm difference between CC and EE females of "on nest". It succeeded in this (data not presented), the rhythm being reduced in EE females when compared with CC females.

C) Observations of maternal behaviour in CC, CE, EE and EC groups:

No significant differences between groups were observed here, either in terms of the total amount of each behaviour or in terms of separate a.m. and p.m. levels (see Figs 3.3-3.32: standard errors have been omitted for clarity's sake. Each group of animals was then examined individually for changes over the 21-day lactation period, looking first at the total amount of each behaviour, and then breaking this down into morning and afternoon levels. Most categories showed significant changes over time (see Table 3.4), and for easy reference diagrams were constructed to represent this (see Figs 3.33-3.35). Where a significant change over time existed the direction of the change was assessed (using Student's t-test comparisons of 7-day or 3-day blocks),

Table 3.1.: Mean frequencies of "On nest":

			Day Postpartum																			
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
TO	CC	a.m.	10.6	9.4	8.1	9.6	6.1	8.8	10.0	10.5	6.5	11.0	7.4	5.7	4.5	6.5	4.2	6.2	8.4	9.1	9.3	8.0	9.4
		p.m.	7.7	8.2	4.6	4.6	3.5	2.9	2.5	1.6	2.1	3.4	3.9	2.0	4.0	2.3	3.6	2.7	3.3	3.2	1.9	0.7	1.7
		Total	18.3	17.6	12.7	14.2	9.6	11.7	12.5	12.1	8.6	14.4	11.3	7.7	8.5	8.8	7.8	8.9	11.7	12.3	11.2	8.7	11.1
	EE	a.m.	11.8	7.0	8.4	10.5	8.0	6.0	4.1	6.7	7.7	4.8	3.9	4.6	4.0	8.0	6.9	6.9	8.9	7.3	7.1	7.8	7.2
		p.m.	9.4	9.3	6.1	6.1	3.9	4.4	5.1	5.0	4.9	3.9	4.8	3.4	3.4	3.7	2.3	3.6	2.3	9.2	4.0	1.4	0.6
		Total	21.2	16.3	14.5	16.6	11.9	10.4	9.2	11.7	12.6	8.7	8.7	8.0	7.4	11.7	9.2	10.5	11.2	15.5	11.1	9.2	7.8
Wild	CC	a.m.	10.3	11.7	10.6	12.0	9.4	10.0	10.4	9.1	9.9	8.6	7.3	10.1	5.7	6.3	9.4	11.3	6.2	7.2	8.1	8.1	9.3
		p.m.	10.2	7.9	7.2	8.0	10.7	7.9	4.7	3.3	3.1	4.8	1.9	3.6	1.7	1.6	1.0	1.6	2.6	1.0	4.6	3.3	1.6
		Total	20.5	19.6	17.8	20.0	20.1	17.9	15.1	12.4	13.0	13.4	9.2	13.7	7.4	7.9	10.4	12.9	8.8	8.2	12.7	11.4	10.9
	EE	a.m.	10.7	10.8	10.0	11.6	10.3	9.4	9.6	6.9	7.4	1.9	10.3	8.7	8.4	7.9	7.7	5.8	7.7	7.7	7.6	9.4	11.0
		p.m.	8.8	7.6	7.3	8.2	4.2	7.0	4.1	5.4	2.8	1.7	3.4	3.1	1.6	2.6	4.3	3.3	2.4	5.0	1.9	4.4	4.6
		Total	19.5	18.4	17.3	19.8	14.5	16.4	13.7	12.3	10.2	3.6	13.7	11.8	10.0	10.5	12.0	9.1	10.1	12.7	9.5	13.8	15.6

TO: CC n=10
EE n=9

Wild: CC n=9
EE n=9

Table 3.2.: Mean frequencies of "Pup-directed activity":

			Day postpartum																			
			1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
TO	CC	a.m.	22.5	15.8	17.0	19.7	12.8	17.4	20.4	20.8	12.4	22.2	14.0	12.3	9.2	12.9	8.0	12.0	14.6	19.1	17.5	7.9	11.7
		p.m.	15.0	12.8	10.2	9.4	7.3	6.0	4.9	3.4	7.7	5.9	8.0	3.9	8.0	4.5	6.6	6.6	5.7	4.8	1.9	0.8	1.8
		Total	37.5	28.6	27.2	29.1	20.1	23.4	25.3	24.2	20.1	28.1	22.0	16.2	17.2	17.4	14.6	18.6	30.3	23.9	19.4	8.7	13.5
	EE	a.m.	22.8	15.0	17.7	20.2	16.6	11.7	8.2	13.3	15.1	13.7	6.8	9.2	7.8	16.0	13.2	12.7	16.9	13.9	10.7	11.3	10.3
		p.m.	18.8	15.3	12.6	11.8	7.8	9.1	10.4	10.0	9.8	6.3	9.6	5.6	5.8	7.3	5.6	8.6	4.6	7.9	7.2	1.9	0.6
		Total	41.6	30.3	30.3	32.0	14.4	20.8	18.6	23.3	14.9	20.0	16.4	14.8	13.6	23.3	18.8	21.3	21.5	21.8	17.9	13.2	10.9
Wild	CC	a.m.	21.3	22.6	21.6	23.6	19.8	19.6	21.3	18.2	20.1	17.0	16.9	20.4	11.8	13.7	18.8	21.0	12.7	12.8	16.7	15.3	17.4
		p.m.	11.3	15.9	14.7	16.2	21.6	15.9	9.3	6.7	6.2	9.8	4.2	7.1	3.3	4.8	2.1	3.9	5.8	3.4	6.8	5.1	2.6
		Total	32.6	38.5	26.2	29.8	41.4	25.5	20.6	24.9	26.3	26.8	21.1	27.5	15.1	18.5	20.9	24.9	18.5	16.2	23.5	20.4	20.0
	EE	a.m.	21.8	21.7	20.6	22.3	20.6	19.2	19.2	14.2	14.1	4.3	20.7	18.0	16.8	15.7	14.9	11.3	13.9	13.2	14.1	17.1	20.7
		p.m.	18.4	15.2	15.6	16.6	8.8	14.1	8.9	10.3	5.6	3.6	6.9	6.2	3.3	5.1	8.7	6.9	5.0	10.8	5.7	8.2	8.6
		Total	40.2	36.9	36.2	38.9	29.4	33.3	28.1	24.5	19.7	7.9	27.6	24.2	20.1	20.8	23.6	18.2	18.9	24.0	19.8	25.3	29.3

TO: CC n=10
EE n=9

Wild: CC n=9
EE n=9

Table 3.3.: Mean frequencies of feeding (total):

		Day postpartum																			
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
TO:	CC	2.7	4.9	12.1	6.4	9.4	7.6	8.1	8.5	11.1	6.7	7.0	11.1	11.2	10.5	13.3	12.0	8.7	7.3	6.6	8.8	6.9
	EE	1.7	5.7	3.4	4.4	6.4	8.2	10.9	9.4	7.6	10.1	10.9	13.1	12.8	5.4	8.4	10.4	10.9	7.0	7.0	8.2	8.7
Wild:	CC	1.4	2.4	4.2	1.6	3.2	3.8	5.1	9.2	7.1	8.2	8.8	7.4	11.0	10.3	10.1	9.4	11.3	10.7	8.3	8.7	7.7
	EE	1.9	3.3	3.2	2.7	8.6	11.0	7.2	8.1	8.9	14.7	8.0	7.7	10.1	9.3	10.0	11.3	9.8	9.0	8.7	7.0	4.4

TO: CC n=10
EE n=9

Wild: CC n=9
EE n=9

Table 3.4.: Results of within-group analysis of variance expressed in terms of probabilities.

		FREQUENCY CHANGES OVER DAYS 1-21			A.M./P.M. DIFFERENCE		
		TOTAL (A.M. + P.M.)	A.M.	P.M.	A.M. vs. P.M.	A.M. vs. P.M. OVER DAYS 1-21	A.M. vs. P.M./DAYS 1-21 INTERACTION
ON NEST	CC	0.0113	NS	0.0010	0.0018	0.0050	NS
	CE	NS	(NS)	NS	0.0012	NS	NS
	EE	0.0029	0.0000	NS	0.0000	0.0000	0.0000
	EC	0.0161	0.0045	0.0097	0.0000	0.0222	0.000
NURSE	CC	0.0062	NS	0.0000	0.0029	0.0011	NS
	CE	NS	NS	NS	0.0038	NS	NS
	EE	0.0029	0.0018	NS	0.0041	0.0022	(NS)
	EC	0.0157	0.0285	0.0039	0.0000	0.0303	0.0040
PDA	CC	0.0000	0.0113	0.0000	0.0011	0.0000	NS
	CE	0.0104	0.0394	NS	0.0024	0.0072	NS
	EE	0.0000	0.0000	(NS)	0.0012	0.0000	NS
	EC	0.0000	0.0000	0.0000	0.0000	0.0000	0.0072
FEED	CC	0.0025	NS	0.0021	0.0171	0.0000	NS
	CE	0.0055	0.0178	NS	0.0241	0.0000	NS
	EE	0.0000	0.0000	NS	0.0274	0.0000	NS
	EC	0.0136	0.0203	NS	0.0029	0.0154	(NS)
OTHER	CC	0.0000	NS	0.0410	0.0087	0.0445	NS
	CE	NS	NS	(NS)	0.0032	NS	NS
	EE	NS	NS	NS	0.0000	NS	NS
	EC	NS	NS	NS	0.0122	NS	NS

(NS) indicates that the result bordered on significance

CC n=10 EE n=9
CE n=9 EC n=7

Fig. 3.1: Mean frequency of "On nest" (±S.E.) for TO groups:

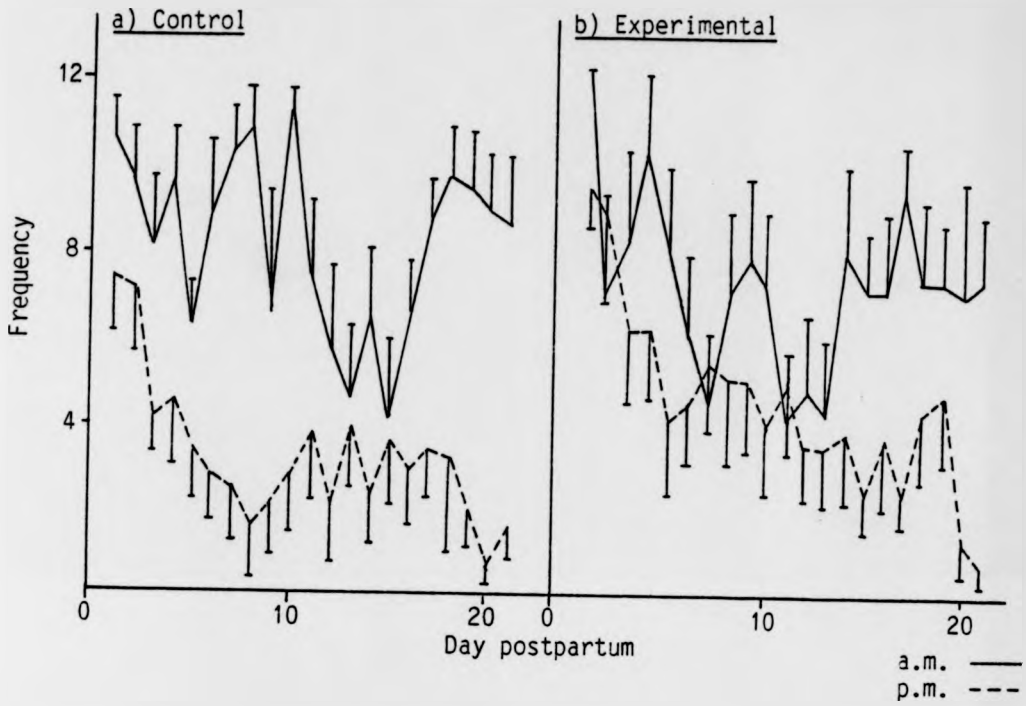

Fig. 3.2: Mean frequency of "On nest" (±S.E.) for Wild groups:

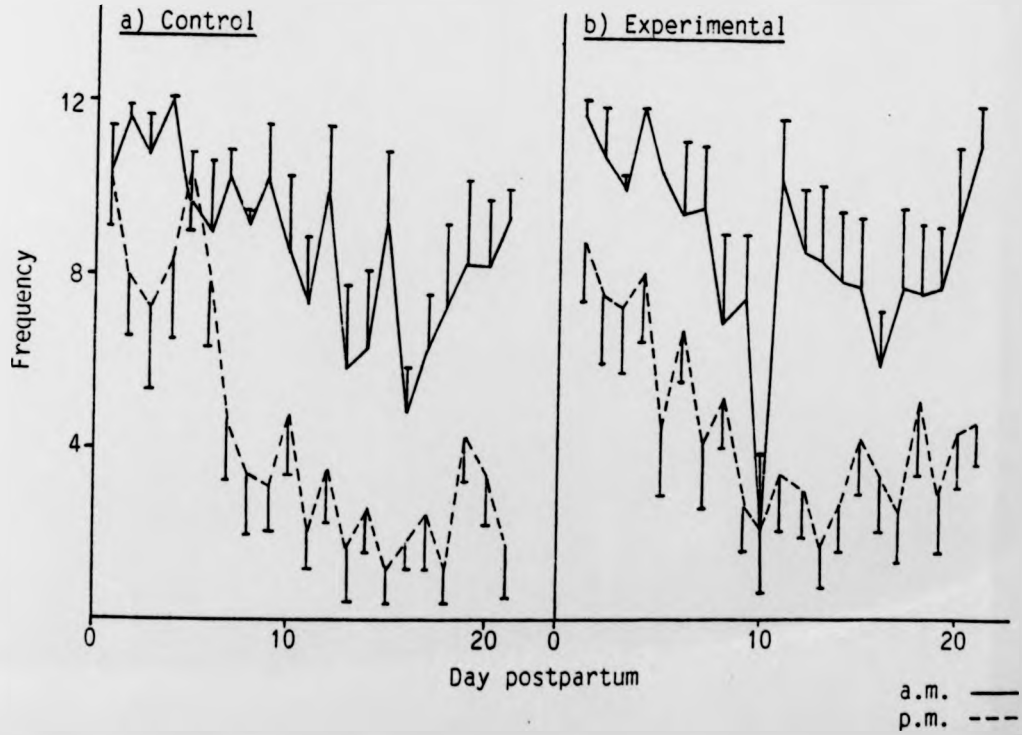

Fig. 3.3: Mean daily frequency of "On nest" (total) - CC and CE groups:

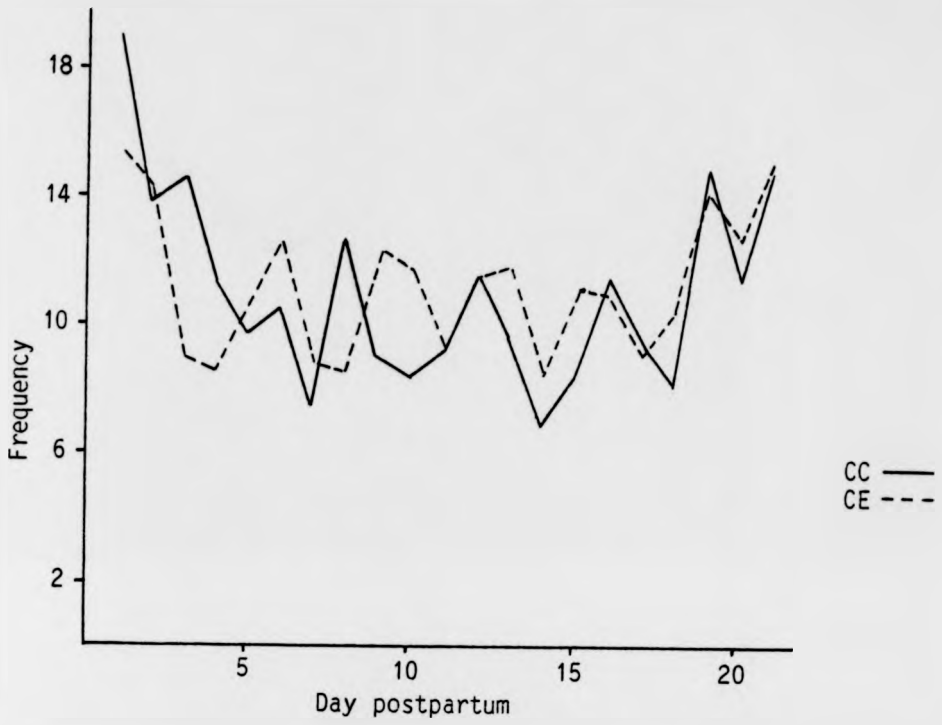

Fig. 3.4: Mean daily frequency of "on nest" (total) - EE and EC groups:

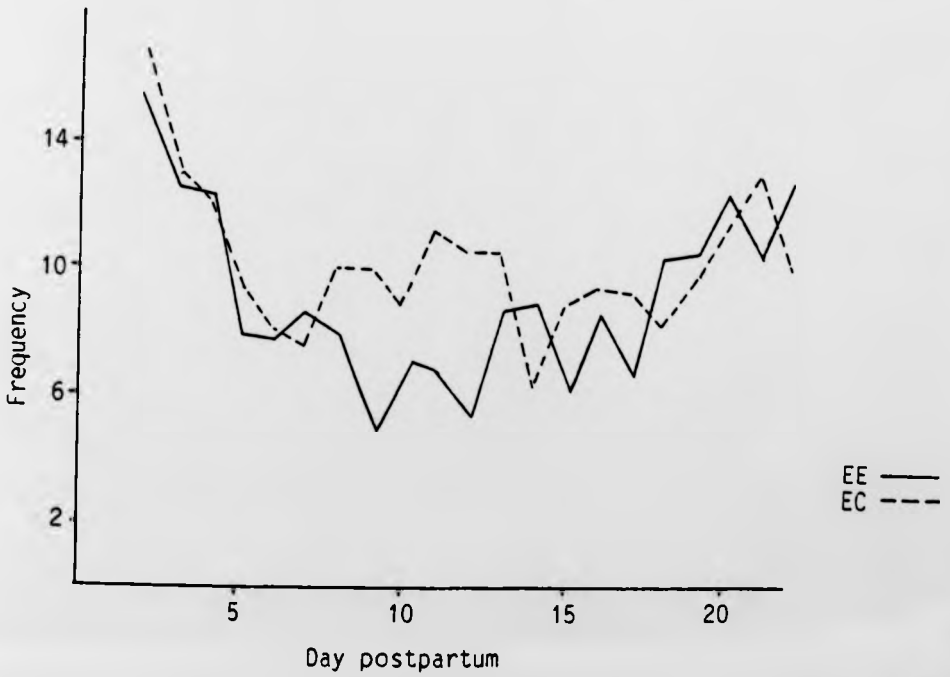

Fig. 3.5: Mean daily frequency of "On nest" (a.m. and p.m.) - CC:

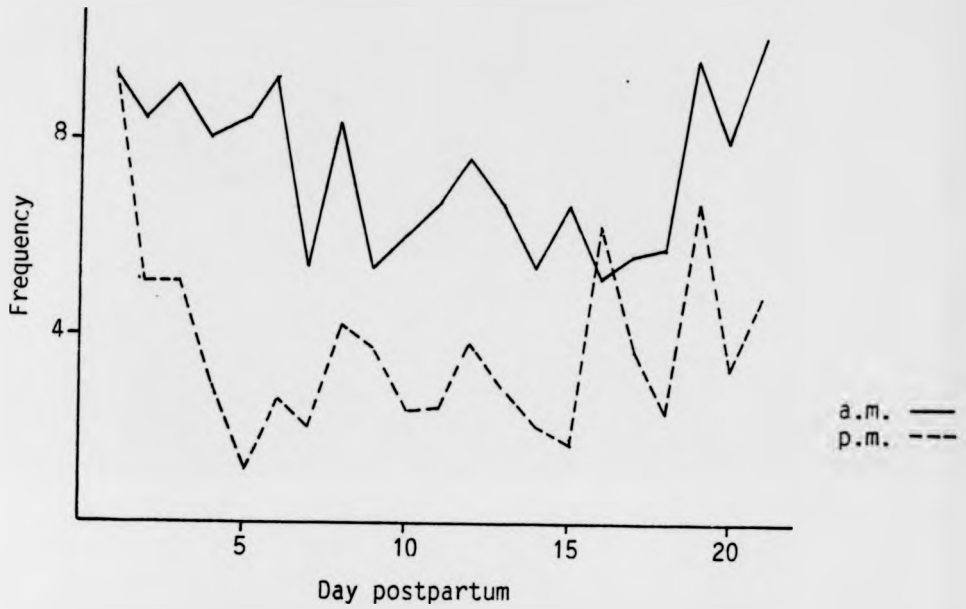

Fig. 3.6: Mean daily frequency of "On nest" (a.m. and p.m.) - CE:

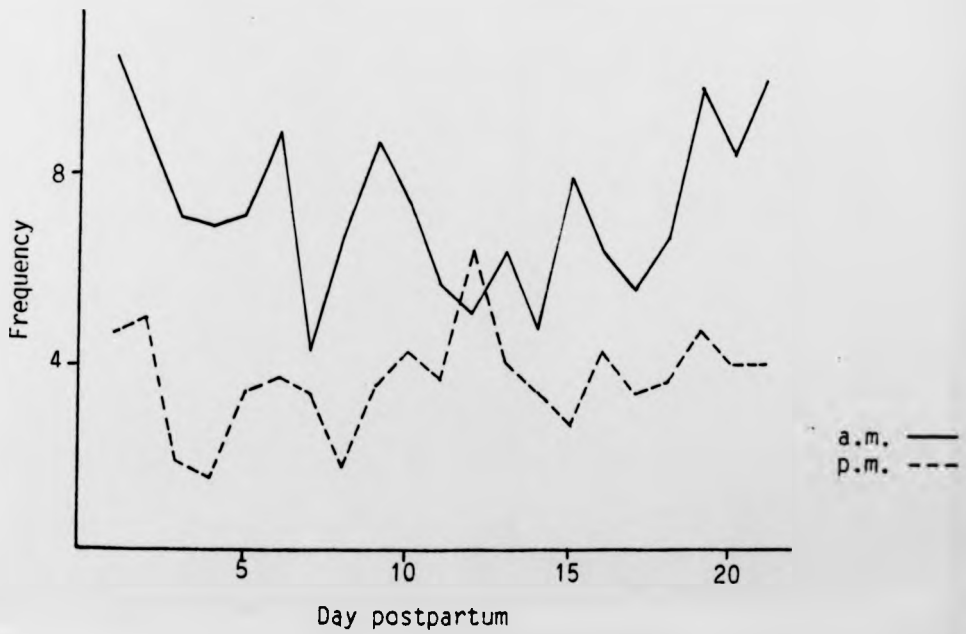

Fig. 3.7: Mean daily frequency of "On nest" (a.m. and p.m.) - EE:

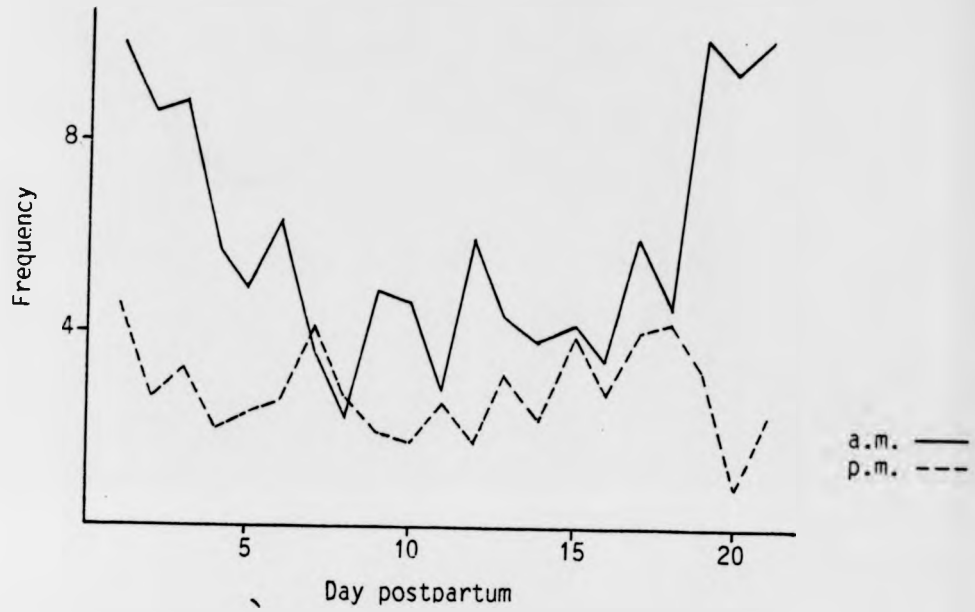

Fig. 3.8: Mean daily frequency of "On nest" (a.m. and p.m.) - EC:

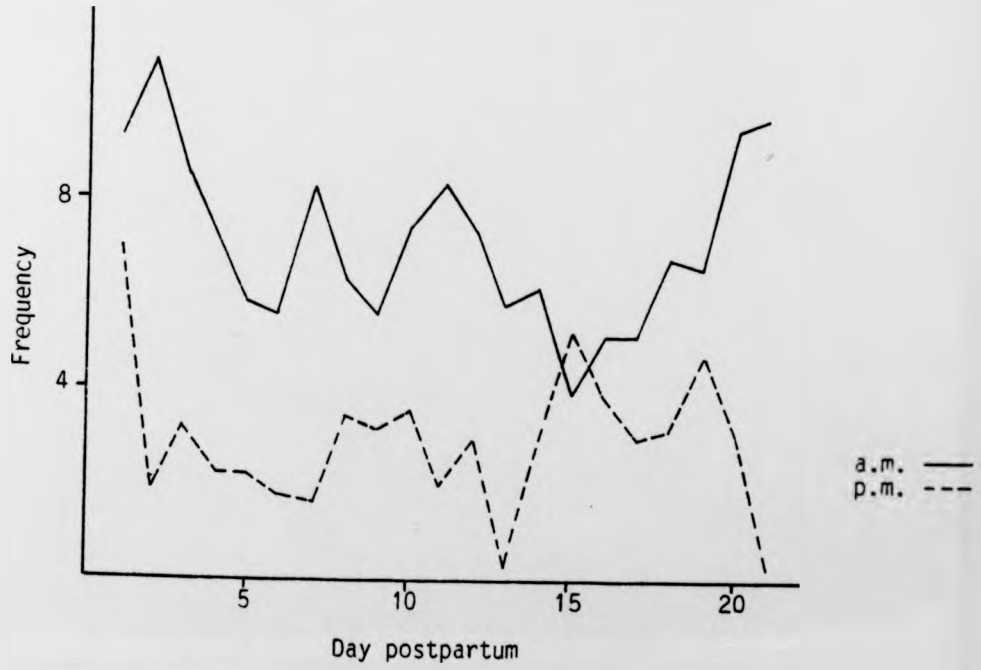

Fig. 3.9: Mean daily frequency of "PDA" (total) - CC and CE groups:

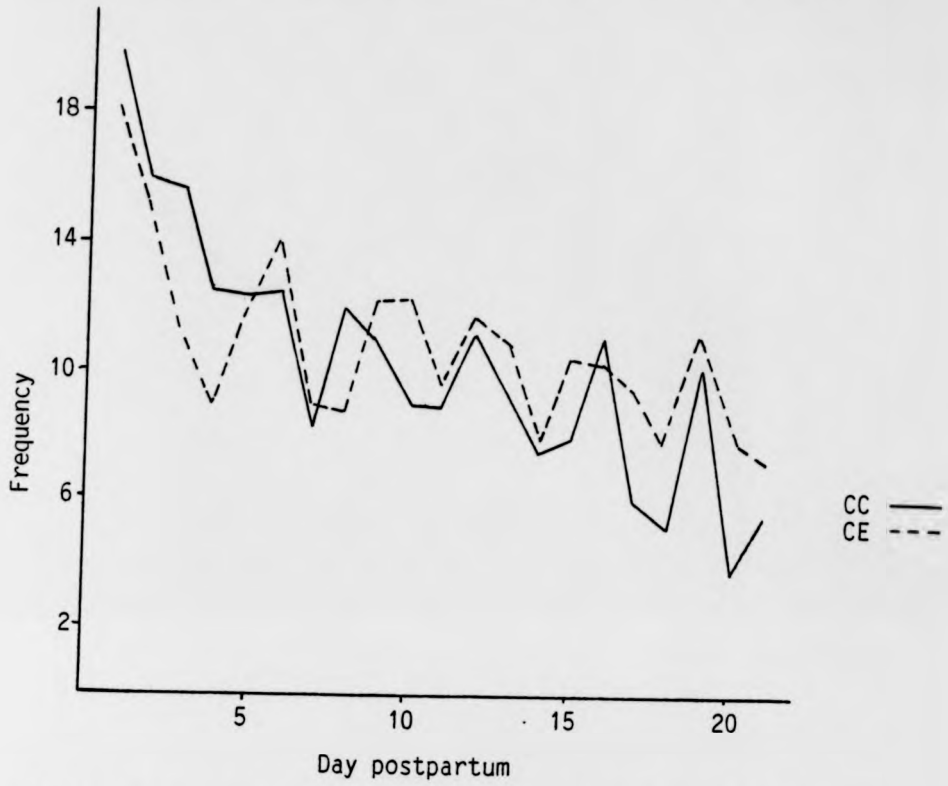

Fig. 3.10: Mean daily frequency of "PDA" (total) - EE and EC groups:

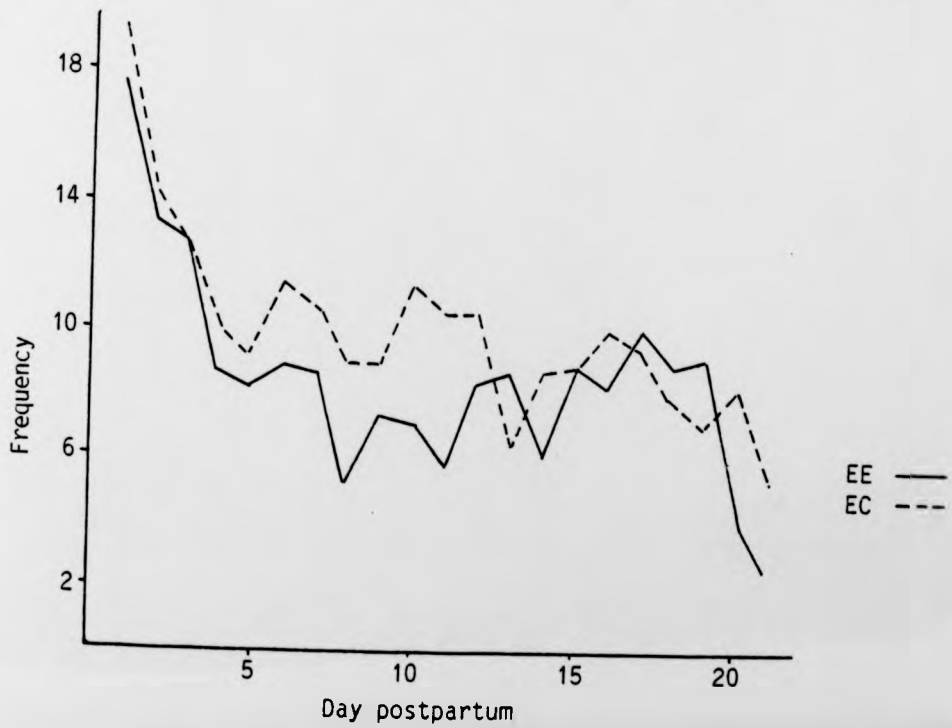

Fig. 3.11: Mean daily frequency of "PDA" (a.m. and p.m.) - CC:

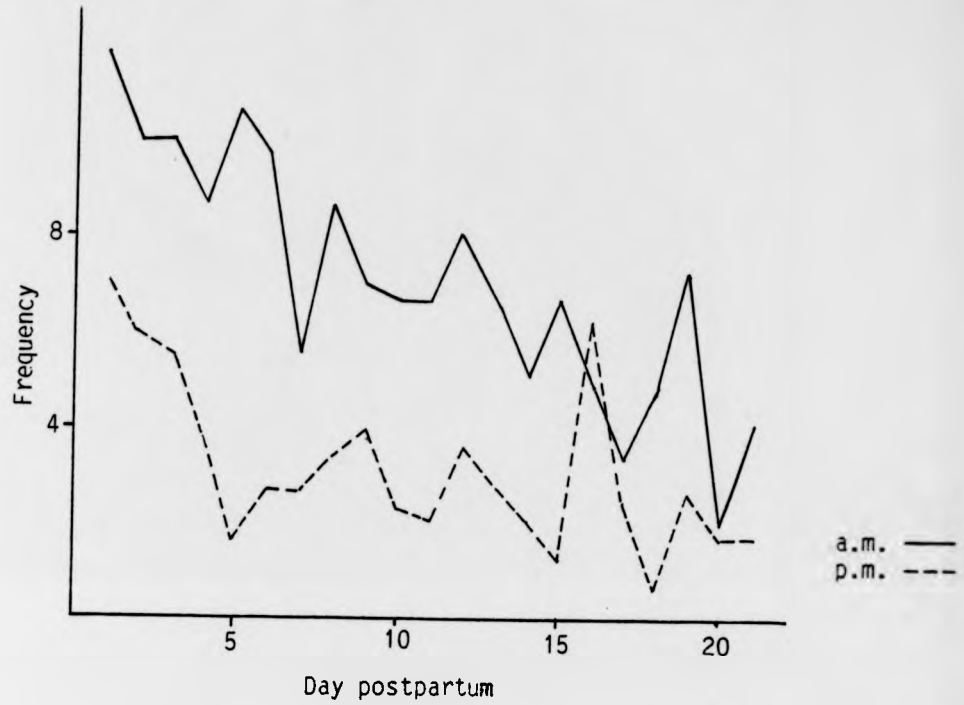

Fig. 3.12: Mean daily frequency of "PDA" (a.m. and p.m.) - CE:

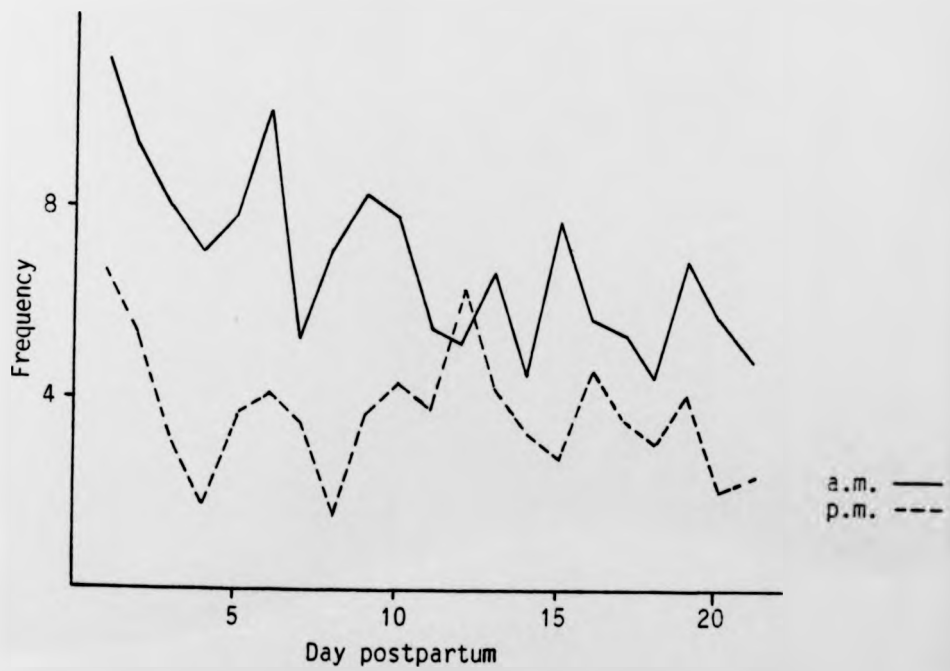

Fig. 3.13: Mean daily frequency of "PDA" (a.m. and p.m.) - EE:

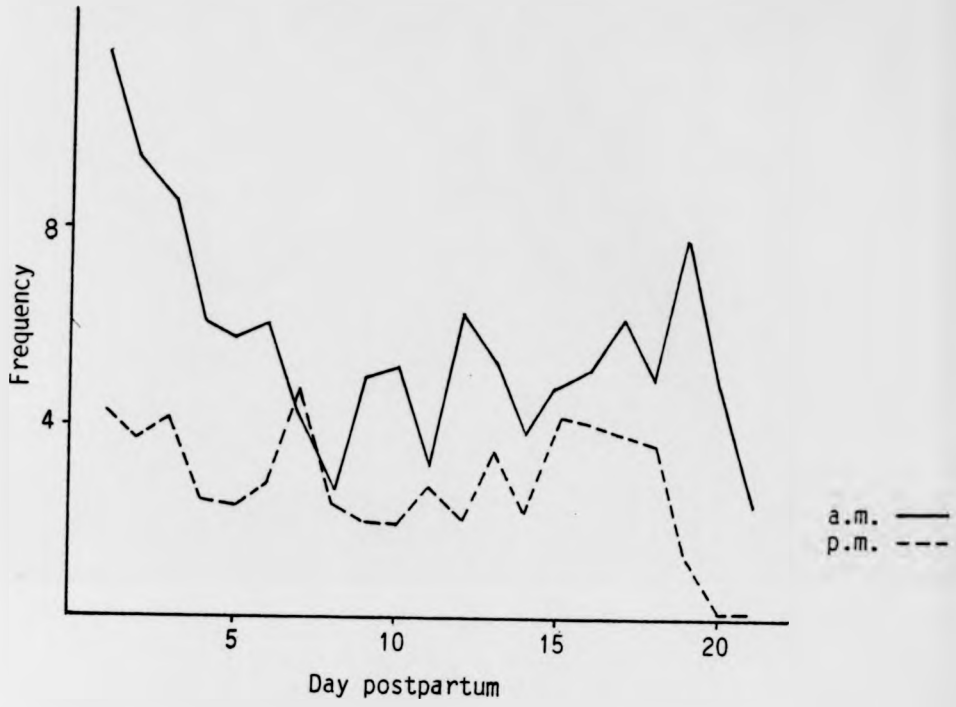

Fig. 3.14: Mean daily frequency of "PDA" (a.m. and p.m.) - EC:

Fig. 3.15: Mean daily frequency of "Feed" (total) - CC and CE groups:

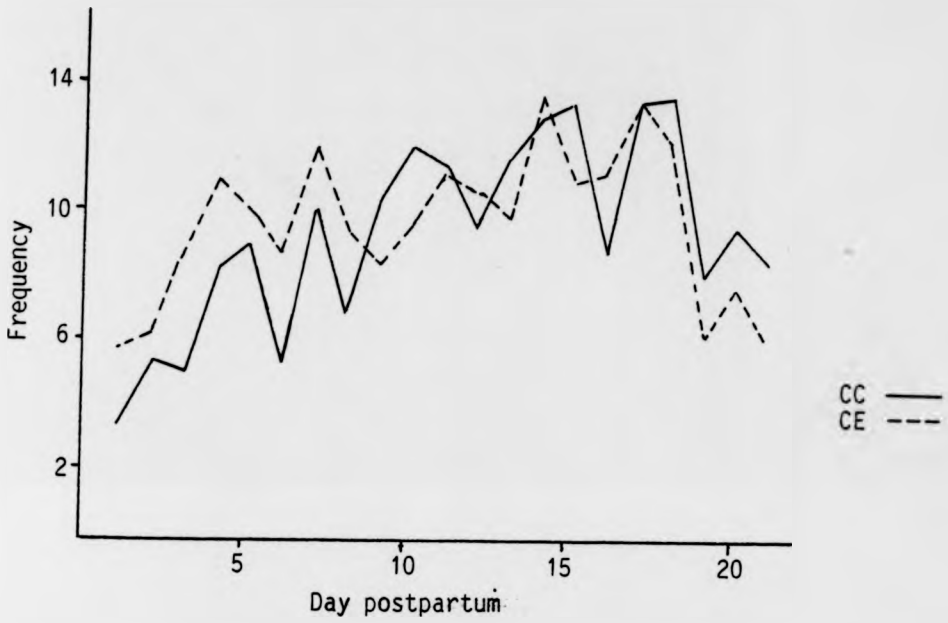

Fig. 3.16: Mean daily frequency of "Feed" (total) - EE and EC groups:

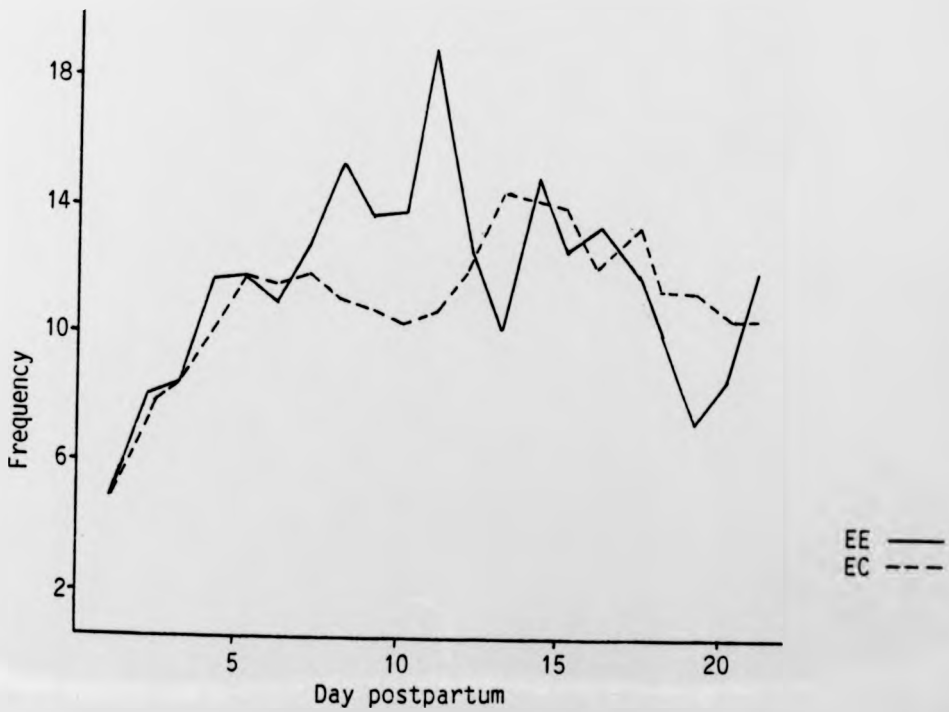

Fig. 3.17: Mean daily frequency of "Feed" (a.m. and p.m.) - CC:

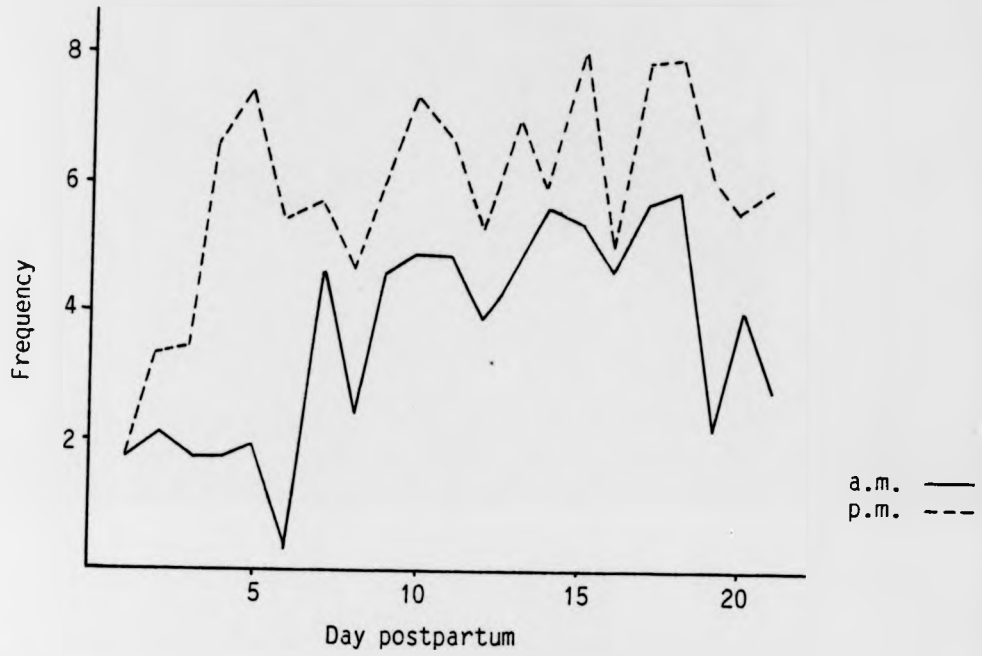

Fig. 3.18: Mean daily frequency of "Feed" (a.m. and p.m.) - CE:

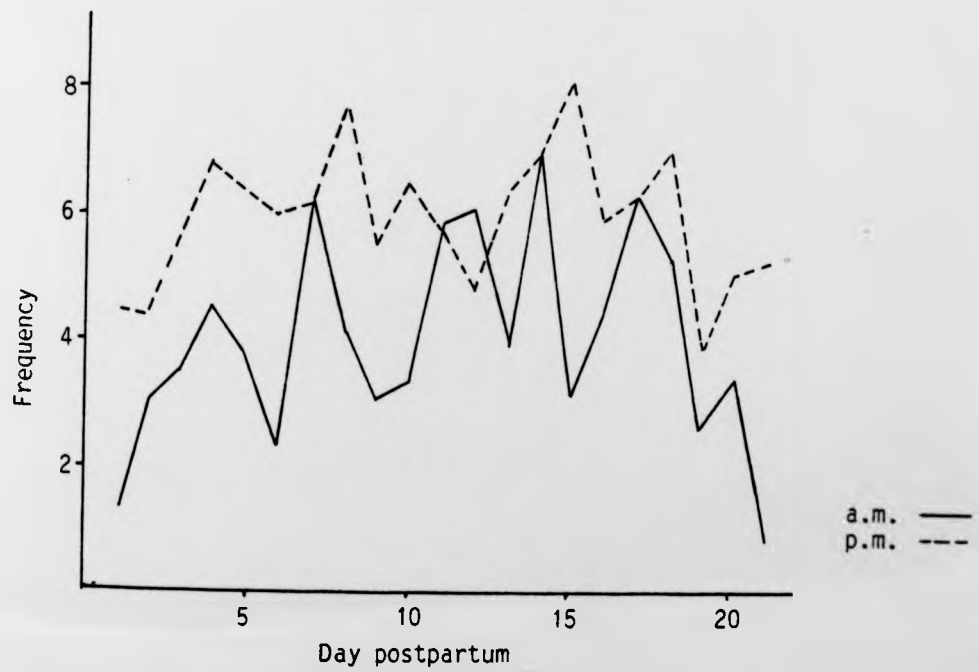

Fig. 3.19: Mean daily frequency of "Feed" (a.m. and p.m.) - EE:

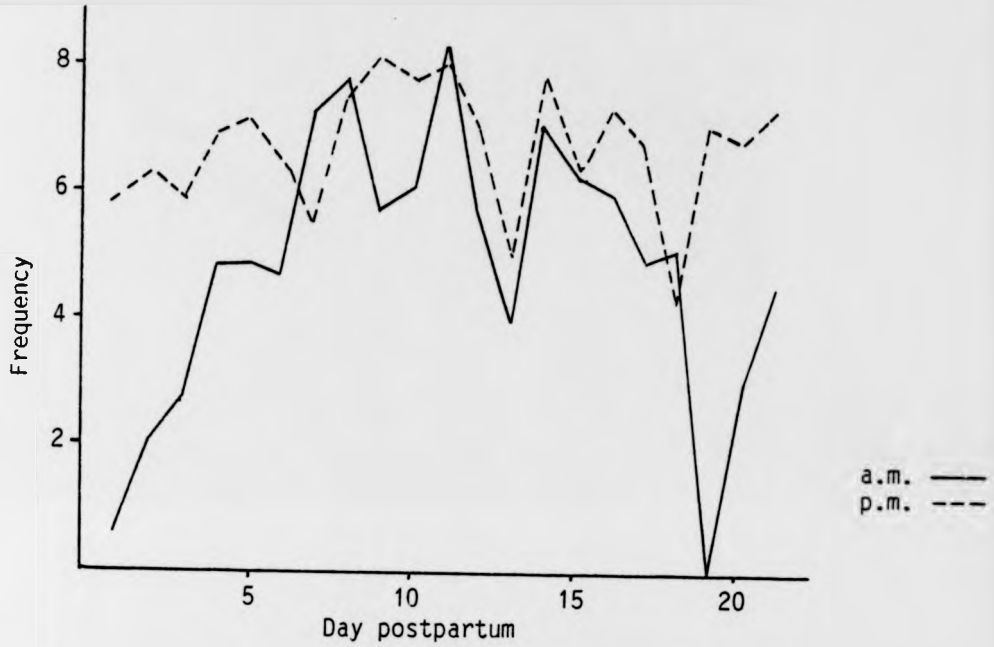

Fig. 3.20: Mean daily frequency of "Feed" (a.m. and p.m.) - EC:

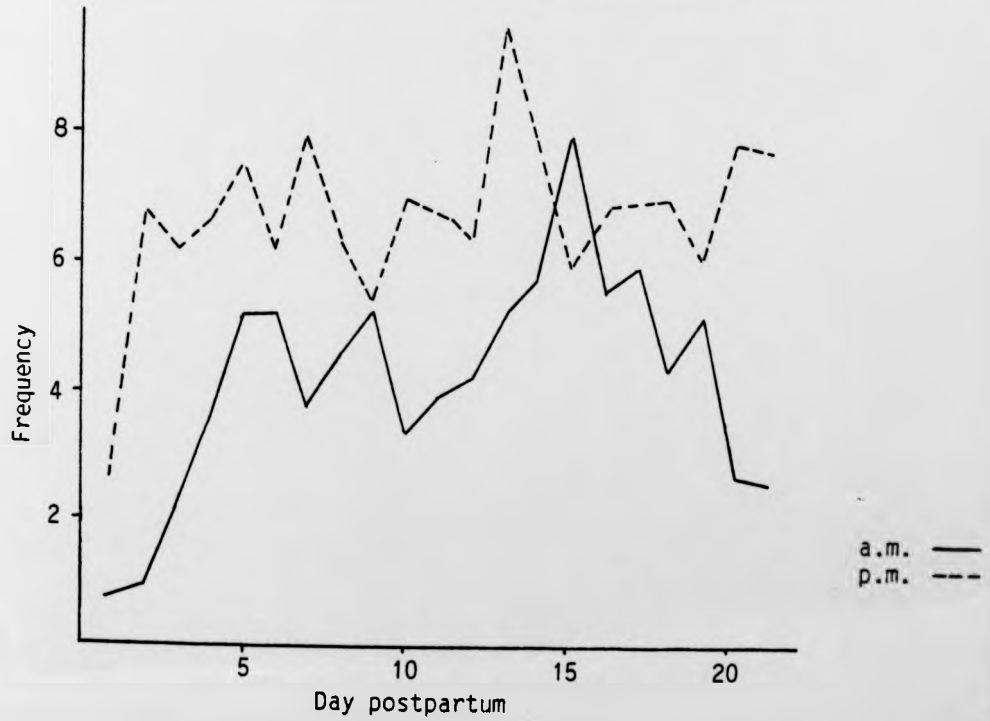

Fig. 3.21: Mean daily frequency of "Nurse" (total) - CC and CE groups:

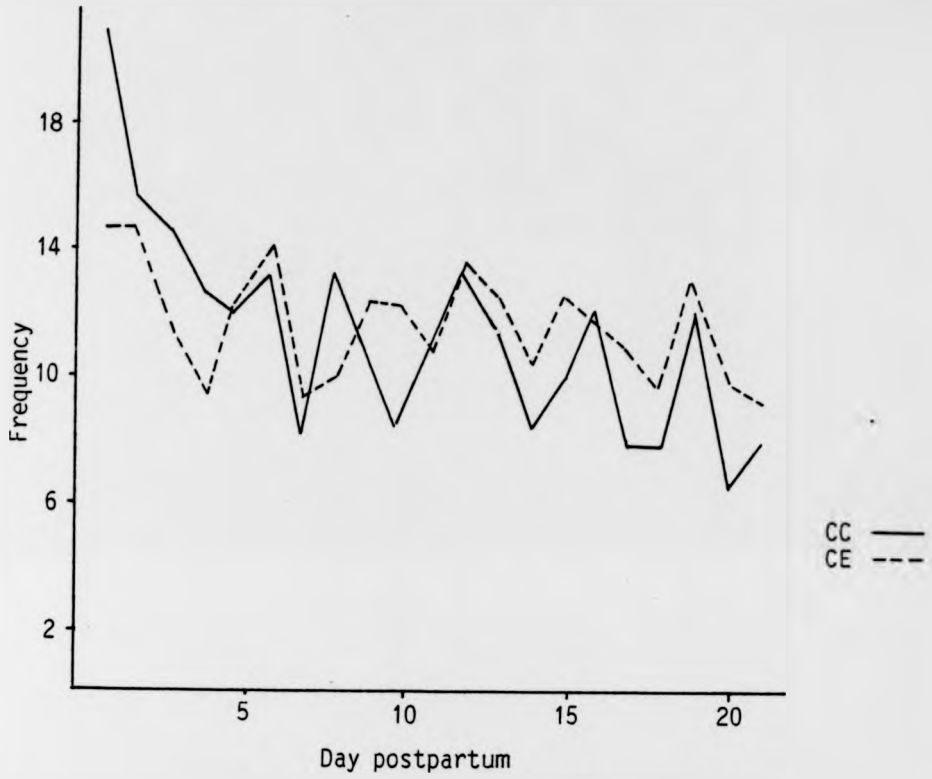

Fig. 3.22: Mean daily frequency of "Nurse" (total) - EE and EC groups:

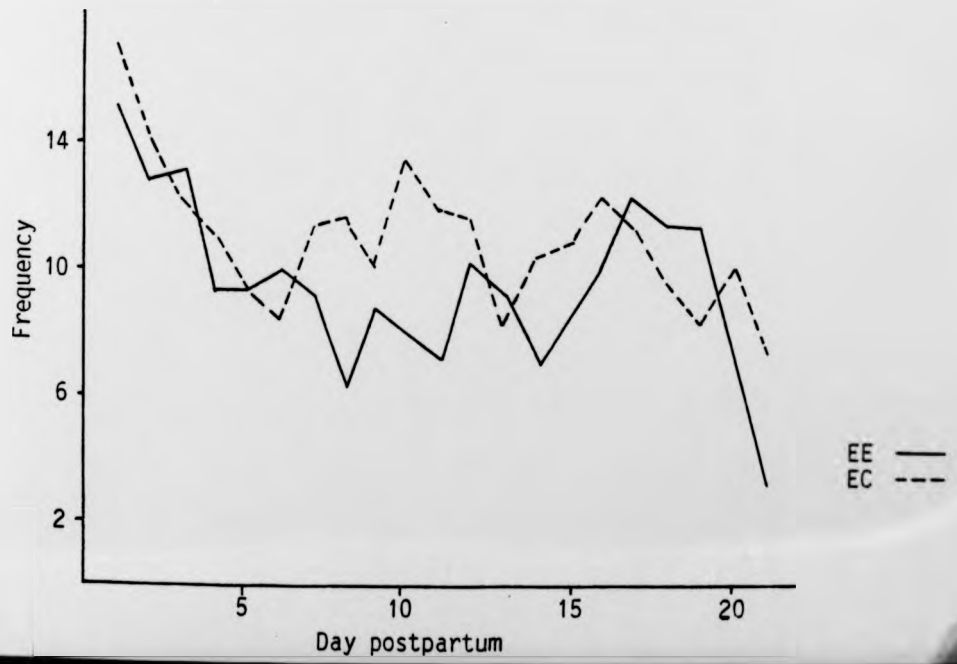

Fig. 3.23: Mean daily frequency of "Nurse" (a.m. and p.m.) - CC:

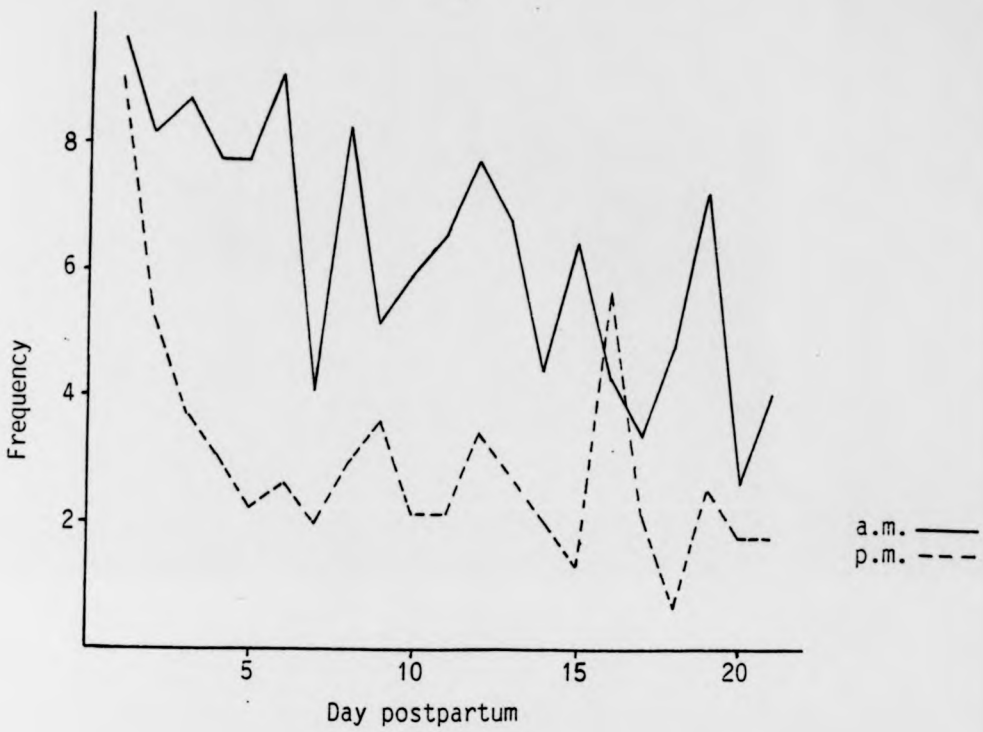

Fig. 3.24: Mean daily frequency of "Nurse" (a.m. and p.m.) - CE:

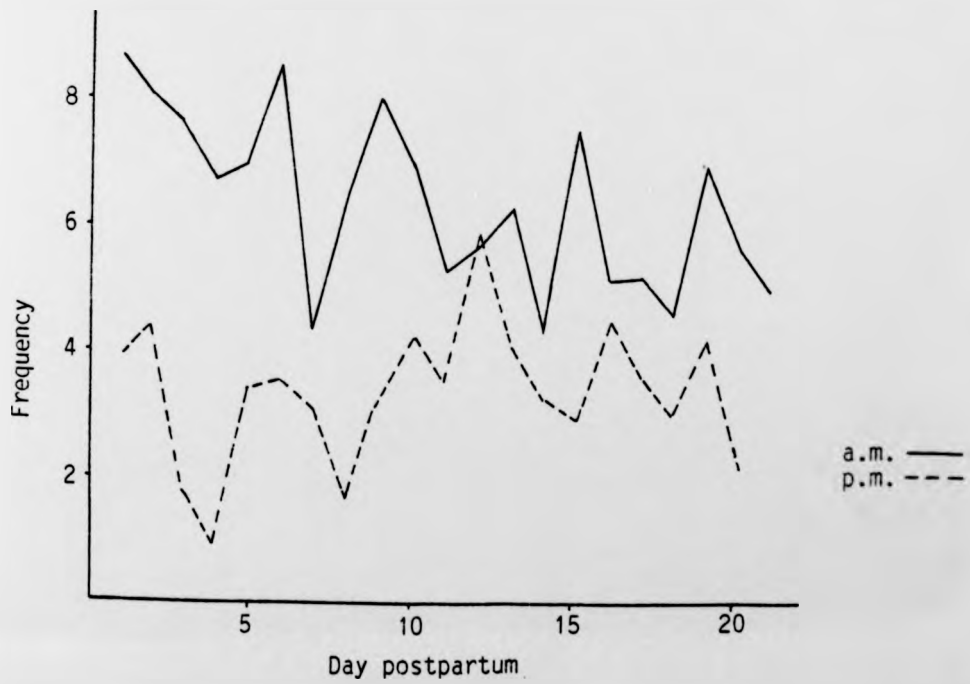

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Fig. 3.25: Mean daily frequency of "Nurse" (a.m. and p.m.) - EE:

Fig. 3.26: Mean daily frequency of "Nurse" (a.m. and p.m.) - EC:

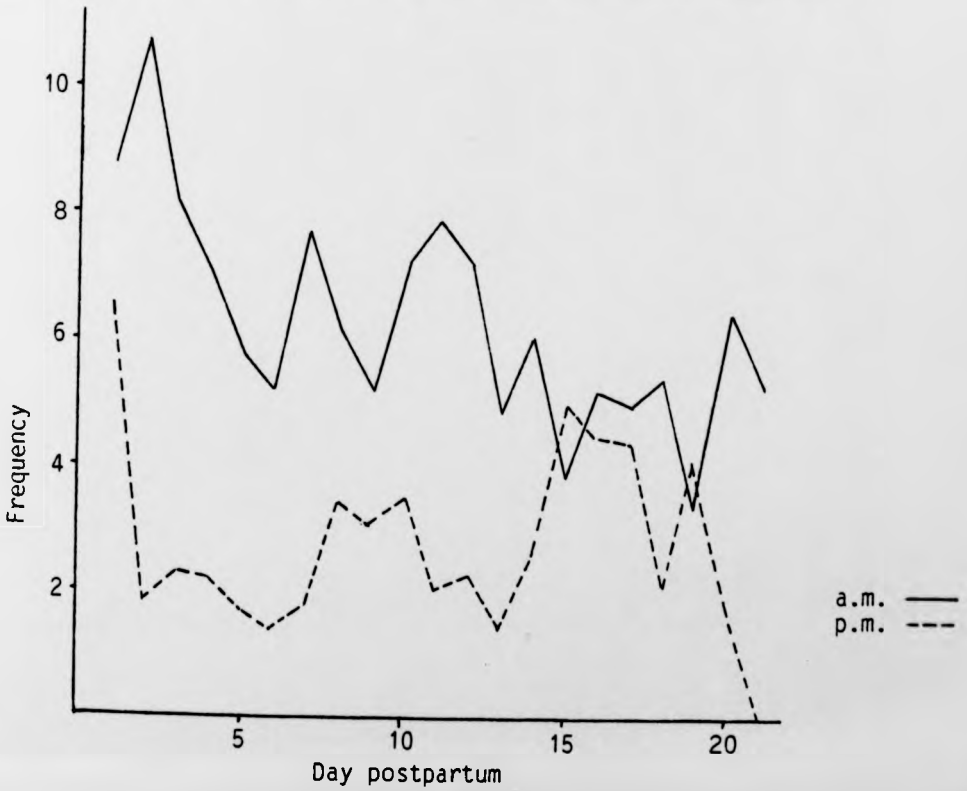


Fig. 3.27: Mean daily frequency of "Other" (total) - CC and CE groups:

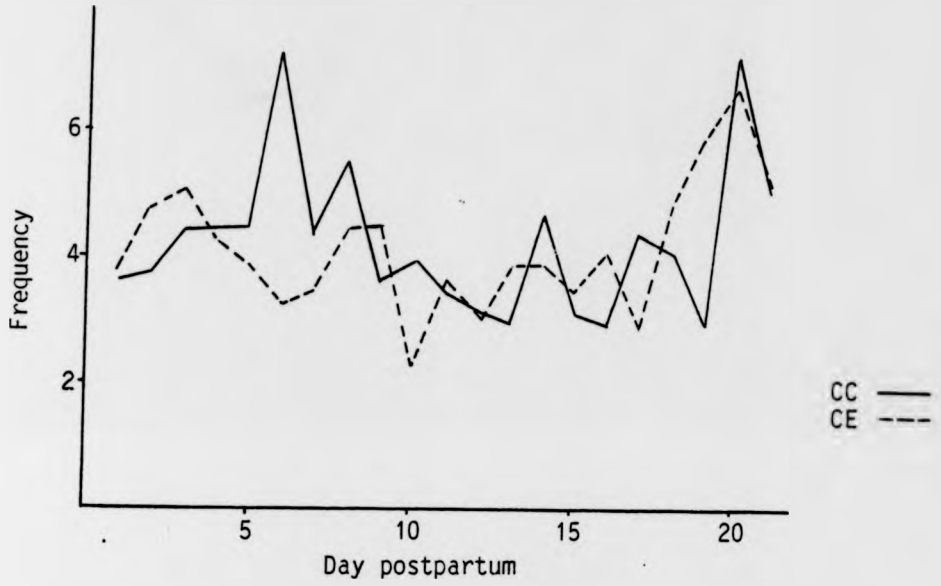


Fig. 3.28: Mean daily frequency of "Other" (total) - EE and EC groups:

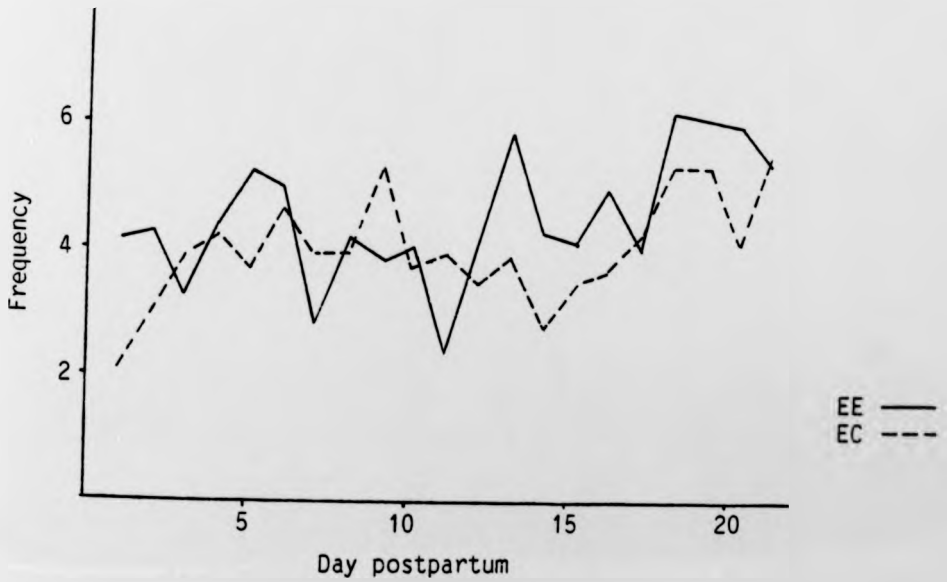


Fig. 3.29: Mean daily frequency of "Other" (a.m. and p.m.) - CC:

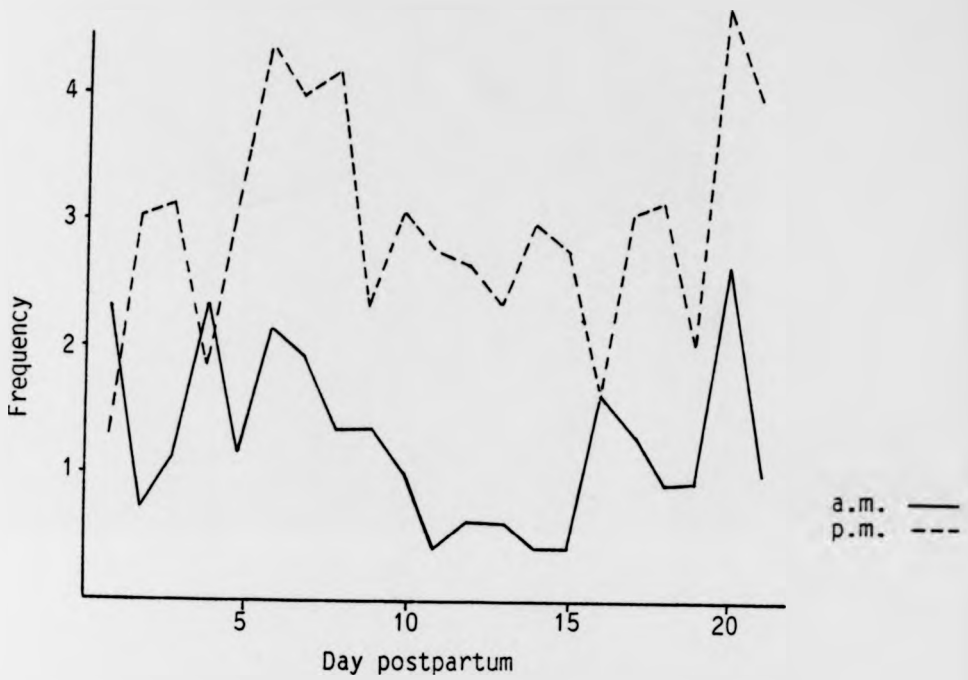


Fig. 3.30: Mean daily frequency of "Other" (a.m. and p.m.) - CE:

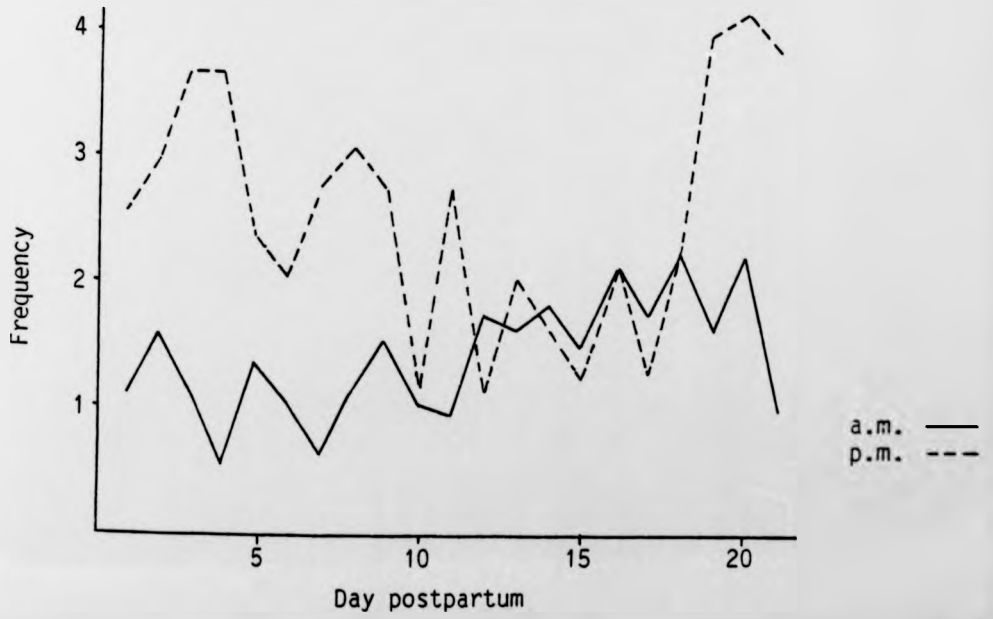


Fig. 3.31: Mean daily frequency of "Other" (a.m. and p.m.) - EE:

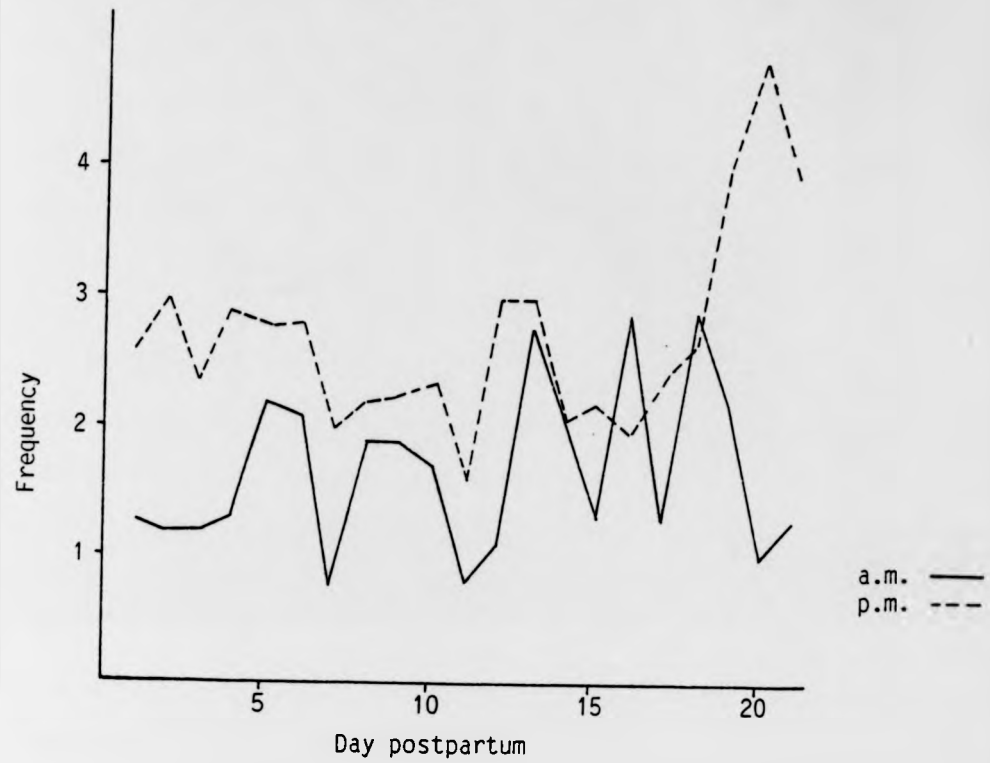
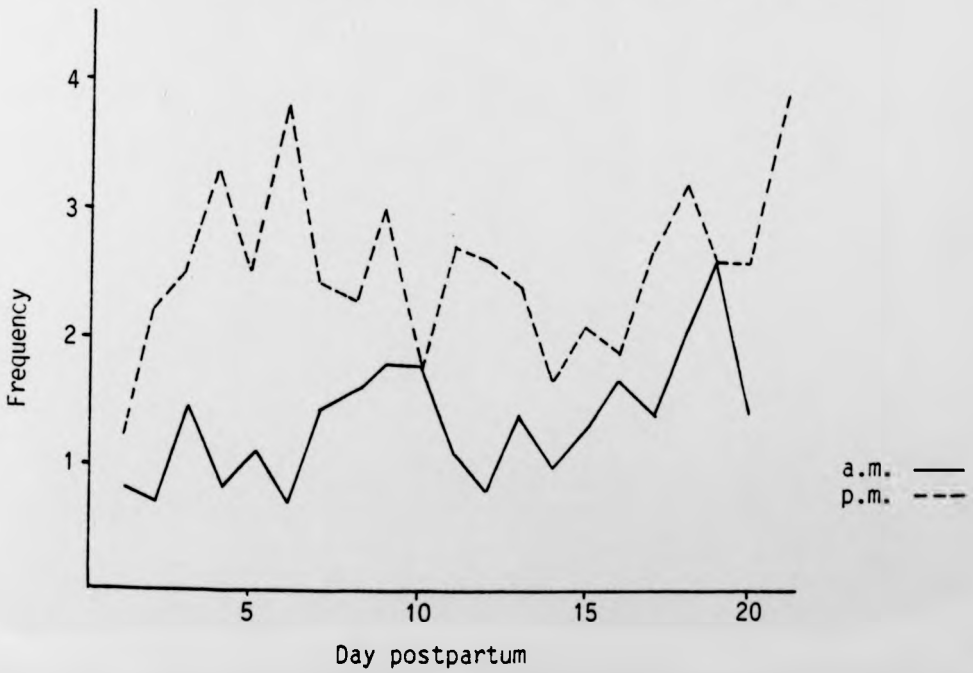


Fig. 3.32: Mean daily frequency of "Other" (a.m. and p.m.) - EC:



and the diagrams thus indicate an increase, decrease, U-shaped or inverse U-shaped change, or a steady unchanging state: in two cases the direction of the change was unclear, and in these cases the change is represented by a fluctuating line. These diagrams do not however represent absolute levels of the behaviour, nor the precise time course of the change, but merely the direction of the change in frequency over time.

Examining these diagrams, effects due either to the mother or to the offspring could be isolated (see Table 3.5). There was a maternal effect on a.m. levels of "on nest" and "nurse", the control mothers showing no change over time while experimental mothers showed a decrease (with a later increase of "on nest" in EE's), irrespective of the offspring's treatment. There was also a maternal effect on p.m. levels of "other" behaviour, where controls showed an increase but experimentals showed no change.

Litter effects were apparent on total (a.m. + p.m.) levels of "feed", and also (albeit less clearly) on p.m. levels of "on nest" and "PDA". Mothers with control litters show an increase in feeding over time whereas mothers with experimental litters showed (after an initial increase in EE's) a decrease. Mothers with experimental pups showed no change over time in p.m. frequencies of "on nest" and "PDA", whereas mothers with control pups clearly changed. Time spent on the nest by the latter increased or decreased over time, depending on whether the mother had herself been crowded during gestation. Pup-directed activity at this time of day decreased over time in CC groups, and "changed" (in a direction not revealed by analysis) in EC groups.

Where other differences occurred there seemed to have been what I have called an "experimental effect". In these cases all groups that had an experimental component, be it mother, litter or both, differed in the same way from the CC group. This "experimental effect" would seem

Fig. 3.33: Total behaviour frequencies over time

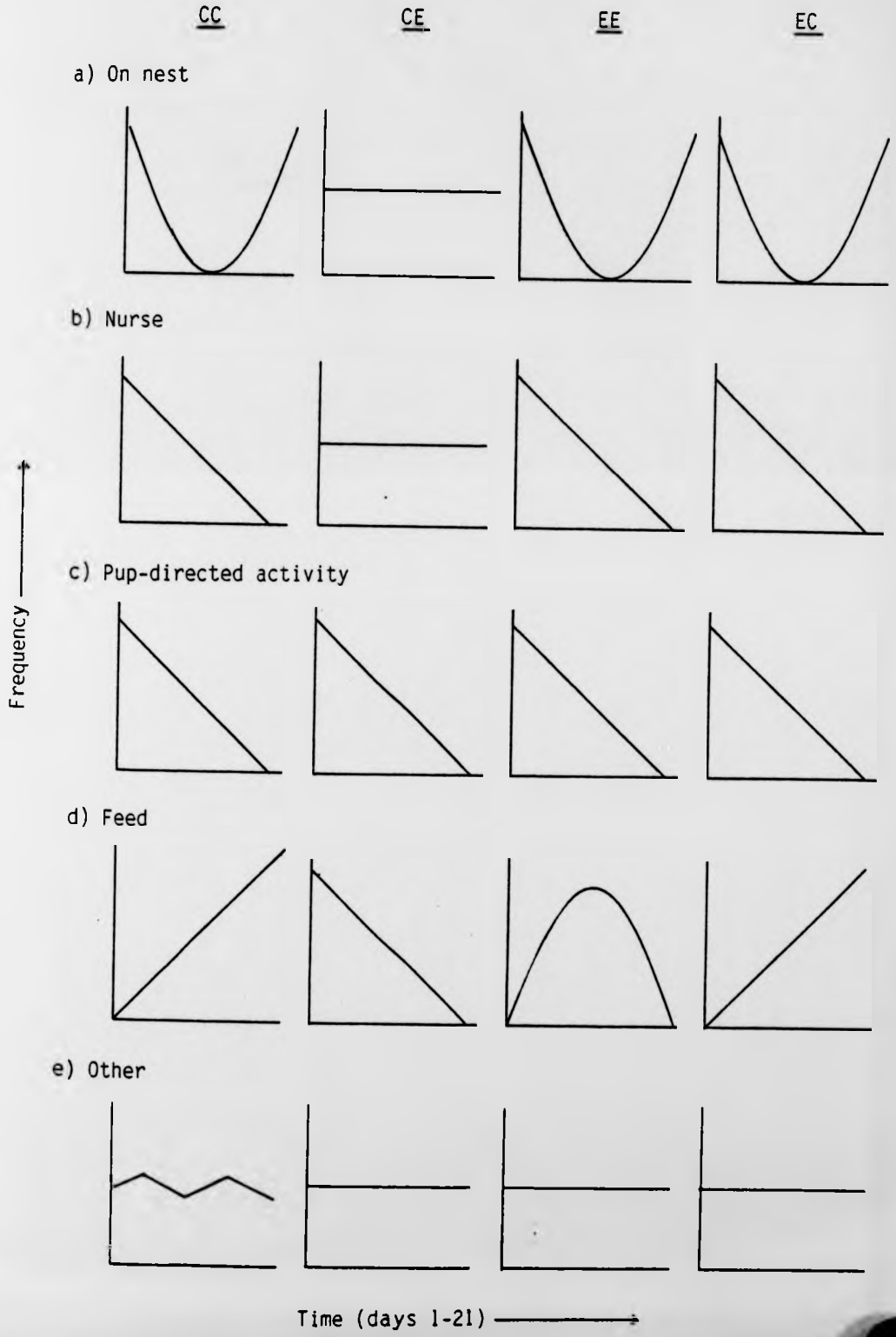


Fig. 3.34: Morning vs. afternoon frequencies of behaviour

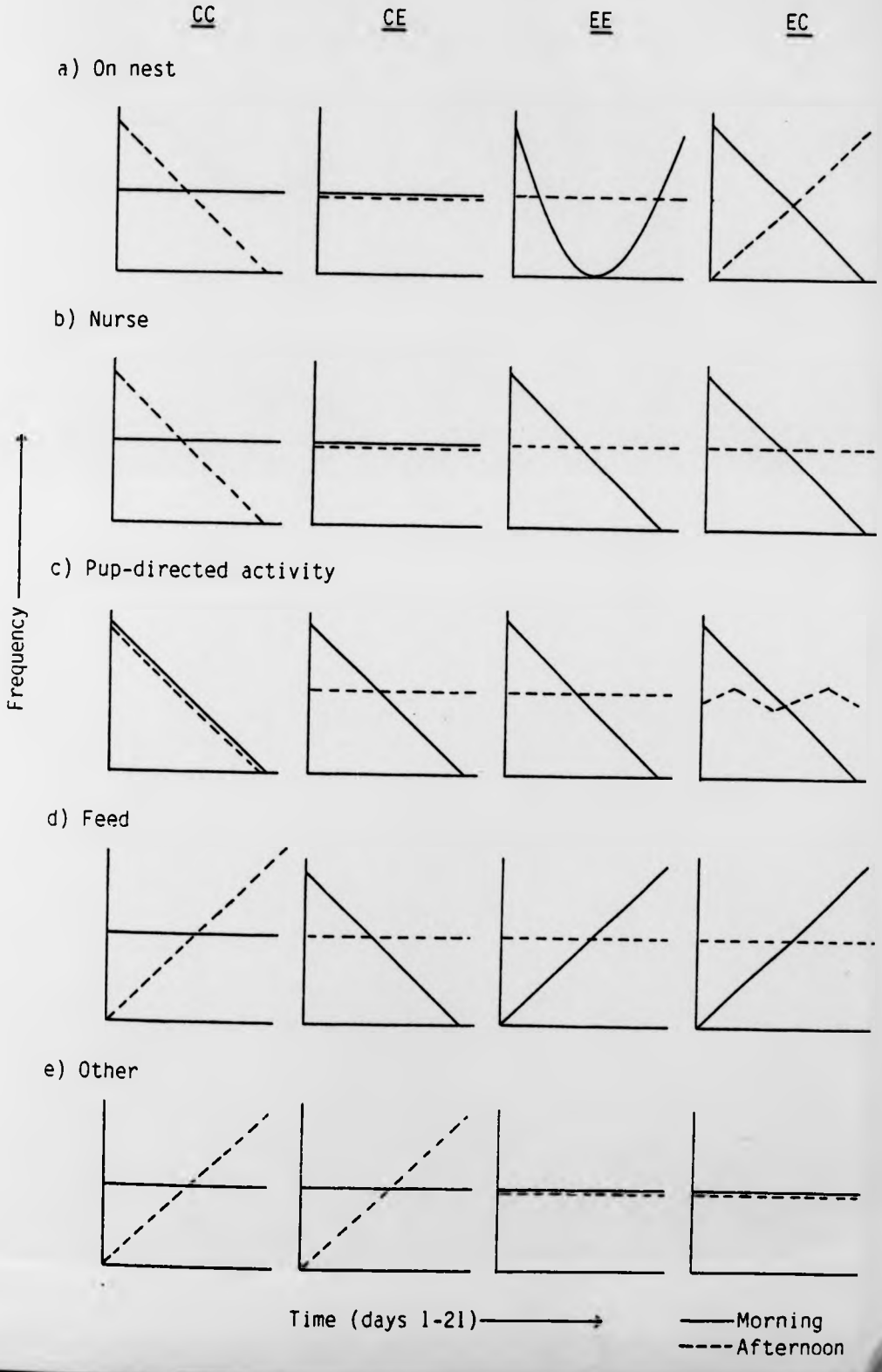


Fig. 3.35: Summary of Fig. 3.34

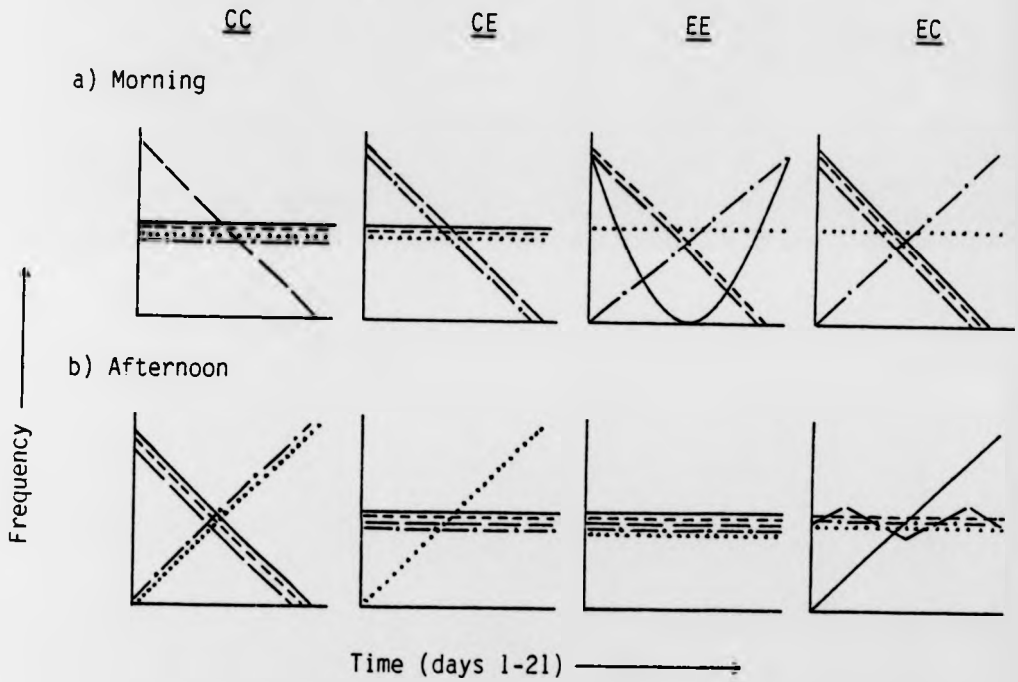


Table 3.5: Summary of difference sources:

	a.m.	p.m.	Total
On nest	M	?P	?
Nurse	M	E	?
PDA	-	?P	-
Feed	E	E (or ?M)	P
Other	-	M	E

M = Maternal effect
P = Pup effect
E = Experimental effect

Maternal Effects:

On nest a.m. - C's are constant, E's show decrease (EE's show later increase)
Nurse a.m. - C's are constant, E's show decrease
Other p.m. - C's show increase, E's are constant

Pup Effects:

On nest p.m. - E's are constant, C's show decrease or increase
PDA p.m. - E's are constant, C's show tendency to decrease? (EC's direction not clear)
Feed total - C's show increase, E's show decrease (EE's show initial increase)

Experimental Effects:

Nurse p.m. - E's are constant, CC's show decrease
Feed a.m. - E's show increase or decrease, CC's are constant
Feed p.m. - E's are constant, CC's show increase
Other total - E's are constant, CC's show "change"

to be some interactive effect of maternal and pup treatments, and was seen in p.m. levels of "nurse", a.m. and p.m. levels of "feed" (for a.m. not in terms of direction but in terms of change vs. no change), and in total levels of "other".

Overall then, while there were no demonstrable differences between the four groups in the total amounts (a.m., p.m., or a.m. + p.m.) of each behaviour during lactation, there were differences in their time courses, and also in the way in which diurnal rhythms of activities changed with time.

d) Observations of maternal behaviour in CC, CE, EE and EC groups, where the crowding period was extended to day 17 prepartum:

As stated earlier, this experiment was carried out to determine whether the extra day's crowding treatment would have any overt effect on maternal behaviour. Hence no detailed breakdown, such as in Expt. 1c. above, was carried out, but simply an analysis of differences between groups in behaviour frequency (total frequency, as well as separate morning and afternoon values). No differences were observed between the groups, either in terms of total behaviour frequencies or in terms of separate morning and afternoon frequencies, on any of the behavioural measures examined. The data are hence not presented.

Experiment 2.:

a) Retrieval tests:

Table 3.5.: Mean latencies (±S.E.'s) in retrieval tests:

		Day postpartum													
		1	2	3	4	5	6	7	8	9	10	11	12	13	14
Latency to emerge from nest (secs).	CC	17.13	12.50	8.00	5.20	6.75	25.50	5.50	6.75	8.13	11.00	4.38	6.00	4.63	4.75
		6.3	2.6	2.4	1.1	1.5	22.5	1.7	1.8	2.4	5.7	1.2	1.5	2.3	2.0
	EE	17.29	9.57	8.14	9.71	4.29	4.00	4.86	3.71	4.29	5.00	3.57	4.57	4.14	3.71
		9.3	4.3	3.4	3.5	1.5	0.8	1.1	0.9	0.6	1.1	0.7	0.8	1.5	1.3
Latency to 1st retrieval (secs).	CC	54.30	41.86	24.75	15.50	31.63	46.50	60.25	29.38	61.65	95.13	105.75	106.13	131.38	275.63
		8.7	12.3	6.7	4.0	15.0	21.8	34.5	9.1	34.7	45.3	45.1	45.0	49.5	24.4
	EE	141.14	34.00	68.29	33.29	22.71	27.71	33.43	71.70	89.14	116.70	132.71	152.43	159.14	168.7
		44.2	9.19	39.3	11.00	4.2	5.8	12.9	38.9	38.9	48.33	53.0	52.8	51.4	50.4
Total retrieval time (secs)	CC	163.29	148.57	130.43	91.86	123.29	144.56	171.57	221.43	236.71	200.86	165.86	213.00	262.71	300.00
		16.3	29.0	24.18	29.8	31.8	41.3	41.6	40.3	38.3	45.4	47.5	42.7	27.0	0
	EE	261.57	127.57	161.14	149.43	189.00	160.29	201.14	175.7	199.57	198.43	22.00	216.00	209.29	252.14
		20.3	17.6	35.2	41.3	36.1	29.5	42.4	35.1	38.0	38.7	35.7	25.1	35.2	35.4

CC n = 8; EE n = 7.

Experiment 2:

a) Retrieval Tests:

No significant differences between the two groups (using two-way analyses of variance - see results table below) were found on latency to emerge from the nest, or on latency to first retrieval. Both latency to first retrieval and total retrieval time varied over the lactation period as a function of pup age ($p=0.00$), although the two groups did not differ in the frequency of these measures. Results from the ultrasound tests and measures of body weight (Expts. 3a & 3c) indicated that physical differences between control and experimental pups might exist only within the first few days of life, so Student's t-tests were carried out on retrival latencies over days 1-7. These revealed a longer total retrieval time on day 1 in EE groups ($p=0.002$), and a longer latency to first retrieval on day 4 in EE groups ($p=0.036$).

Results of Two-way Analyses of Variance on Retrieval Latencies:

	Source	df	F-ratio	Probability
a) Latency to 1st Retrieval:	Trt. Groups	1	0.0069	0.9351
	Time	13	8.4370	0.0000
	Trt. x Time interaction	13	1.1311	0.3370
b) Latency to emerge:	Trt. Groups	1	1.9885	0.1839
	Time	13	0.9761	0.4773
	Trt. x Time interaction	13	0.7438	0.7177
c) Total Retrieval Time:	Trt. Groups	1	0.1859	0.6740
	Time	13	4.7376	0.0000
	Trt. x Time interaction	13	1.6408	0.0794

b) Nest Ratings:

Table 3.6.: Median (±95% confidence limits) nest ratings over the lactation period.

Day Postpartum	Treatment CC	CE	EE	EC
1	7.0 (6.0-9.0)	9.0 (6.0-9.0)	9.0 (5.0-9.0)	9.0 (8.0-9.0)
2	6.5 (6.0-9.0)	8.0 (6.0-9.0)	9.0 (6.0-9.0)	9.0 (9.0-9.0)
3	7.0 (3.0-9.0)	9.0 (8.0-9.0)	9.0 (8.0-9.0)	9.0 (8.0-9.0)
4	6.0 (3.0-9.0)	9.0 (8.0-9.0)	9.0 (5.0-9.0)	9.0 (7.0-9.0)
5	6.0 (3.0-8.0)	8.0 (3.0-9.0)	8.0 (6.0-9.0)	9.0 (8.0-9.0)
6	6.0 (4.0-6.0)	8.0 (6.0-9.0)	8.0 (4.0-9.0)	8.0 (6.0-9.0)
7	6.0 (3.0-6.0)	8.0 (6.0-9.0)	8.0 (3.0-9.0)	6.0 (6.0-9.0)
8	6.0 (3.0-6.0)	7.0 (6.0-9.0)	8.0 (6.0-9.0)	6.0 (6.0-9.0)
9	5.5 (3.0-6.0)	6.0 (6.0-9.0)	9.0 (4.0-9.0)	6.0 (6.0-9.0)
10	3.5 (3.0-8.0)	7.0 (4.0-9.0)	7.0 (3.0-9.0)	7.0 (5.0-9.0)
11	6.5 (3.0-9.0)	6.0 (3.0-8.0)	6.0 (6.0-8.0)	6.0 (6.0-9.0)
12	5.0 (3.0-6.0)	6.0 (3.0-7.0)	6.0 (6.0-9.0)	6.0 (6.0-9.0)
13	5.0 (2.0-5.0)	6.0 (3.0-8.0)	6.0 (4.0-9.0)	6.0 (5.0-9.0)
14	6.0 (3.0-6.0)	6.0 (6.0-6.0)	6.0 (4.0-7.0)	6.0 (6.0-9.0)
15	5.0 (3.0-6.0)	6.0 (3.0-6.0)	6.0 (6.0-6.0)	6.0 (3.0-8.0)
16	3.0 (3.0-6.0)	6.0 (3.0-6.0)	6.0 (3.0-7.0)	6.0 (4.0-6.0)
17	3.0 (2.0-6.0)	3.0 (2.0-4.0)	6.0 (2.0-6.0)	6.0 (3.0-6.0)
18	3.0 (1.0-6.0)	1.0 (1.0-3.0)	3.0 (1.0-6.0)	5.0 (3.0-6.0)
19	2.0 (1.0-5.0)	1.0 (1.0-3.0)	3.0 (1.0-6.0)	3.0 (1.0-6.0)
20	1.5 (0.5-2.0)	1.0 (1.0-2.0)	3.0 (1.0-6.0)	3.0 (1.0-6.0)
21	1.0 (0.0-3.0)	1.0 (0.0-2.0)	3.0 (1.0-6.0)	3.0 (1.0-3.0)
n	10	9	7	11

The data were initially analysed using Kruskal-Wallis one-way analyses of variance each day's ratings: where differences were obtained further analysis was carried out using Mann-Whitney U-tests to compare individual groups. The results of these tests were as follows:

a) Maternal effects:

Day 2 : CC<EC, $p < 0.02$
Day 4 : CC<EC, $p < 0.02$
Day 5 : CC<EC, $p < 0.02$
Day 6 : CC<EC, $p < 0.05$
Day 7 : CC<EC, $p < 0.02$
Day 8 : CC<EC, $p < 0.02$
Day 9 : CC<EC, $p < 0.02$
Day 12 : CC<EC, $p < 0.02$
Day 17 : EE>CE, $p < 0.05$

b) Pup effects:

Day 4 : CC<CE, $p < 0.05$
Day 6 : CC<CE, $p < 0.02$
Day 7 : CC<CE, $p < 0.02$
Day 8 : CC<CE, $p < 0.05$

c) Maternal and/or pup effects:

Day 6 : CC<EE, $p < 0.05$
Day 8 : CC<EE, $p < 0.02$
Day 9 : CC<EE, $p < 0.02$
Day 12 : CC<EE, $p < 0.02$.

This analysis revealed a fairly consistent maternal effect between CC and EC groups over the first half of lactation, with CC females building nests of inferior quality. The CC group also built nests that were inferior to those of CE groups for a short period, by the end of which their nests were also inferior to those of EE groups. Almost all of the differences were confined to the first twelve days of lactation.

Experiment 3a. Age at eye-opening:

Table 3.7: Percentage of pups with eyes open:

Age of pups (days)	Treatment group: CC	Treatment group: EE
12	0	0
13	53	41
14	98	93
15	100	100

CC n=64
EE n=56

No significant difference using X^2 analysis were obtained between the groups on any of days 12-15.

Experiment 3b. Pup weights:

Table 3.8: Mean litter birth weights *(±S.E.):

	Litter treatment: C	Litter treatment: E
Weight (g)	1.67 (±0.03)	1.54 (±0.03)
n	20	18

These weights were significantly different ($p<0.01$) on a Student's t-test.

Table 3.9: Mean litter weights*at 11 days postpartum:

	Treatment group: CC	Treatment group: CE	Treatment group: EE	Treatment group: EC
Weight (g)	7.24 (±0.17)	7.23 (±0.23)	7.20 (±0.17)	6.80 (±0.43)
n	10	10	10	8

No significant differences were seen here between any of the four groups (Student's t-tests).

* See note overleaf

Table 3.10: Mean litter weights* at weaning:

	CC	CE	EE	EC
Weight (g)	11.01 (±0.37)	11.46 (±0.46)	10.73 (±0.57)	11.81 (±0.51)
n	10	10	10	8

No significant differences were obtained on Student's t-tests.

These results indicate that whereas prenatal stress does have an effect on birth weight, the difference in body weight between control and experimental animals has been eliminated by day 11 and is not seen at weaning.

* Where "litter weights" are quoted, the weight referred to is that of the mean pup weight in each litter. Hence the "mean litter weights" represent mean pup weights, using the litter as the unit of analysis.

Experiment 3c: Pup Vocalisations.

Table 3.11: Median number of calls over 2-minute test period (with 95% confidence limits):

	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13
CC.	102.5 (62-116)	149 (107-176)	107 (68-129)	121 (102-131)	10.5 (4-49)	0.5 (0-7)
EE	19 (4-53)	132 (107-147)	100 (43-121)	129.5 (109-184)	42.5 (14-72)	1.5 (1-58)

n=10 in both groups.

On Mann-Whitney 'U' test:

Day 3 frequencies: CC>EE, $p<0.001$.

The only CC/EE difference seen in this test is a reduced frequency of ultrasonic calling in EE animals on day 3. This suggests a delayed development of ultrasonic calling. Both groups show a peak rate of calling on day 5, and there is no difference in frequency at this peak. Calling declines after day 5, and after a second smaller peak on day 9, falls to very low levels by day 13.

Discussion

One major theme of this project was to determine whether the effects of prenatal maternal stress, some of which may be profound and long-lasting, can be related to postnatal factors associated with mothering. The most direct way to answer this question is to observe maternal behaviour of stressed and unstressed females following parturition. Two reasons were given for possible alterations to maternal behaviour. Firstly, that deficits in maternal care of experimental females might arise owing to disruption of the normal endocrine status (and/or behaviour) during the period leading up to parturition. Secondly, prenatally stressed offspring commonly have lower birth weights and show delays in reflex development (Chevins, 1981, unpublished), and there is evidence (eg. Hennessy et al., 1977) to suggest that underdeveloped pups may be less arousing to the mother: hence prenatal stress could affect the quality of maternal care as a consequence of inadequate cues from the pups to the mother.

Measures of birth weight indicated that the prenatal crowding treatment had indeed had an effect on the offspring, and this agrees with work by others (eg. Herrenkohl & Whitney, 1976) in this field, although weights were similar at 11 days and at weaning. Pup vocalisation was reduced in experimental pups at 3 days of age, but thereafter the two groups did not differ. The peak in ultrasonic calling occured in both groups at day 5 postpartum, which is earlier than Elwood and Keeling (1982)'s peak at day 7. This could however be due to strain differences (they used CS1 mice), as could the fact that their animals showed only one peak in ultrasonic calling, whereas here a second, albeit smaller, peak was seen on day 9. Another difference between the two studies was that in litter size. In the work presented here litters were culled to 8, whereas Elwood

and Keeling culled their litters to 4 pups: litter size might influence both nutrition and nest warmth, and both of these factors may contribute to physical development.

It should be noted that the data presented here only allow for a limited resolution: the difference in weight disappeared somewhere between days 0 and 11, and that in vocalisation on day 3 or day 4. The indication is that prenatal stress only retards very early development, and that the difference is soon made up. On the other hand, since developmental differences between control and experimental offspring occur at later stages in life, for example in the age at adrenal rhythm onset (see Chapter 6) and at puberty (see Chapter 5), it is likely that differences do exist between these groups at later stages of lactation, perhaps detectable by the mother but not by these tests.

The differences in litter development seen here were not reflected in maternal retrieval tests. This was unexpected in that maternal retrieval is known to be affected by ultrasonic calling (eg. Hennessy et al., 1980). However the retrieval tests and ultrasonic measurements were carried out on two different sets of litters, and it may be that one group was less affected by the stress procedure, although again the maternal responsiveness of one strain may differ from that of another and so this very general prediction may not be applicable.

Having demonstrated that there is an effect of prenatal crowding on offspring developmental characteristics, maternal care as a function of pup treatment was assessed. At first sight there did not appear to be any effect, other than "on nest" rhythm differences between CC and EE mothers in Expts. 1a and 1b. This effect was not repeated in Expts. 1c and 1d, although it is not clear why. A more detailed analysis was carried out in Expt. 1c and examined changes over time. On this analysis total levels of pup-directed

activity were unaffected by the experimental treatment, and in all groups a general decline was seen over lactation as the pups became increasingly independent. Neither were the total frequencies of "on nest" consistently affected by pup treatment (although a CC vs. CE difference was seen), the normal pattern being a general decline over time ending in an increased frequency late in lactation. This U-shaped pattern could be explained in terms of pup needs over the lactation period. The initial decrease may reflect a decreased need for the mother in her role as a source of warmth and the later increase an increased demand on suckling time: when pups are mobile enough they frequently follow the mother off the nest and attempt to suckle, and when this happens the mother often abandons whatever other activity she was engaged in and returns to the nest, with or without the litter. Effects on nursing (whose normal pattern is a decline over lactation) were generally the same as those for "on nest". The clearest litter effects are seen in total (a.m. + p.m.) levels of maternal feeding (see Fig. 3.33d), where mothers raising control litters showed an increase, while mothers raising experimental offspring (after an intial increase in EE's) showed a decrease. This may again reflect pup demand, experimental litters perhaps needing comparatively more nutrition early in life, and later on, having "caught up", needing less. This implies an earlier independence of the maternal food supply, and could reflect the earlier attainment of puberty and the adrenal rhythm that may be seen in experimental animals (see Chapters 5 and 6).

Closer examination (in terms of separate a.m. and p.m. levels) of the categories "on nest" and "PDA" also revealed litter effects. When "PDA" is examined in the dark (p.m.) phase (Fig. 3.34c), the decline over lactation shown by dams raising control litters was replaced by an unchanging frequency if the litters were experimental.

The retarded development of these litters may be preventing the normal decline of "PDA" although the results only reach significance in the dark phase of the cycle. The same observation applies to the increase in "on nest" in the dark phase of the cycle seen towards the end of lactation (Fig. 3.34a) for control litters but not for experimental litters.

Maternal effects can be seen in a.m. levels of "on nest" and "nurse", where control mothers showed constant levels of each behaviour but experimentals show a decrease. This decrease was followed by a late increase in EE's perhaps in part reflecting possible restricted access to food during the crowding period of gestation. The reason for this difference between C and E mothers is unclear, as is the cause of the difference between C and E mothers in p.m. levels of "other" behaviour: controls showed a general increase while experimental showed no change. However, it should be remembered that no one behaviour category is independent of another when considered in terms of time devoted to it per day. Hence time changes in "other" behaviour probably reflects time changes in alternative maternal activities, which taken singly may or may not show significant changes.

In Expt. 1a) and b) an alteration in rhythmicity of the amount of time spent on the nest was observed, although it was not clear whether the rhythm was reduced or was merely shifted in phase. Disruption of the normal daily cycles, although not seen in terms of differences of rhythm as in 1a) and b), could also be detected in Expt. 1c. This can be most clearly seen by comparing CC and EE for "on nest" where the changes over time in both a.m. and p.m. levels are exactly reversed. Much the same is true for "nurse". Indeed, when one looks at Fig. 3.35, where all behaviours are represented together, the general impression is that, in terms of behaviour

frequencies, for the CC group the morning is the most stable phase, whereas for the other three groups it is on the whole the afternoon, i.e. the dark phase, and normally the most active phase, that is more stable. However, since all three groups are affected similarly, this may be an example of what I have called an "experimental effect". Rhythmicity in maternal activity has also been demonstrated by Grota and Ader (1970). Rhythmicity of maternal activity may influence the phase setting of the infant's biological clock (Reppert & Schwartz, 1983). Circadian rhythmicity in corticosterone secretion may also be set before birth (Reppert & Schwartz, 1983), although foster mothers can also exert an influence (Takahashi et al., 1982; Hiroshige et al., 1982). During the crowding phase in experimental treatment it is possible that maternal circadian activity is suppressed due to a general disturbance caused by constant fighting amongst males, and by attempts of the males to mount the females. If this disruption was to persist to any extent after parturition, postnatal maternal behaviour could affect the offspring's rhythmicity. Suckling periodicity can also set the pup's circadian rhythm (eg. Miyabo et al., 1980; Hiroshige et al., 1982), and hence if the amount of time the mother spends nursing is altered or shifted in phase the pup's circadian periodicity could be affected. Both prenatal and early postnatal treatments have been shown to affect the onset of the circadian rhythm of corticosterone secretion (eg. Ader, 1969; Lorenz, 1974), and these effects may in some part be mediated by differential maternal behaviour towards the litters. Since adrenal rhythmicity has been shown to affect fertility (Paris & Ramaley, 1974) this provides yet another means in which maternal behaviour could influence offspring development. The circadian rhythm of corticosterone secretion will be discussed in Chapter 6, and female fertility in Chapter 5.

There appear to be both maternal and litter influences on nest-building, though differences are only seen during early lactation. When both had control litters, experimental females built better quality nests than did control females. Control mothers built better nests, for a short period, if they had experimental litters. This latter could again reflect the pups' need for warmth - if control pups developed a thermoregulatory ability earlier than experimentals, nest quality would not need to be maintained as long.

In conclusion, the effects of prenatal crowding on maternal behaviour are complex. Their extent is only revealed by separate examination of the activities of the mothers in the light and dark phases of the daily cycle, examining how these alter as the pups develop. Some of these effects appear to be caused by maternal treatment, some by litter treatment, and others by some interaction between maternal and offspring factors - an "experimental effect". Furthermore, some alterations are interpretable in terms of retarded pup development. The only real generalisation to emerge is that it is mainly the rhythm of the activities that is altered, rather than their absolute frequency, and this has implication for offspring development.

Chapter 4: Emotionality

The reasons for studying emotionality have already been outlined in Chapter 1. The term "emotionality" itself was first coined by Hall in 1934, who defined it as "the state of being emotional". He then went on to say that "This state consists of a group of organic, experiential and expressive reactions and denotes a general upset or excited condition of the animal. Emotionality can be thought of as a trait since animals and men differ in the intensity of emotional reactions displayed". The concept of emotionality is, however, open to anthropomorphic interpretation, and indeed is often thought of in terms of fear, timidity and arousal. Because of this there may be differences in the choice of parameters measured in tests of emotionality, and indeed in the interpretation of these parameters. One example is the use of ambulation in the open-field as an indicator of emotionality: an "emotional" animal may sit still or show limited activity, but might it not also run around frantically, in an attempt to escape from the situation? It is in fact the case that whereas most workers take low activity to be indicative of emotionality (e.g. Brain & Nowell 1969), there are some who take the opposite view (e.g. Sutton et al., 1982). Because of the difficulties associated with any concept not defined in specific terms, there is a case here for re-definition of "emotionality", if only so as to introduce some coherence into its measurement.

Emotionality is usually assessed in terms of an animal's responses to novel or "frightening" situations. Tests designed to measure this response include novel environment tests, in which an animal's activity (and, frequently, defecation) are measured, the so-called "timidity tests", in which the latency to emerge from a familiar, safe environment is measured, and a variety of maze tests,

runway tests and conditioned avoidance tests. There have also been one or two rather obscure tests, which will not be discussed here, such as the "water-wading-defecation test", and the latency to descend from a pole by means of a ladder.

The novel environment tests are the most popular as indicators of emotionality, and the most familiar of these is the open-field test. This test was introduced by Hall in 1934 and has remained popular because it provides a method for rapid measurement of specific aspects of behaviour with simple apparatus. In addition certain aspects of the behaviour measured are sensitive to a wide range of genetic, experiential, physiological and pharmacological manipulations and are reliable enough under standardised conditions to give repeatable measures on a wide range of independent variables. However, having said this, it must be added that among open-field tests apparatus, techniques, subjects, parameters and interpretations have varied enormously, while conflicting interpretation and wide generalisations continue to be made on them.

Another novel environment test, less common but becoming more so, is the holeboard apparatus. This was introduced by Boissier and Simon in 1962 and has been widely used for assessing drug effects. The holeboard, like the open-field, consists of a large arena, but differs in that there are holes (the number of which may vary between tests) in the floor through which the animal may poke its head (although not its whole body). Head-dipping is taken as a measure of exploration (e.g. File and Wardill, 1975_b).

There are two common types of "timidity tests": one is the reaction-to-handling test, in which vocalisation and escape attempts are usually measured (e.g. Ader & Plaut, 1968), and the other is the home-cage emergence test, in which time taken to emerge from the home cage into either a runway, a maze, or simply "the open" is recorded (e.g. Keeley, 1962).

Other tests typically involve some degree of learning on the part of the animal. The maze-learning and runway tests both require that the animal learns to reach a food reward located somewhere within the apparatus, and the latency to reach this goal is taken as an index of the animal's learning ability (see Young, 1963; Howarth, 1962). Conditioned avoidance tests also require a learned response from the animal, but here the "reward" is the avoidance of electric shock (e.g. Joffe, 1965_b). These learning tests have all been used as indicators of emotionality, where longer latencies are taken to represent increased emotionality.

It is clear that a great variety of behavioural tests for emotionality exist. These tests may measure common parameters, but equally may not. The tests themselves reflect different demands on the animal: open-field tests look at an animal's readiness to explore, maze tests at its ability to learn (for a food reward, when hungry), and conditioned avoidance tests look not only at learning, but perhaps also at the degree to which the animal wishes to avoid pain. The animal's motivation in all of these tests may be very different, and so it is left to question whether in fact a) these tests are truly tests of emotionality, and b) again whether "emotionality" should not be redefined, in terms of more specific responses to novel situations. One theme which would seem to be common to many studies is the readiness of an animal to explore in unfamiliar surroundings. But authors cannot even agree on this: some (e.g. Halliday, 1967) regard exploration as being closely related to emotionality, while others (e.g. Whimbey & Denenberg, 1967_a) see the two concepts as being completely independent. And again, whether activity is truly representative of a readiness to explore is itself open to dispute. And while interpretation of activity varies, so does that of defecation, for the relationship between them (high open

field activity in conjunction with low defecation denoting low emotionality) does not always hold: inverse relationships are sometimes seen, leading one to question their validity as measures of a common state. Defecation does not necessarily correlate with heart-rate response, which is presumably also under sympathetic nervous control. There is also a suggestion that defecation is a situationally-determined response and varies in different types of test situations: Tobach and Schneirla (1962) found that defecation measures taken in different tests were not significantly correlated with each other for mice, but tended to increase or decrease according to the nature of the test situation and according to each individual's developmental history, rather than remaining on a level typical of that individual's responses.

Defecation, especially in mice, is seen by some (e.g. Bruell, 1969; Brain & Nowell, 1969) to represent territorial marking. Hall, in 1934, warned against interpreting emotionality as a thing or a faculty, suggesting it merely as a convenient concept for describing a complex of factors. Since then emotionality has come to be regarded as a specific state, capable of being measured along a continuum by any one of a number of behavioural measures, all of which are supposedly similarly related to sympathetic activity. Archer (1973) has proposed two alternative approaches to studying the types of behavioural response occurring in emotionality tests. The first of these assesses an animal's adaptation to novelty, measuring the decline or rise of certain aspects of behaviour over a period of time, while the other approach involves the measurement, over a shorter time-period, of several emotional responses within one test, rather than just one or two. Neither approach makes any assumption of a single unitary emotionality state, nor indeed ascribes any kind of motivation to the animal's responses. Both approaches could prove

valuable.

A major problem in reviewing work on emotionality lies in the number of variables now known to influence its expression. Principal among these variables is the sex of the animal being tested. Archer (1975), in a review of sex differences in open-field behaviour, has shown that males typically defecate more than do females, but that females are more active. This is more true of rats than mice (although this difference is seen in mice, it is less consistent), which again suggests caution should be taken when generalising from one species to another. Early experiences may have different effects on the two sexes, depending on the nature of the treatment. Early "noxious stimulation" yields the typical sex differences in behaviour (higher defecation and lower ambulation in males), where early cold stress results in higher defecation by females and no difference in ambulation (Henderson 1967_a). There is also evidence (e.g. Birke and Archer, 1975) that ambulation is higher and defecation lower in females at oestrus than dioestrus. Rearing in a maze is also more frequent at oestrus. In the majority of sex difference studies, one must assume (there being no evidence to the contrary) that the stage of the oestrous cycle was not controlled for, and this could perhaps explain some of the more equivocal results. Sex differences have also been found in emergence tests, conditioned avoidance tests, responses to shock, and heart rate response to stressors (see Archer, 1975). Again, the picture is clearer for rats: for mice the results are more variable, some studies showing the same pattern as for rats, others showing no differences. On the whole however, females ambulate, rear and sniff more in a novel situation than do males, emerge sooner from the home cage, and acquire a conditioned avoidance response sooner. Females' flinch and jump responses to shock are lower, and the heart rate response is higher in response to the open-field or handling.

Housing is another variable that can affect emotionality. Denenberg and Morton (1962) showed that being raised in a free environment as opposed to a laboratory cage reduced emotionality in rats, acting to augment the effects of early handling. He also demonstrated that the effect of housing handled and non-handled subjects together in a free environment was "beneficial" for handled animals, especially females, but "detrimental" for non-handled subjects. Priestnall (1973) examined the effects of pre- and post-weaning grouping on open-field behaviour. Animals were raised in small or large litters (2 or 8 pups respectively), and at weaning either isolated or housed in bisexual groups of 8-10. The results indicated that animals raised in small litters had higher exploration scores, isolated animals showed more exploratory behaviour and less eating, grooming and defecation, and that males obtained generally lower scores than females. In other words, emotionality was greater in animals reared in larger litters, in animals grouped at weaning, and in males. Gentsch et al. (1981) also demonstrated a decrease in emotional response as a result of isolation. These effects are not necessary reflected physiologically: Armario and Balasch (1981) examined the corticosterone response to stressors in animals housed in a number of ways, without finding any differences in response between the groups. This may in part be due to the fact that their housing conditions were imposed on the animals either twenty hours prior to the experiment or just beforehand : neither would have the same effect as long term housing conditions. Stress prior to testing can lead to reduced pituitary-adrenal responses. The age and strain of the animal may also determine its emotional responses. Dixon and DeFries (1968) tested two strains of mice and their crosses over ten ages, ranging from 15 to 120 days. They found that increased activity was also associated with increased age in all groups, but that the

magnitude of the effect depended on the genotype. Initially increased defecation was also associated with increased age, but at later ages defecation patterns were dissimilar. Streng (1971) also examined strain differences in mice, and found significant differences between four strains, with respect to frequency of behaviour in the open-field. Frequency changes of some aspects of behaviour over time were also strain-dependent.

Early nutrition may also affect an animal's response to novelty. As Smart (1979) has pointed out, permanent deficits in brain size and distortion of brain structure may be caused by undernutrition, as can be altered endocrine function and retarded development. The work on effects of early undernourishment on emotionality have produced a by now familiar body of conflicting results, but Wiener et al. (1983) have produced some interesting results with rats. They found that while perinatal malnourishment did not affect either behavioural or plasma corticosterone responses to the open-field, head-dip frequency and duration in the holeboard test were decreased in perinatally-malnourished subjects. The latency to drink and the amount of fluid drunk in a novel environment did not differ, but the perinatally-malnourished animals were unable to modulate their pituitary-adrenal response to the situation by the consummatory behaviour of drinking.

The open-field behaviour of an animal may also be affected by maternal factors. Quadagno and Banks (1970) demonstrated, by cross-fostering two species, a postnatal maternal determinant of open-field activity. However, Ottinger et al. (1963), examining the association between maternal and offspring emotionality in rats, found that offspring emotionality is independently related to both prenatal and postnatal emotionality of the mother. They also showed that multiple mothering (rotating two mothers between their litters)

significantly increases the offspring's emotionality. Denenberg and Rosenberg (1968) studied open-field behaviour not only as a function of maternal charactersitics, but also as a function of rearing environment and sexual experience. Mothers were either handled or not during pregnancy, and the offspring were either brought up in maternity/laboratory cages or in free environments. Offspring of handled mothers were more emotional, but sexual experience (bearing and raising a litter) interacted with this : inexperienced females were more active if raised by non-handled mothers, while experienced females were more active if raised by handled mothers. Generally, experienced females were more active and defecated more.

Overall then, there are an enormous number of factors influencing the outcome of emotionality tests. It is thus perhaps not surprising that the results of studies on the effects of prenatal stress on emotionality are so varied. These results have already been discussed in Chapter 1. That the variables influencing the outcome of emotionality tests may interact with prenatal manipulation has been amply demonstrated by several workers. For example, early postnatal handling will augment the effects of conditioned avoidance during pregnancy, significantly increasing offspring emotional reactivity (i.e. defecation) over that following either type of stimulation alone (Porter & Wehmer, 1969). This study also demonstrates several two-way interactions of maternal and offspring treatments on open-field behaviour. DeFries et al. (1967) also demonstrated that maternal and foetal genotypes were important in determining the outcome of prenatal maternal treatments. An alternative means of explaining the observed variation in prenatal stress effects was postulated by Chapman and Stern (1977), who found significant litter effects for almost every behavioural and morphological measure in the open-field and in cage-emergence tests. They observed a

sex-difference (females being more active and defecating less in an open-field), but found no reliable effect of prenatal stress on males, and have suggested that failure to control for the litter variable may account for previously reported effects of prenatal stress on emotionality.

One aim of this study was to examine emotionality in a strain of mouse that has hitherto (as far as can be ascertained) not been used in this field. In an attempt to draw general conclusions about the animal's fearfulness, or responses to novel situations, both in terms of prenatal stress and in terms of the postnatal maternal environment, a number of tests have been used. This was seen to be more valuable than using a single test given the variability of results within any one test. Three tests were used : the open-field test, which despite the fact that it has come under heavy criticism in recent years (e.g. Archer, 1973) remains the most commonly used as an index of emotionality; the holeboard test, now becoming more widely used; and passive avoidance conditioning. In addition, the readiness to investigate a novel object was examined, as was the response to graded novel environments. Because of the original supposed link between emotional behaviour and pituitary-adrenal activity, variously supported or refuted in the literature, the plasma corticosterone response to graded novelty was also assessed. An attempt was made to study free (as opposed to forced) home-cage emergence into the open-field, but this was unsuccessful.

Another area of research, developed primarily in the late 1970's, which relates to pituitary-adrenal activation and might have implications in prenatal stress studies is the interaction between ACTH and opioid peptides. It is known that corticotrophin releasing factor stimulates the release of both corticotrophin and β-endorphin. ACTH, β-endorphin and enkephalin are synthesised from a

common precursor, and ACTH and β-endorphin are concomittantly secreted by the pituitary gland in response to acute stress or long-term adrenalectomy (Guillemin et al., 1977). This latter effect can be blocked by dexamethasone. It has also been demonstrated (Mandenoff et al., 1982) that endogenous opioid - mediated analgesic mechanisms are readily activated by situations involving "biologically significant" forms of stress. Mousa et al., (1981) state that analgesia mediated through endorphin/enkephalin systems may be modulated by corticosteroid levels which in turn can be affected by either dexamethasone or metyrapone. Hence treatments which increase or decrease plasma ACTH levels should also produce an increase or decrease in plasma β-endorphin levels. In prenatal treatment studies the possibility that some effects may be in part mediated via the opioid system should not be ignored : Davis and Lin (1972) have demonstrated a decrease in birth weight and increase in perinatal mortality of rat offspring as a consequence of prenatal morphine treatment. Effects were also found in the open-field, in that increased ambulation and rearing (although not defecation) were seen in prenatally-treated offspring. Because of the implied interactions of corticosteroids with pain-inhibiting systems, pain thresholds were examined here in prenatally-stressed mice. This is especially relevant in view of recent opinions on the hormonal correlates of emotionality and may bear directly on avoidance learning tests. It has been shown (Katz & Gelbart, 1978) that naloxone (an opiate inhibitor) will depress holeboard exploratory activity and entry into a novel environment and that there is a direct correlation between exploratory behaviour and enkephalin-induced behavioural activation. Roth and Katz (1979) have suggested that the response to stress be seen as several parallel but distinctive means of coping. The hypothalamo-pituitary-adrenal system

is involved in stress and physiological regulation, and a second system is postulated, based on one or more endogenous opiates and controlling several aspects of behavioural coping. If differences can be found between prenatally-stressed and unstressed animals in pain-thresholds then some credence might be lent to this view.

Materials and Methods

Subjects were the natural offspring of prenatally crowded or control females, fostered at birth to either crowded or control dams. The naming of these groups (CC, CE, EE and EC) has already been explained in Chapter 2. All animals*were tested between 25-35 days of age: this age was chosen partly because Keeley (1962) had found prenatal crowding effects in animals of this age, and partly to reduce the hormonal variables that influence adult female behaviour: the females used in these studies were prepubertal. Testing was carried out in a room adjoining the animal housing room, and was done under red light between 2 and 6 hours after "lights-off". Animals were tested in a random order within each cage. Unless otherwise stated all timing was carried out with stopclocks and stopwatches. The test procedures were as follows:

i)Open-field test:

The open field arena consisted of a $60cm^2$ arena with 30cm high walls. This apparatus was made out of chipboard with a washable surface. The floor of the arena was marked into 25 squares 12cm x 12cm, so dividing the space into outer, inner and central regions, each one square "deep". The animal to be tested was placed in a specific corner square and the latency to move from the start square recorded. Latencies to move into the inner area and centre square

* Except those used in Expt. (vi)

were also taken, as well as the frequency of grooming, number of faecal boli, and the total number of squares entered with all four feet. Each animal was tested for five minutes, then replaced in the home cage. The arena was cleaned with a mild disinfectant between each test so as to reduce distraction by odours. Animals were tested in a random order.

Two open-field experiments were carried out. For operational reasons, the first tested animals of CC, CE and EC groups only. The second examined open-field performance of animals from CC, CE, EE and EC groups. Both males and females were tested.

ii) Holeboard:

The holeboard consisted of a perspex platform measuring 45 x 25cm, with four holes (1.2cm diameter), each of which was 5.5cm from the long edge of this platform and 11.5cm from the shorter edge. The distances between neighbouring holes were either 19.5cm (along the long edge) or 18.3cm (shorter edge). The platform was supported within a wooden frame, with walls 25cm high on three sides, and a clear perspex fronting to facilitate observations. The animal to be tested was placed in a specific corner at the start of the test period, and the number of rears, number of faecal boli and the number of times the animal poked its nose through one of the holes (to below the base fo the platform) were recorded. Each test period lasted three minutes, and again the apparatus was cleaned with mild disinfectant between each test. This test was carried out three times; the first, again for operational reasons, was only carried out on CC, CE and EC animals; the second comprised part of the study of responses to graded novelty and examined only CC's and EE's; the third test examined all four treatment groups. Both sexes were studied.

iii) Graded Novelty:

This experiment was founded on work by Bindra and Spinner(1958) and Hennessy and Levine (1978). Bindra and Spinner demonstrated graded behavioural responses to environments of differing degrees of novelty. Hennessy and Levine demonstrated a graded plasma corticosterone response to three graded novel environments. This experiment aimed to combine facets of both of these experiments, and to assess whether a graded behavioural response to novelty could be associated with a graded hormonal response. Three novel environments differing in their degree of novelty were chosen, and behavioural and plasma corticosterone responses to these were assessed. The first novel environment (Environment 1) consisted of a 42 x 25 x 11cm cage, of the same type as the home cage, containing clean bedding but with no food or water in the metal top. The second environment (Environment 2) consisted merely of an empty cage of the same size - no bedding, food or water. The third novel environment (Environment 3) consisted of the holeboard apparatus (see above). Beacause of the dissimilarities between the first two of these environments and the last, behavioural comparisons could only be made between Environments 1 and 2, although the plasma corticosterone response was compared in all three environments.

For Environments 1 and 2, animals were placed in the cage in a specific corner, and then observed over a ten-minute period. The behaviour in progress was recorded at 6-second intervals, and later sorted into the following mutually-exclusive categories:

1. Walk: any activity involving locomotion, with at least two paws not in contact with the ground.
2. Rear: hind feet on the ground, front feet raised.

3. Climb: all feet off the ground, animal gripping bars with one or more paws.
4. Groom: stationary; washing or scratching.
5. Attention to floor: stationary; digging or sniffing at the floor, or chewing bedding.
6. Other: stationary; includes freezing, stretching (as in hesitance to move). No other overt behaviour.

After the ten-minute observation period animals were left in the apparatus for a further ten minutes (to achieve a maximal corticosterone stress response: Levine & Treiman, 1969) before being removed for blood-sampling.

The holeboard test was carried out as in (ii) above, the animal being left in the apparatus after the 3-minute test period for a further twenty minutes before blood-sampling.

The animals studied were males and females of CC and EE groups. No mouse was tested in more than one environment. All environments were cleaned between tests.

iv) Novel object investigation:

This work was based on studies by Misslin and Ropartz (1980, 1981) who examined the responses of mice to novel objects, and to the effects of the object being placed in a novel as opposed to a familiar environment. This experiment examined investigations of a novel object in novel and familiar environments by CC and EE mice. Males only were studied, as investigation and general activity in females may be strongly influenced by the ovarian cycle (Birke, 1979). Observations were made over a five-minute period of novel investigation in:

a) a familar environment (a large (42 x 25 x 11cm) cage lived in for 48 hours)

b) an unfamiliar environment (a large cage with clean bedding, and no food or water).

The "novel object" was a cylindrical block of resin, 6cm in diameter and 3.5cm in height. The animal to be tested was removed from its home cage and placed in a smaller cage while the resin block was placed in the centre of the testing cage. The animal was then placed (or replaced) in the testing cage in a specific corner, the lid was replaced and testing commenced. The total amount of time spent investigating the object was recorded for each minute of the test period, as was the total number of investigations. An investigation comprised sniffing at the object (with the nose not more than 2cm away from it), touching it or climbing onto it.

v) Passive avoidance:

The performance of male CC, CF, EE and EC offspring in a passive avoidance test was examined. The apparatus consisted of two adjoining chambers: the "safe" chamber measured 12 x 8 x 12 cm, while the "unsafe" chamber (with an electrified metal grille base) measured 13 x 10 x 12.5cm. A circular hole in the wall common to both linked the two chambers. A lead ran from the metal grille to a control box, via which shock delivery could be instigated and regulated. This apparatus had been adapted from a Skinner Box apparatus supplied by Techserv Inc. Maryland, U.S.A.

Passive avoidance requires that the animal remain in the safe area to avoid shock - as opposed to active avoidance, where an active response is necessary. Because of reports (from this laboratory) of long latencies to enter the shock chamber, animals were given a habituation run one hour before testing where each animal was placed

in the apparatus until it had emerged into the shock chamber. This latency was recorded but no shock treatment was given on this trial. To further encourage animals to move into the shock chamber the floor of the safe chamber was heavily sprayed with wet disinfectant. It was hoped too that the shock delivery would be more uniform if the animals had wet paws. On the test run the animal was placed in the safe chamber, and when it emerged into the shock chamber, was given an electric shock for 5 seconds at 2 second intervals until the animal returned to the safe chamber.

The intensity of shock given varied from one animal to another and was determined by overt distress thresholds. This method was employed for three main reasons:

a) Most investigators use constant current settings when delivering shock, although it is known that neither voltage nor current, but current density determines the degree of pain inflicted. This is impossible to control, as it depends on the area of the animal's body in contact with the shock grid.

b) Different areas of the body and different individuals certainly vary in their sensitivity to shock.

c) Only a constant voltage device was available and it was decided that working with constant voltage was inadequate.

Hence the shock intensity used for each animal was one to which the animal could be seen to respond. A great variation was seen in the response thresholds: on a intensity dial graded in arbitrary voltage units, some animals responded to as little as 65 units, while others only responded to higher intensities, in some cases as high as 130 units. The shock given was initially low, and then raised until a distressed response was seen (e.g. escape attempts and/or vocalisation).

One hour after the test run the animals were retested and the latency to enter the shock chamber was measured although no shock was

given.

vi)Pain thresholds:

Pain thresholds were assessed in CC and EE males aged 196 days, using a hotplate apparatus supplied by Technilab Instruments Inc., New Jersey, U.S.A. This consisted of a platform ($28cm^2$) which could be heated to specific temperatures, with removable clear perspex walls (15.5cm in height) and roof. The apparatus incorporated a timing device that could be operated manually or via a foot-pedal. The temperature was set at $52^{o}C$, after Harvey (in preparation) and stabilised at $51.3\pm0.5^{o}C$. The animal to be tested was placed on the platform, and the latencies to lick the paws twice were recorded. This was carried out three times, at fifteen-minute intervals. If the animal did not respond in the required manner within 60 seconds of test commencement it was removed from the apparatus.

No animal was used more than once, either within any one test or on different tests. This procedure attempted to control for the effects of prior experience.

Summary of Experiments

(i) Open Field:		
Expt. 1 - CC, CE, EC	♂ & ♀	Stressed days 12-17
Expt. 2 - CC, CE, EE, EC	♂ & ♀	Stressed days 12-16
(ii) Holeboard:		
Expt. 1 - CC, CE, EC	♂ & ♀	Stressed days 12-17
Expt. 2 - CC, EE (part of Expt. iii).	♂ & ♀	Stressed days 12-16
Expt. 3 - CC, CE, EE, EC	♂ & ♀	Stressed days 12-16
(iii) Graded Novelty:		
CC, EE	♂ & ♀	Stressed days 12-16
(iv) Novel Object Investigation:		
CC, EE	♂	Stressed days 12-16
(v) Passive Avoidance:		
CC, CE, EE, EC	♂	Stressed days 12-16
(vi) Pain thresholds:		
CC, EE	♂	Stressed days 12-17

Animals in Expts. (i) - (v) were aged 25-35 days at the time of testing.

Animals in Expt. (vi) were aged 196 days.

Results

1) Open-Field

Table 4.1.: Median scores (with 95% confidence limits) obtained on Open-Field test (expt. 1)

	CC		CE		EC	
	♂	♀	♂	♀	♂	♀
	9	9	7	7.5	6	8.5
Latency to leave start (secs)	(5-17)	(4-26)	(1-48)	(0-15)	(2-68)	(0-16)
Latency to inner area (secs)	50.8 (20.2-94.6)	29.8 (14.8-142.2)	37 (23.6-186.5)	70.1 (42.8-127.1)	89.4 (41.8-219.6)	49.2 (3.8-125.1)
Latency to centre area (secs)	112.2 (57.5-211.4)	122.9 (44.4-233.2)	112.2 (32.8-300)	237.5 (150-300)	207.8 (140.5-300)	147.7 (42-252.1)
No. of squares entered	196 (128-201)	170.5 (81-262)	196 (154-218)	155.5 (116-224)	142 (79-185)	147.5 (106-169)
No. of faecal pellets	2 (0-5)	2.5 (0-8)	2 (0-4)	3 (1-7)	3 (0-5)	3.5 (0-6)
No. of grooms	1 (1-2)	1 (1-2)	1 (1-2)	1.5 (1-2)	1 (1-2)	2 (1-3)
N	11	10	11	10	12	10

On Fisher Exact-Probability tests:

Maternal effects:

Latency to enter centre area: ♂ CC<EC, p=0.014

No. of squares entered : ♂ CC>EC, p=0.02

Pup effects:

Latency to enter centre area: ♀ CC<CE, p=0.03

No. of squares entered : ♀ CC>CE, p=0.04

Other effects?

Latency to centre area : ♂ EC<CE, p=0.014

As the comparison of CE vs. EC is near impossible to interpret directly in terms of maternal or pup treatments, it hwas not been made in future analyses, although differences may exist between these groups.

Male/Female differences:

Defecation : CE ♀ > ♂ , p<0.05

Latency to inner area : EC ♀ > ♂ , p<0.05

Latency to centre area : EC ♀ > ♂ , p<0.05

Table 4.2.: Median scores (with 95% confidence limits) obtained on Open-Field test (Expt. 2).

	CC		CE		EE		EC	
	♂	♀	♂	♀	♂	♀	♂	♀
Latency to leave start (secs)	12 (3-16)	6 (2-12)	10.5 (7-14)	11.5 (5-25)	16 (5-39)	14 (4-27)	13 (5-21)	7 (6-15)
Latency to inner area (secs)	25.7 (6.2-46.6)	25.3 (9.3-57.8)	31.7 (13.4-98.6)	56.5 (12-111.5)	77.3 (12.4-300^{+})	32.8 (18.5-106.8)	32.2 (17-137.1)	39.4 (20.4-105.9)
Latency to centre area (secs)	91.1 (16.2-136.2)	43.2 (15.6-300)	80.9 (30.2-224)	113.5 (56.9-152.5)	173.8 (39.9-300^{+})	170.7 (67.7-300^{+})	122.3 (27.2-300^{+})	98.6 (62.9-242.4)
No. of squares entered	139 (81-179)	162 (139-218)	152.5 (130-174)	143 (116-166)	114 (65-155)	146 (103-165)	110.5 (32-159)	155 (123-170)
No. of faecal pellets	2 (0-8)	6 (2-10)	4 (1-7)	2.5 (0-5)	4 (0-6)	3 (0-7)	4.5 (0-6)	4.5 (3-8)
n	10	9	10	10	10	9	10	10

On Mann-Whitney 'U' tests:

Maternal effects: No. of squares entered: ♂CE>EE, $p<0.02$
Maternal and/or pup effects: Latency to inner area: ♂CC<EE, $p=0.02$

The results indicate a greater effect of prenatal stress on open-field behaviour of males than on females. Differences lie in the latencies to move into the inner and central areas and in the amount of ambulation CC males showed shorter latencies than EC ♂'s to the centre area in Expt. 1, and shorter latencies than EE males to the inner area in Expt. 2. CC males also entered more squares than EC males in Expt. 1 while CE males entered more squares than EE males in Expt. 2. This could be interpreted as a maternal effect, but the results are not clear-cut in this respect. In females differences were only seen between CC and CE animals, CC animals having shorter latencies to reach the centre area, and ambulating more. This effect would seem to be a prenatal effect in that in both cases the foster mother was a control female. The latencies to move into the inner and centre areas should reflect a decreased timidity in a readiness to move away from the walls and into the open. High activity is also generally interpreted as being indicative of decreased timidity (although this was discussed earlier). In this respect the results from the females would seem to be consistent with each other. Open-field performance would seem to depend more on postnatal experience in males, and on prenatal experience in females.

ii) Holeboard

Table 4.3.: Median scores (with 95% confidence limits) obtained on holeboard test (Expt. 1).

	CC		CE		EC	
	♂	♀	♂	♀	♂	♀
No. of pokes	13 (8-19)	3.5 (0-14)	10 (5-13)	12.5 (7-16)	8 (4-18)	6 (2-11)
No. of rears	26 (13-28)	20.5 (6-28)	18 (10-26)	14 (4-26)	17 (15-21)	27 (15-32)
No. of grooms	1 (1-1)	1 (1-1)	1 (0-1)	1 (0-2)	1 (0-1)	1 (1-1)
No. of faecal pellets	1 (0-2)	1 (0-5)	0 (0-2)	0 (0-2)	0 (0-4)	0 (0-2)
n	17	10	15	10	15	10

On Fisher Exact-Probability tests:

Maternal effects:

No. of pokes: ♂ CC > EC, p=0.04

No. of rears: ♂ CC > EC, p=0.04

Pup effects:

No. of pokes: ♀ CC < CE, p=0.02

'Other' effects:

No. of rears: ♀ EC > CE, p=0.03

Male/Female differences:

No. of rears: EC ♀ > ♂ , p<0.05

Table 4.4.: Median values (with 95% confidence limits) obtained on holeboard test (Expt. 2).

	CC		EE	
	♂	♀	♂	♀
No. of pokes	13 (0-19)	13 (6-23)	12 (4-28)	20 (0-25)
No. of rears	18.5 (8-23)	16 (12-32)	21 (12-27)	17 (10-27)
No. of grooms	1 (0-2)	0.5 (0-1)	0 (0-1)	1 (0-1)
No. of faecal	1 (0-1)	0 (0-1)	1 (0-2)	1 (0-1)

n = 10 in all groups

No significant differences were obtained on any measures in this experiment.

Table 4.5.: Median values (with 95% confidence limits) obtained on holeboard test (Expt. 3).

	CC		CE		EE		EC	
	♂	♀	♂	♀	♂	♀	♂	♀
No. of pokes	57 (52-80)	42 (31-50)	60.5 (36-69)	36.5 (23-47)	37 (10-53)	35 (19-51)	28.5 (17-63)	43.5 (19-63
No. of rears	19 (3-34)	18 (4-3)	12 (5-31)	22 (7-44)	20.5 (11-39)	45 (14-61)	12.5 (2-35)	24 (5-41)
No. of faecal pellets	4 (3-5)	0 (0-5)	0 (0-3)	3 (2-6)	3.5 (0-6)	5 (3-7)	5 (3-8)	5.5 (4-9)
n	10	9	10	10	10	9	10	10

On Mann-Whitney 'U' tests:

Maternal effects:

No. of pokes: ♂ CC > EC, $p<0.02$

EE > CE, $p<0.02$

Defecation: ♀ CC < EC, $p<0.02$

Pup effects:

Defecation: ♂ CC > CE, $p<0.02$

Maternal and/or pup effects:

No. of pokes: ♂ CC > EE, $p<0.02$

No. of rears: ♀ CC < EE, $p<0.02$

Defecation: ♀ CC < EE, $p<0.05$

Male/Female differences:

No. of pokes: CC ♂ > ♀ $p<0.01$

CE ♂ > ♀ $p<0.05$

The results of the holeboard tests would again seem to indicate more effects of the stress treatment on males than on females. On the whole, effects are seen in measures of exploratory or active behaviour, that is, in the number of nose-pokes and amount of rearing observed. CC males nose-poked more than EC males in both Expt. 1. and Expt. 3. In Expt. 3. CC males showed a greater incidence of nose-poking than EE males also, and EE males scored higher on this measure than CE males. In Expt. 1. CC males also reared more than EC males. This would again seem to indicate a maternal factor influencing activity in males, with higher activity in those reared by control mothers. Females show less consistent differences in the incidence of nose-poking and rearing, but again the differences are more readily interpreted in terms of prenatal experience, CC animals showing less nose-poking than CE animals in Expt. 1, and showing less rearing than EE animals in Expt. 3. Less rearing is also seen in EC females than CE females in Expt. 1, although the EC/CE comparison is harder to interpret and could be used to support either hypothesis (prenatal vs. postnatal influences). Less defecation was observed in CC females than in either CE or EE females: this would again appear to depend on the treatment of the offspring rather than the treatment of the mother. The reason for the lack of any significant differences in Expt. 2 is unclear, but may have been due to the extra disturbance caused by running three different types of novel environment tests simultaneously. In general, the effects of stress would seem to be to decrease activity in females while increasing actvitity in males, the influences being mediated prenatally in females but postnatally in males.

iii) Graded novelty.

a) Behaviour

Table 4.6.: Median scores (with 95% confidence limits) obtained for behavioural measures in Env. 1 and Env. 2

(Env. 3 results are given in previous section on holeboard test results):

	Env. 1				Env. 2			
	CC		EE		CC		EE	
	♂	♀	♂	♀	♂	♀	♂	♀
Walk	21 (15-35)	22 (17-31)	21 (17-27)	26 (20-29)	28.5 (27-35)	29 (25-34)	28 (21-29)	28 (21-35)
Rear	57 (41-52)	30 (24-39)	47 (34-45)	30 (26-39)	45 (38-51)	32 (21-34)	43 (23-49)	40 (23-39)
Climb	13.5 (7-25)	30 (9-53)	14 (6-17)	9.5 (5-19)	8.5 (3-17)	14 (4-25)	10 (3-15)	7 (2-11)
Groom	5.5 (4-10)	4 (2-8)	6.5 (4-9)	7.5 (5-14)	6.5 (3-13)	5 (2-13)	5 (3-10)	8 (3-20)
Attention to floor	6 (2-16)	11 (4-17)	9 (7-15)	18.5 (10-23)	5 (2-7)	7 (2-7)	3 (2-7)	7.5 (6-14)
Other	1 (0-3)	2 (1-6)	4 (3-5)	2.5 (1-4)	0.5 (0-6)	14 (4-26)	9 (6-15)	14.5 (7-25)
n	10	9	8	8	10	9	9	10

On Mann-Whitney 'U' tests:

Env. 1.

Groom: ♀ CC < EE, p=0.05

Other: ♂ CC < EE, p<0.02

Env. 2.

Attention to floor: ♀ CC < EE, p<0.05

Other: ♂ CC < EE, p<0.002.

Env. 1 vs. Env. 2

Walk: C ♂ Env. 2 > Env. 1, p<0.05

Attention to floor: C ♀ Env. 2 < Env. 1, p=0.02

Other: E ♂ Env. 2 > Env. 1, p=0.002

C ♀ Env. 2 > Env. 1, p=0.002

E ♀ Env. 2 > Env. 1, p<0.02

Male-Female differences:

Env. 1.

Rear: C ♂ > ♀, p<0.02; E ♂ > ♀, p<0.02

Walk: E ♀ > ♂, p<0.03

Attention to floor: E ♀ > ♂, p=0.01

Env. 2.

Rear: C ♂ > ♀, p<0.02; E ♂ > ♀, p<0.05

Attention to floor: E ♀ > ♂, p<0.01

Other: C ♀ > ♂, p<0.002

b) Plasma Corticosterone Levels:

Table 4.7: Mean plasma corticosterone levels (±S.E.) in response to graded novelty.

	CC		EE	
	♂	♀	♂	♀
Env. 1	110.6 ±14.8 (n=9)	138.2 ±23.3 (n=8)	122.4 ±12.6 (n=8)	149.9 ±23.0 (n=8)
Env. 2	114.4 ±14.8 (n=10)	216.8 ±42.3 (n=8)	132.6 ±14.6 (n=9)	281.9 ±55.7 (n=8)
Env. 3	107.4 ±15.8 (n=10)	174.1 ±18.8 (n=9)	138.4 ±5.2 (n=7)	271.0 ±31.5 (n=8)

On Student's t-tests EE♀'s showed a differential response to Environments 1 and 2 ($p<0.05$) and to Environments 1 and 3 ($p<0.01$). No other differential response was seen. However, since the hormone assay was at this time subject to technical problems, the corticosterone response to graded novelty was re-examined at a later date (see Chapter 5).

In this experiment no attempt was being made to distinguish pre- and postnatal influences. The aim here was to see whether behaviour varied with the degree of novelty, and whether behavioural differences were reflected in plasma corticosterone levels. The only case in which this was seen was in the reduced activity and increased corticosterone levels shown by EE♀'s in Env. 2 as compared with Env. 1. A few other behavioural parameters reflected differential responses to Env.'s 1 and 2: CC males were more active (in terms of walking) in Env. 2, and CC females paid less attention to the floor and were more inactive in Env. 2.

iv) Novel object investigation:

Table 4.8: Median values (with 95% confidence limits) obtained in novel object investigation tests.

		Number of investigations	Total time spent investigating (secs)	Mean time per investigation (secs)
A) Familiar env.	CC	26.5 (16-31) **	25.8 (10.2-47.7) *	0.92 (0.76-1.84)
	EE	17 (14-22)	21.4 (17.2-22.7)	1.23 (0.87-1.51)
B) Unfamiliar env.	CC	16 (13-20) *	8.45 (5.9-18.3)	0.50 (0.45-1.14)
	EE	18 (16-23)	16.4 (11.2-19.5)	0.85 (0.63-0.97)
Familiar vs. Unfamiliar environments	CC	$p<0.01$	$p<0.025$	$P<0.01$
	EE	N.S.	$P<0.025$	$P<0.01$

CC n=10, EE n=9.

* $p<0.05$
** $p<0.01$
} Refers to CC vs. EE difference on Mann-Whitney 'U' test.

All animals investigate the object more (in terms of total investigation time and in terms of the mean duration of each individual investigation) if it is presented in a familiar environment. Control animals investigate the object more frequently in a familiar environment than in an unfamiliar environment, while experimental animals show no difference on this measure. Control animals investigate the novel object more frequently than do experimental animals when the object is presented in a familiar environment, but the situation is reversed when the object is presented in an unfamiliar environment. Control animals also spend more total time investigating the object in a familiar environment than do experimental animals.

v) Passive avoidance

Table 4.9.: Median scores (with 95% confidence limits) obtained in passive avoidance tests.

	CC	CE	EE	EC
Habituation latency (secs)	24.5 (8-62)	36.5 (21-68)	36.5 (26-132)	28.0 (18-81)
Test latency (secs)	15.6 (9-22)	17.5 (9.5-59.5)	10.3 (4-18.4)	22.5 (11-60)
Retest latency (secs)	235.0 (52-300^{+})	147.5 (49-300^{+})	190.0 (38-264)	254.0 (138-300^{+})
Habituation/test latency differences (secs)	13.5 (-12-53.5)	19.0 (4-29)	29.0 (17.6-119)	5.5 (-1-54)
Test/retest latency differences (secs)	221.0 (34-268^{+})	134.5 (39-279^{+})	168.5 (25-260)	226.0 (119-275^{+})
No. of faecal pellets	1.5 (0-3)	3.0 (0-4)	2.5 (0-6)	2.0 (0-4)
n	10	10	10	5

On Mann-Whitney 'U' test:

Maternal effects:

Test latency: EE < CE, $p<0.05$

Pup effects:

Test latency: EE < EC, $p<0.05$

Significant results on Mann-Whitney 'U' tests were obtained for test latencies only. Here EE animals showed significantly shorter latencies to enter the shock chamber than did EC or CE animals. EE animals were the only ones to show a significantly different latency ($p<0.05$) between the habituation and test runs. This result could be interpreted in terms of decreased timidity, or in terms of superior "remembrance" that the second chamber had been dry and safe on the habituation run.

vi) Pain thresholds:

Table 4.10: Mean scores (+S.E.) obtained in the hotplate test.

	CC		EE	
	1st paw-lick latency (secs)	2nd paw-lick latency (secs)	1st paw-lick latency (secs)	2nd paw-lick latency (secs)
Test 1	14.84 ±1.75	22.97 ±3.90	18.06 ±1.71	26.12 ±5.00
Test 2	21.52 ±3.50	31.17 ±2.30	16.06 ±3.69	25.67 ±3.77
Test 3	10.42 ** ±3.47	18.29 * ±2.17	20.41 ±2.72	27.54 ±9.18

n = 9 in both groups.

Asterisks refer to CC vs. EE differences: *$p<0.05$, **$p<0.01$ (see below).

A one-way analysis of variance was carried out on the three tests in each group of animals: a change in thresholds over time (both 1st and 2nd paw-lick latencies) was observed in CC animals but not in EE animals. This could reflect analgesia in CC but not in EE animals. There were also significant differences (Student's t-tests) on Test 3 between CC and EE groups, on both first ($p<0.01$) and second ($p<0.05$) paw-lick latencies. This could again reflect analgesia in CC animals.

Discussion

Although the picture given by the results of these tests is on the face of it rather confusing, a few generalisations can be made. In the tests which examine the animal's response to novelty, namely the holeboard, novel object investigation, and responses to graded novelty a number of parallels can be drawn. Both the open-field and holeboard suggest a) that the effects of prenatal stress are differentially mediated in males and females, and b) that these effects may differ in direction. This is in agreement with work by Ader & Conklin (1963), who found that the effects of prenatal maternal handling varied both as a function of sex and postnatal treatment (fostering or being reared by own mothers). They showed that females from the experimental group showed shorter latencies than control offspring only if reared by their own mothers: male experimental animals only showed shorter latencies than controls if reared by foster mothers. Thompson et al. (1962) found that experimental females took longer to run a water maze than control females, but that the reverse was true for males. Weir and DeFries (1964) found that in prenatally stressed animals of a low-active strain, males showed increased open-field ambulation, whereas females showed decreased ambulation. Here the result suggest that the effect of prenatal stress on males is to increase emotionality (in terms of decreased activity), and that this is mediated postnatally. In females the effect would appear to be mediated prenatally, although the direction of the effect is less certain: higher rearing and poking scores by prenatally stressed females in the holeboard imply decreased emotionality whereas the longer latencies and lower ambulation observed in the open-field suggest the opposite. Differences in defecation were not observed. Ader and Plaut (1968) and Thompson and Quinby (1964) found that the differences between

prenatally-stressed and control offspring were more pronounced for females. It may be that this is also the case here, and the effects on the males are weak enough to be overridden by postnatal maternal treatment effects.

On the tests of response to differing degrees of novelty (reflected in behaviour in two different novel environments) experimental animals generally show more inactivity ("other") than control animals and show more inactivity in the more novel of the two environments. Bindra and Spinner (1958) showed a (non-significant) increase in ambulation with the degree of novelty, along with an (again non-significant) increase in "freezing" behaviour and a significant decrease in sniffing between environments 1 and 3. In this study, control male mice seem to reflect the increase in ambulation with degree of novelty, although experimental males do not show differential ambulation. An increase in inactivity (similar to "freezing") is seen only in experimental males and in females. "Sniffing" could be seen as being similar to "attention to floor", although only control females show a decrease in this behaviour as novelty increases. While the similarities between this and Bindra and Spinner's work seem limited, it should be remembered that it is unwise to generalise between species, let alone between sexes.

The reduced activity in experimental males is in agreement with the hypothesis, derived from the open-field and holeboard tests, that emotionality is increased in males as a function of parental stress. The data is again less clear for females. Both control and experimental females show more inactivity in the more novel environment, but show no activity differences within each individual environment. Experimental and control females differ only in that experimental animals exhibit more grooming in the less novel environment than do controls, and pay more attention to the floor in

the more novel environment than do controls. These results conflict to some extent: grooming is more often associated with nervousness or indecision, while investigation of the floor could be regarded as exploratory. The higher incidence of grooming in stressed females might suggest that emotionality is in fact increased in females as a function of prenatal stress, although the absence of any other significant differences in grooming on other tests makes this a somewhat tenuous support for this theory. To support the alternative theory - that prenatal stress decreases emotionality in females - a greater degree of activity or exploratory behaviour is required of experimental females. With regard to "attention to floor" in Env. 2, experimental females paying more attention to the floor than controls, this theory would seem to have some support. Attention to the floor actually decreases (from Env. 1 to Env. 2) in controls and whereas experimental animals also show a decrease in this respect it is not significant.

One fact that emerges is that degrees of novelty may be differentially responded to by control and experimental animals. As already mentioned, in the graded novel environment tests, control males showed an increase in ambulation while experimental males did not. Likewise control but not experimental females show a decrease in the amount of attention paid to the floor as novelty increases. This differential response is also reflected in the novel object investigation tests. Control animals will investigate an object more frequently if it is presented in a familiar environment, whereas experimental animals do not appear to differentiate in this respect.

The responses to novel objects seen here did not tally with those obtained by Misslin and Ropartz (1981). Using Swiss albino mice, they found that a novel object placed in a familiar environment released avoidance and burying responses, while the same object

placed in a novel environment increased contacts with it and reduced burying. Burying was only observed once in this study presented here, and the object appeared to be less aversive in the familiar environment. The experiments did differ in two major respects however. Misslin and Ropartz's mice were tested over a ten-minute period whereas these animals were tested for five minutes. The dimensions of the environments also differed: the environments measured 42 x 25 x 11 cm in this study, whereas Misslin and Ropartz's environments measured 30 x 10 x 20cm. The reduced dimensions of Misslin and Ropartz's environment probably forced the animals into closer contact with the object (which was 10 cm in diameter) and this itself may have prompted the burying responses, as burying is seen as species-typical defensive behaviour (Pinel & Treit, 1978). The greater size of the environment in this study enabled the animal to avoid the object more easily, and hence the measures taken are more likely to reflect the animal's readiness to investigate novelty, and hence more interpretable in terms of fearfulness or emotionality. Since (in a familiar environment) the control animals spend significantly more time investigating the novel object, and investigate it more frequently, than do experimentals, the effect of stress would again appear to increase emotionality in males. In the unfamiliar environment the experimental animals appear to investigate the object more frequently than controls, although no differences exist on total investigation time or mean time per investigation. It would seem that experimental animals are less affected by environmental changes, as mentioned above.

The results of the passive avoidance tests do not agree with those of Joffe (1965_b), who found that in rats prenatally-stressed offspring had significantly shorter avoidance latencies than control offspring. This could have been interpreted in terms of treated

(maternal conditioned avoidance) offspring showing a greater degree of fear or anxiety in the avoidance situation, and this facilitating the learning of the avoidance response. In this study, EE animals would appear to have learned better than EC or CE animals that the shock chamber was safe and dry, but no differnces were seen in latencies to enter this chamber following shock treatment. Little work other than that by Joffe has been carried out on the effects of prenatal or even early postnatal treatment on passive avoidance. If increased emotionality can be associated with increased fear and hence facilitated learning, then one might indeed predict (following the hypothesis that stress effects are postnatally mediated and result in increased emotionality in males) that EE animals should show lower latencies than CE animals. The EE vs. EC difference does not fall in with this prediction: it could be that the number of EC animals used is too small to provide a truly representative result, or else that there is some prenatal factor operating here as well. It may be that no latency differences are observed following shock-exposure because the shock was too severe a stimulus. It has been suggested (Joffe, 1965_b) that a high level of stress or stimulation may inhibit activity while a moderate degree may facilitate it. Another explanation for the fact that EE animals show shorter latencies than EC and CE animals may be provided by a derivation of an hypothesis given by Joffe (1965b). Looking at the effects of premating and gestational stress, he suggested that the two stresses could be regarded as different "dosages" of stress, one level affecting open-field behaviour, and another avoidance conditioning. If gestational stress and mothering could act as two different "dosages" of early stimulation then while open-field behaviour may be affected in one direction, the picture may be less clear for passive avoidance learning.

The results from the hotplate test are interesting in that they suggest that control and experimental animals exhibit differential responses (in terms of paw-lick latencies) to repeated exposure to the hotplate. Given that analgesic mechanisms are readily activated by situations involving "biologically significant" forms of stress (Mandenoff et al., 1982), and may be modulated by corticosteroid levels (Mousa et al., 1981), it would seem that the experimental animals respond less, perhaps in terms of corticosterone levels, to the hotplate situation. The study that might have helped to confirm or disprove this possibility - the corticosterone response to graded novelty - unfortunately did not support this theory, and indeed if anything suggested the opposite. However the hormone assay was at this stage subject to technical problems and so the test has had to be repeated. The results will be discussed in relation to analgesia modulation in Chapter 5. If experimental animals do differ from controls in their analgesic systems, then the theory (advanced by Roth & Katz, 1979) that the behavioural and physiological responses to stress may be differentially mediated by the opiate and hypothalamo-pituitary-adrenal systems would seem to be supported. This would help to explain why on many tests of emotionality behabioural and physiological parameters (e.g. activity vs. defaecation) are not consistently correlated. vs. defecation) are not consistently correlated.

The differential responses of control and experimental animals to degree of novelty may reflect differences in general levels of "arousal" or "activation" in novel situations. The implication here would be that experimental animals are less aroused/activated by increasing novelty, perhaps having higher "activation thresholds" than controls. The postweaning environment has been shown to affect pain thresholds: Fessler and Beatty (1976) demonstrated that rearing

in an enriched environment elevated shock thresholds in rats, and Rodgers and Hendrie (1983) showed that hotplate thresholds in mice could be elevated by isolation housing. If the postweaning environment can influence pain thresholds in this way, then perhaps so too can pre-weaning environmental variables.

Because of evidence to suggest that males are "demasculinised", possibly "feminised" by prenatal stress (e.g. Dahlöf et al., 1977) and that females may be "defeminised" (e.g. Politch & Herrenkohl, 1984), one might predict that prenatal stress would erode (or perhaps reverse) sex differences. Differences in emotional responsiveness could be due to altered sex-typical behaviour. One might even suggest that prenatal factors act on sexual differentiation while postnatal mothering acts on activation levels (responsiveness) of the offspring. The results from this chapter do not indicate an erosion of sex differences, however, and on the whole results obtained of sex differences in emotional reactivity do not agree with the statement by Archer (1975) that females tend to be more active in novel situations and to defecate less than males. However it has already been pointed out that different treatments can have different effects, early "noxious stimulation" resulting in the typical sex difference in behaviour while early cold stress results in higher defecation in females than in males, with no significant ambulation differences (Henderson, 1967_a). In these experiments females (CE and EC) have shown higher defecation and longer latencies in the open-field than males. The fact that this effect was not seen in CC animals suggests that prenatal stress is here in some way creating a sex difference in behaviour. On holeboard tests EC females did show higher activity with respect to rearing but CC and CE females both showed less activity with regard to nose-poking. In the novel environment tests experimental females showed higher ambulation than

males in Env. 1, but all females had lower rearing scores than males in both environments. Control females showed more inactivity in the more novel environment, and experimental females paid more attention to the floor than males in both conditions. Overall then, it would seem that where sex differences exist, females are showing less activity than males. The discrepancy between this and the general trend could be due to one of four main factors. Firstly, the species and strain of the animal: mice show a much more varied response in terms of sex differences in emotional behaviour, and while in rats the results of e.g. open-field tests tend to show the typical sex difference or no difference, in mice the sex difference is sometimes reversed. Unfortunately no relevant data is available for "TO" mice. Secondly, the prenatal treatment as opposed to mothering (postnatal) may have affected the test outcome: it is worth noting that in the cases where sex differences existed that conformed to the expected "typical" sex difference, the groups affected were either FC (holeboard) or EE (novel environments). Thirdly, the females in these tests were prepubertal, but it may be that hormonal factors acting around this period influence a female's general predisposition for exploration and general activity. This is certainly true of adult females, when activity and exploration increase at oestrus (Birke, 1979) and, at least in rats, olfactory thresholds are reduced at oestrus (Petras & Moulton, 1974). It is possible that oestrogen induces a general increase in sensory acuity at oestrus, and hence the behaviour of near-pubertal mice, perhaps about to enter into first oestrus, may be altered as a consequence. Leading from this, the fourth factor is age - it may be that the "typical sex-difference" is only seen in adult animals, although available evidence suggests that the sex difference in defecation appears at puberty in rats (Broadhurst, 1957), and perhaps a bit earlier in mice (Candland &

Nagy, 1969). Activity and exploration may take longer to settle into adult patterns, though. Effects of prenatal stress can depend on mothering, and sex differences can be seen in this field too. Ader and Conklin (1963) found that effects of prenatal handling varied as a function of both sex and postnatal treatment (fostering). In their study experimental females showed shorter latencies in the open field only if raised by their own mothers whereas males only showed shorter latencies if raised by foster mothers. Thompson et al. (1962) reported that cross-fostering decreased activity in controls and increased activity in experimental animals, whereas fostering led to increased activity in both groups. Hockman (1961) also demonstrated decreased open-field activity only in animals reared by foster mothers. This data helps to support the theory that in this work the nature of the maternal environment may have different effects on the two sexes.

Overall then, despite the fact that the results are not always clear-cut a general picture can be pieced together from information derived from the various tests carried out. The effects of prenatal maternal crowding on emotional behaviour in "TO" mice would appear to be differentially mediated in males and females. In males the major influence seems to be postnatal, in terms of the postnatal maternal environment, while in females the strongest influence seems to be the prenatal maternal environment. Emotional responsiveness appears to be altered by prenatal stress, the result being an increased emotionality in males, and, on balance, a reduced emotionality in females. The finding of increased emotionality in males agrees with the results of Keeley (1962) who found greater latencies in emergence tests for animals that had been prenatally crowded, and indeed with the overall tendency (though there are certain exceptions) for prenatal stress to increase emotionality. Although the term "emotionality" has been used throughout this work, it should nevertheless be

remembered that it is merely a convenient blanket term to describe an animal's responses to novel situations. In interpreting two different parameters such as rearing and ambulation as measures of activity, one is making perhaps unjustified assumptions about the animal's motivations. Here the term "emotionality" has been used for consistency's sake, as an attempt was being made to relate this work to that of others, but the fact remains that various aspects of "emotionality" may have different causal mechanisms and that emotionality should still be regarded as Hall's "complex of factors", to whatever extent interrelated.

Chapter 5: Adrenocortical Function

This chapter examines the effects of prenatal maternal stress on offspring adrenocortical function (as reflected by plasma corticosterone levels), and uses evidence from three basic areas of research as a foundation for the experimental work presented here.

The adrenal cortex itself produces three classes of steroid hormones. The *zona glomerulosa* releases mineralocorticoids, which promote sodium retention and potassium excretion, in response to decreased sodium levels. The *zona fasiculata* produces glucocorticoids (in mice, principally corticosterone)which have a predominantly catabolic effect, providing energy for use in stressful situations. These glucocorticords are controlled by ACTH secretion from the anterior pituitary. So too are the adrenal sex hormones, which are produced in the *zona reticularis* and appear to act synergistically with gonadal sex hormones. The secretion of glucocorticoids and sex steriods is under the control of ACTH, but mineralocorticoids, although not entirely free of ACTH influence, are predominantly regulated by the renin-angiotensin system. ACTH secretion is stimulated by the hypothalamic peptide CRF, and inhibited by glucocorticoid feedback. This negative feedback which achieves the stable "basal" levels of circulating glucocorticoids is complex, and will not be reviewed in detail here. It is sufficient to note that it acts both at the level of the hypothalamus (on CRF) and of the pituitary (on ACTH): it is sensitive both to the absolute level of free steroid in the plasma, and to its rate of increase (Jones et al., 1972).

Superimposed on this steady basal secretion of corticosterone are two other systems influencing its plasma levels: a circadian rhythm and a "stress" response. The rhythm of steroid concentration

in the blood generally coincides with the activity of a species, and in rodents reaches its peak at, or just before, dusk. The sharp rise to this peak is followed by a slower fall to a trough level several hours later (Nichols & Chevins, 1981_b), but the rhythm can, and does easily entrain to other regular signals, such as regular presentation of food. The "stress" response can be elicited at any time of day, and by almost any form of disturbance, whether noxious or not. It was for a long time believed to be an all or nothing response, but Hennessy and Levine (1978) have shown that in mice it is possible to demonstrate a graded corticosterone response to mild novel stimuli. The response is fast, showing in plasma corticosterone levels in 2-3 minutes, and reaches a peak in 15-20 minutes (Levine & Treiman, 1969). These aspects of adrenocortical function - basal levels, rhythm amplitude, and the stress response - have been examined here, together with the onset of the diurnal rhythm, in relation to prenatal stress. In addition, because the corticosterone rhythm shows marked fluctuations in amplitude with stages in the oestrous cycle, a study of peak and trough levels at two points in this cycle was made.

The first of the areas on which this work is based is that of the association between the mother and her offspring, especially before birth, and the way in which the mother can influence the normal development of the foetal pituitary-adrenocortical system. The growth and differentiation of the foetal adrenal glands in the rat depend on the adrenocorticotrophic activity of the foetal pituitary (Milković & Milković, 1966), which begins between days 17 and 18 of gestation (Milković et al., 1973). The relationship between maternal and foetal pituitary-adrenocortical systems is a close one (eg. Zarrow et al., 1970). Milković et al. (1970) have demonstrated that high maternal adrenal activity may inhibit foetal ACTH, as a significant negative correlation was found to exist between maternal

and foetal adrenal weights. This negative correlation disappeared at maternal adrenal weights of 100mg/g body weight or more, when foetal adrenal weight remained constant at an atrophic level. Hence high maternal adrenal activity can influence offspring adrenal development and function. That this maternal effect can persist to adulthood has been demonstrated by Wood and Shire (1983) in *in vitro* studies of isolated adrenal cells: in reciprocal crosses of strains with high or low corticosterone production, mothers of a high-production strain produced offspring that had a low corticosterone output when adult, and vice versa. The maternal influence on adult corticosterone levels was extended to postnatal influences in a study by Denenberg et al. (1968), examining the effects of cross-species fostering in mice and rats on plasma corticosterone levels. Mice reared by mice had higher levels than rats reared by rats, but mice reared by rats had lower levels than the other groups. Mice were also less active in the open-field if reared by rats rather than by mice.

Another way in which the mother can influence her offspring's adrenal system is in the setting of the diurnal rhythm. This rhythm may be affected by both prenatal and postnatal maternal influences, as well as by external environmental factors such as the light:dark cycle and time of feeding. This has been to a large extent demonstrated in studies which have examined the effects of blinding the mother, the offspring or both on the development of and/or setting of the diurnal corticosterone rhythm. This approach has been taken because of the known importance of the light:dark cycle in entraining the rhythm: as already mentioned, in the absence of other significant *zeitgebers*, corticosterone levels in adult rats reach a peak just prior to the dark phase of the photoperiod, and thence fall, reaching a trough prior to or during the early part of the light phase (eg. Halberg et al., 1959). In immature rats a

corticosterone rhythm is first seen before weaning (Ramaley, 1972). The nature of the rhythm changes during the first few days, becoming adult in pattern by about day 26 (Ramaley, 1972) although some workers set this date to be as late as day 30-32 (eg. Allen & Kendall, 1966). The variation among different studies probably in part reflects differences in the sensitivities of assay techniques used. There may also be strain and species differences: Diez et al. (1976) found genetic variation between two mouse strains in the onset of the stress response. Levin et al. (1976) have shown that blind offspring reared by blind mothers do not normally show daily corticosterone periodicity, whereas blind pups reared by intact mothers do, as too do intact offspring, whether reared by blind or intact mothers. Takahashi et al. (1979) showed that in blinded rat pups the rhythm was not so much non-existent as free-running, although initially the phasing of the rhythm was similar to that of the mother and only started to shift by the 4th week of life. Mother rats can also affect the offspring's circadian phase if they show periodicity in nursing bouts. If a restricted suckling regime is imposed (on either blind or sighted pups) a corticosterone rhythm that peaks just before feeding time emerges (eg. Miyabo et al., 1980; Hiroshige et al., 1982). This effect is, however, only transient. That both the natural mother and the foster mother are important was demonstrated by Hiroshige et al. (1982): examining DL pups (those born of mothers under a reverse-lighting regime) reared by LD mothers (under a normal lighting regime), and vice versa, he found that in each case about half of the pups showed the phase angle of the natural mother, and half showed the phase angle of the foster mother. This suggests that the phase is in fact set prenatally but that it may be modifiable during early postnatal life. More recent work (Reppert & Schwartz, 1983) has confirmed that the "clock" is set

prenatally, as demonstrated by synchronous metabolic activity of the suprachiasmatic nuclei of both mother and foetus. The role of the SCN has been further demonstrated by Saito and Ibuka (1983), who have shown that rats with SCN lesions do not show the feeding rhythm seen in control-lesioned animals, although both groups ate similar amounts of food. However, adiurnal feeding schedules decreased food intake in both groups, so implying an endogenous time-keeping mechanism not necessarily incorporating the SCN. But in any case, the importance of the mother in setting the phase of the offspring's circadian rhythmicity has been established, as is the fact that there is potential for both prenatal and postnatal influences. Rhythmicity in maternal behaviour (eg. Grota & Ader, 1970) may influence the setting of the offspring's clock. So indeed may the attenuated maternal circadian rhythm of corticosteroids seen during lactation (Stern et al., 1973), especially if corticosteroids are transmitted in the mother's milk to the pups.

The second area of research on which the work presented here is based is that of the effects of early manipulations on aspects of adrenal activity. Ader (1969) demonstrated that early daily handling or electric shock could accelerate the development of an adrenal rhythm: in control rats the rhythm was established by 21-25 days, whereas in experimental animals it developed at as early as 16 days. Lorenz et al. (1974) examined the effects of postnatal ACTH treatment over three consecutive days starting on days 2, 7, 12, 17, 22 or 27. The corticosterone rhythm was suppressed in adult animals that had been injected on days 7-9 and 17-19. Days 7-9 correspond to the time when the hypothalamo-hypophyseal portal system is developing, and days 17-19 to the time when hypothalamic differentiation is complete and neural cell proliferation and differentiation is occuring in the forebrain. Hypothalamic and forebrain regions have both been

implicated in the central control of the circadian pituitary-adrenal rhythm in adult rats (cited by Lorenz et al., 1974), and so alterations in ACTH-mediated corticosteriod levels and/or ACTH itself may have been modifying the subsequent development of the pituitary-adrenal circadian rhythm at these periods.

Not all early treatments have the same effect on the onset of adrenal periodicity. Krieger (1974) demonstrated a delayed onset of the adrenal rhythm in rats given hydrocortisone at 2 days of age, although the corticosterone response to stress and exogenous ACTH was unimpared. Cost and Mann (1976) similarly demonstrated a delayed onset of corticosterone and progesterone rhythms in rats injected with either cortisol, dexamethasone or progesterone at 3 days. In both cases the delay was temporary. One main difference between these studies and those above seems to be in the timing of the neonatal treatment, being longer-term (and hence, possibly imposing a rhythm of sorts on the pups) in the studies where accelerated rhythm development was observed.

Various forms of nutritional deprivation have also been shown to influence adrenal activity. Adlard and Smart (1972) demonstrated that undernourished rats had higher plasma corticosterone levels than controls during the period of nutritional deprivation, and that although after a normal nutritional regime was restored at weaning plasma corticosterone levels between the two groups did not differ, the adrenocortical response to stress was permanently reduced. Wiener and Levine (1977) studied the effects of handling and malnourishment. Handled malnourished pups developed an earlier plasma corticosterone stress response. These workers found that although malnourished pups had elevated corticosterone levels compared to controls, post-stress levels were also higher, and so the stress response was not impaired. However, later work (Wiener et al., 1983) has indicated that the

ability to modulate the stress response (with a consummatory behaviour) is impaired. Although methodological differences may in part account for the different results obtained for stress responses, Shoemaker and Dallman (1972), examining malnourished offspring, reported a depression of the stress response in malnourished animals similar to that of Adlard and Smart (1972).

The final area to be discussed concerns the known effects of prenatal stress and the relationship between aspects of these effects and adrenal function. There are known effects of prenatal stress which might be caused by altered adrenocortical functions. Given that exposure to novel or stressful situations evokes pituitary-adrenal activation, one might expect that behavioural differences in emotionality tests would be reflected in the corticosterone response; although there are many who think that behavioural and adrenocortical reactivity are dissociated (eg. Grota & Ader, 1973; Stern et al., 1973). Roth and Katz (1979) have suggested that endogenous opiates are more associated with behavioural responses than is the hypothalamo-pituitary axis, but there is nevertheless some evidence for adrenocortical involvement (eg. Veldhuis et al., 1982). Puberty too may be affected by altered adrenocortical function: the presence of a rhythm is thought to be necessary for puberty to occur (Ramaley, 1973; Cost & Mann, 1976) and hence a delay (or acceleration) of adrenal rhythm develop may likewise delay or accelerate puberty. Altered adrenal responsivesness may influence pheromonal responsiveness, and adrenal rhythmicity may influence the ovarian cycle (Ramaley, 1975). Even retarded development may be related to hypoactivity of the pituitary-adrenal system prior to birth.

Some of the parameters measured in prenatal stress studies are also influenced by adrenal status. In some strains of rats, for example spontaneous hypertensive (SHR) compared with normotensive

controls (WKY), behavioural response to the open-field may depend on the time of day tested. Kraüchi et al. (1983) showed that SHR rats were less "emotional" than WKY rats in both light and dark phases, but habituation rate depended on the time of day: SHR rats showed higher baseline motor activity, higher motor responses and a greater corticosterone response to "mild" stress in the light phase only. Performance on passive avoidance tests is also influenced by adrenal status. Hypophysectomised male rats show less "fear" in a passive avoidance test than do intact males, and adrenalectomised males show more "fear" (Weiss et al., 1969). The author argued that this was probably because hypophysectomised animals lack ACTH (which they state leads to increased arousal or emotionality), whereas adrenalectomised males lack adrenal steroids to shut down excitatory effects. Behavioural responses to novelty may to some extent be mirrored in the corticosterone response to novelty. Bindra and Spinner (1958) demonstrated a graded behavioural response to different degrees of novelty in rats, while Hennessy and Levine (1978), and Misslin et al. (1982) have demonstrated graded corticosterone responses in mice to different degrees of novelty. Misslin et al. (1982) further demonstrated a higher corticosterone response in mice that were forced into exploring a novel environment than in those animals which were allowed to move freely between the home and novel environments.

Neonatal injections of cortisol, dexamethasone or progesterone not only desynchronise puberty events but also delay the onset of corticosterone and progesterone rhythms (Cost & Mann, 1976). Prenatal maternal treatment with corticosterone or ACTH have been shown to affect male sexual behaviour (Harvey & Chevins, 1984), although this effect could be interpreted in terms of altered adrenal status or in terms of prenatal stress. Female puberty and fertility is also

influenced by adrenal status. If animals are stressed (eg. Hagino, 1968) or injected with ACTH or corticosteroids (eg. Hagino et al., 1969) on a continuous basis, puberty is delayed and the ovulation response to PMS blocked. Daily stress (lead exposure, immobilisation or ether stress) for seven days has been shown to differentially affect females according to their maturity (Paris & Ramaley, 1972; Paris et al., 1973). Stress induced before puberty delayed puberty and reduced fertility once puberty was achieved. In old non-cycling females the heretofore absent daily corticosterone rhythm was reinstated as a result of the seven-day period of stress. In cycling adults, the rhythm inverted, bringing peak levels of corticosterone to coincide with the period of gonadotrophin secretion on the day of proestrous. In immature rats the rhythm was depressed or eliminated, causing a critical drop in serum corticosterone during the proestrous periods. Adrenalectomy does not abolish fertility in well-maintained female rats, but the timing of the ovulatory cycle may be slightly disrupted, resulting in longer oestrous cycles (Thoman et al., 1970).

Leading from the subject of reproductive functioning which is influenced by both prenatal stess (see Chapter 5) and adrenal status, is the subject of housing. Housing conditions are known to influence the reproductive status of both male and female rodents (see Chapter 5), and also influence the adrenal status of mice. Researchers have variously found that singly-housed animals have lower basal plasma corticosterone levels than group-housed animals (eg. Solem, 1966; Brain & Nowell, 1971_b, 1971_c), that single-housed animals have higher levels than animals housed in pairs (Schwartz et al., 1974), and that there are no differences in basal plasma corticosterone levels of singly and group-housed animals (eg. Champlin, 1969; Goldsmith et al., 1977; Misslin et al., 1982). Nichols (Doctoral thesis, 1980) demonstrated no effect of housing conditions on basal plasma

corticosterone levels in TO females, and it may be that strain differences account for the variability in results. Pheromonal influences on reproductive status may also be reflected in adrenal status. When male urine was applied to female bedding, in a study by Nichols and Chevins (1981_a) an increase in basal corticosterone levels was observed, and was greater in singly-housed females than group housed females exposed daily to male urine. The increase was also greater in singly-housed females than in females housed, either singly or in groups, with males.

Hence it has been demonstrated that the close relationship between mother and offspring, both prenatally and postnatally, may influence the development and nature of the offspring's adrenal activity and responsiveness. It has also been shown that several of the parameters measured in prenatal stress studies that are known to be affected by prenatal stress, are also influenced by adrenal status. Hence there is a possibility that some effects of prenatal stress may, at least in part, be caused by changes in the adrenal function of prenatally-treated animals. Ader and Deitchman (1970) have demonstrated that prenatal maternal handling can advance the development of the 24-hour rhythm of corticosterone (from the normal day 25 to day 18) in rat pups. The work presented in this chapter hence examines three aspects of the adrenal profile in control and prenatally-stressed animals. Firstly, basal levels of corticosterone at peak and trough periods have been examined in both males and females. A preliminary study in this laboratory (Grose, 1980) had indicated that the corticosterone rhythm amplitude may be reduced in prenatally-stressed animals. Because corticosterone levels in the female mouse vary according to the stage of the oestrous cycle, with significantly elevated levels at proestrus and oestrus (Nichols and Chevins, 1981_a), corticosterone levels in females were examined both

at proestrus and dioestrus. Secondly, the onset of the corticosterone rhythm was examined: this is known to be affected by both postnatal handling and prenatal maternal handling (Ader, 1969; Ader and Deitchman, 1970) but no known work has assessed whether prenatal stress has an influence. Thirdly, the corticosterone response to graded novelty was assessed: this was to some extent a repetition of the graded novelty described in Chapter 4, but does not include an assessment of the behavioural response. Grose's work (1980) had also indicated that the stress response may be amplified in prenatally-stressed subjects, so this work was also directed towards seeing if this effect could be confirmed. The aim of these studies was to assess the effects of prenatal stress on these aspects of adrenal function, and to determine whether these could be related to differences observed elsewhere in this work.

Materials and Methods

Except in Experiment 2, all animals used were adult TO males or females, aged 10-12 weeks and housed in groups of 8-10. The procedure for blood-sampling and analysis is given in Chapter 2. Blood-Sampling of peak corticosterone levels was carried out during the hour before the onset of the dark period (12.00-13.00 hrs). Trough sampling was carried out between 19.00-20.00 hrs: previous work (Nichols & Chevins, 1981_a) has demonstrated that from this time in the diurnal cycle plasma corticosterone levels remain more or less constant until they start to rise again at about 03.00 hrs. No animal was sampled more than once.

Experiment 1:

This experiment sought to determine whether the diurnal corticosterone rhythm was reduced (in amplitude) by prenatal stress, as indicated by previous work. It also sought to determine whether rhythm differences could be observed in females that might reflect differences in reproductive functioning. Males and females, aged 10-12 weeks, were sampled at peak and trough times of the diurnal rhythm. The oestrous cycles of the females were monitored according to the procedure given in Chapter 2, and females were sampled at proestrus or at dioestrus. No animal was sampled more than once. CC and EE offspring only were examined.

Experiment 2:

This experiment sought to determine the age of onset of the diurnal corticosterone rhythm. This date was said to be the one at which a significant difference between samples taken at peak and trough times was first seen. Two separate studies were carried out: The first of these (Expt. 2a) was a pilot study and examined CC and EE litters raised in small (30 x 13 x 11cm) cages. Blood samples were taken on days 21, 23, 25, 27 and 29. The second of these studies (Expt. 2b) examined the age of rhythm onset in CC, CE and EC litters. These litters were raised in large (42 x 25 x11cm) cages, and since Expt. 1b had indicated that the rhythm was already present in the EE animals at 21 days, assessed corticosterone levels on days 16, 18, 23 and 25 in an attempt to further track down the age of onset in experimental animals. The maternal influence was also examined in this second study. Males only were studied here.

Experiment 3:

In this experiment the corticosterone response of CC and EE males to three different degrees of novelty was examined. The first of the novel environments to which animals were exposed consisted of a large cage with clean bedding, but without food or water in the metal top of the cage. The second novel environment consisted of an empty large cage, without food, water, bedding or metal top. The third novel environment consisted of an inverted plastic litter tray measuring 40 x 25 cm, supported off the ground at each corner by metal food hoppers 16 cm high. Each "environment" was cleaned thoroughly with disinfectant between each use. Testing took place between 14.00 hrs and 16.00 hrs each day, in a room adjacent to that in which the animals were housed and maintained under the same temperature and lighting regimes. Different animals were tested in each of the three environments, so as to avoid possible effects of prior experience on the response. Each animal was placed in the designated environment and left for twenty minutes before being blood-sampled. This time period was employed to achieve a maximal stress response (after Levine & Treiman, 1969). Once all animals from one particular cage had been tested the cage was returned to the animal housing room. Animals within each cage were designated randomly to one of the three environments, so that each cage was represented in each environment. This aimed to control for possible housing effects.

Summary of Experiments

Expt. 1: Diurnal Corticosterone Rhythm:

CC, EE	♂ & ♀	Stressed days 12-16

Expt. 2: Age of onset of Rhythm:

a) CC, EE	♂	Stressed days 12-16
b) CC, CE, EC	♂	Stressed days 12-16

Expt. 3: Graded Novelty Response:

CC, EE	♂	Stressed days 12-16

Results

Experiment 1.

Table 5.1: Mean (±S.E.) basal corticosterone levels in adult males:

	Treatment Group	
	CC	EE
Peak	52.54 (±8.4) n=12	77.7 (±9.6) n=12
Trough	25.43 (±2.1) n=11	21.5 (±3.7) n=10

On Student's t-tests significant differences were obtained for peak vs. trough comparisons in both CC ($p<0.01$) and EE ($p<0.001$) groups. No significant differences existed between CC and EE groups.

Table 5.2: Mean (±S.E.) basal corticosterone levels in adult females:

	Treatment Group			
	CC		EE	
	Proestrus	Dioestrus	Proestrus	Dioestrus
Peak	161.04 (±23.7) n=5	144.1 (±22.5) n=8	143.61 (±18.9) n=8	151.67 (±24.1) n=9
Trough	93.69 (±15.5) n=9	64.08 (±22.7) n=6	82.43 (±23.9) n=8	68.66 (±15.6) n=7

On Student's t-tests significant peak vs. trough differences were seen in all cases ($p<0.05$, except EE dioestrus where $p<0.01$).

Experiment 2a.

Table 5.3.: Mean (±S.E.) basal corticosterone levels (ng/ml):

Treatment group		Age (days) 21 Peak	21 Trough	23 Peak	23 Trough	25 Peak	25 Trough	27 Peak	27 Trough	29 Peak	29 Trough
	CC	126.3 (±12.8) n=12	95.0 (±13.3) n=9	95.4 (±10.1) n=13	76.9 (±11.6) n=11	136.9 (±18.9) n=14	56.3 (±9.9) n=14	145.1 (±18.0) n=13	47.0 (±11.6) n=15	91.0 (±10.7) n=15	27.8 (±7.4) n=12
	EE	159.0 (±15.6) n=11	63.5 (±10.6) n=10	152.2 (±17.0) n=13	52.0 (±8.3) n=12	84.8 (±8.9) n=11	37.9 (±7.3) n=11	94.0 (±8.5) n=12	28.7 (±3.8) n=13	145.2 (±11.8) n=12	23.2 (±4.0) n=12

Data were analysed using Student's t-tests. The results were as follows:

Peak vs. trough differences:	Day 21: EE $p<0.001$
	Day 23: EE $p<0.001$
	Day 25: EE $p<0.001$; CC $p<0.001$
	Day 27: EE $p<0.001$; CC $p<0.002$
	Day 29: EE $p<0.001$; CC $p<0.001$
Intergroup differences:	Day 23 peak levels: CC<EE, $p<0.01$
	Day 25 peak levels: CC>EE, $p<0.01$
	Day 27 peak levels: CC>EE, $p<0.05$
	Day 29 peak levels: CC<EE, $p<0.01$

These results indicate that by the start of the experiment a corticosterone rhythm was already present in experimental animals, whereas in control animals the rhythm was not seen until day 25. Differences between the two groups only occurred in peak samples, perhaps indicating differences in rhythm amplitude.

Experiment 2b.

Table 5.4.: Mean (±S.E.) corticosterone levels:

		Treatment groups					
		CC		CE		EC	
		Peak	Trough	Peak	Trough	Peak	Trough
Age (days)	16	64.42 (±8.1) n=11	62.38 (±6.7) n=10	65.9 (±11.1) n=10	54.82 (±5.0) n=9	61.49 (±5.6) n=9	44.5 (±6.8) n=10
	18	87.74 (±14.6) n=10	62.96 (±6.7) n=8	89.54 (±14.8) n=14	50.08 (±7.1) n=11	89.88 (±14.2) n=9	83.85 (±11.7) n=8
	23	106.56 (±33.0) n=8	63.64 (±13.4) n=11	123.11 (±11.6) n=14	49.48 (±17.6) n=5	112.06 (±13.4) n=7	84.78 (±9.93) n=9
	25	98.67 (±13.5) n=8	61.17 (±16.8) n=9	93.48 (±14.7) n=5	62.35 (±13.8) n=8	109.24 (±21.6) n=9	75.72 (±10.1) n=13

On Student's t-tests peak vs. trough differences were observed on days 18, 23 and 25 in CE animals. No peak vs. trough differences were observed at any of the ages tested in CC or EC groups. Hence CE animals show an accelerated corticosterone rhythm onset in comparison to the other two groups. This only agrees with the earlier study in that experimental pups show an accelerated rhythmicity: it does not support the earlier finding of an established rhythm in CC animals by day 25.

Experiment 3.

Table 5.5.: Mean (±S.E.) corticosterone levels (ng/ml):

	Treatment	
	CC	EE
Environment 1	128.16 (±20.3)	135.3 (±13.3)
Environment 2	171.4 (±26.1)	179.9 (±37.0)
Environment 3	224.6 (±27.5)	212.2 (±20.8)
n	10	10

Data were analysed using Student's t-tests. No CC vs. EE differences were obtained. The only significant differences were between the corticosterone responses to Environments 1 and 3:

For CC, $p<0.02$

For EE, $p<0.01$

This indicates that under these conditions both groups show a differential response to degrees of novelty, although they do not differ in their responses per se.

Discussion

In this study three aspects of adrenal function were studied: the onset of the adrenal rhythm, the adult rhythm, and the corticosterone response to graded novelty (interpretable in terms of responses to mild stress). The first of these, adrenal rhythm onset, was found to be advanced in experimental litters. This was found to be the case in both of the studies carried out, and while the two housing conditions employed prevent the studies from being directly comparable, taken together they indicate that experimental pups start to show a diurnal rhythm of corticosterone secretion at 17 or 18 days of age, whereas in control animals this periodicity may start to be apparent at 25 days. The reason for the fact that a rhythm is present in control animals at 25 days in the first study, but is still absent at the same age in the second study, may simply reflect the fact that the age of rhythm onset is approximately 25 days and hence subject to some variation. Extending the age range examined in the second study could have confirmed this, but practical problems prevented this. What the combined results of the two studies do indicate, however, is that the earlier onset of the corticosterone rhythm is due to the treatment of the pup rather than to the treatment of the mother. This agrees with the work by Ader and Deitchman (1970) who showed that prenatal maternal handling accelerates the circadian rhythm onset in rats from 23 days to 18 days. Ader (1969) also showed that postnatal handling also accelerates the rhythm, from 25 days to 18 days. The effects of early hormone treatment appear to have an opposite effect: Krieger (1972), Cost and Mann (1976) and Turner and Taylor (1976) have all demonstrated a delay in rhythm onset as a result of postnatal corticosterone administration. The implication is that the prenatal and postnatal treatments act in different ways on circadian

periodicity. The hypothalamus, pituitary and adrenals all have independent rhythms which are synchronised, at least in the adult (eg. Szafarczyk et al., 1980_b). Prenatal treatments may act at a stage in development when synchronisation has not yet been initiated, and hence the only effect is an acceleration in rhythm onset. Administering corticosterone during postnatal life may interfere with the feedback links between hypothalamus, pituitary and adrenals, and hence temporarily disrupt the synchronisation process. This could then lead to delayed puberty: Ramaley (1974) suggests that synchronisation of rhythmic processes is necessary for puberty onset. This further suggests that the early handling effects described by Ader (1969) should be reinterpreted: it may be here that the daily handling procedure itself provides a rhythm to which other rhythms can entrain, at an earlier stage in development than would normally be the case. Undernutriton has been shown to retard rhythm onset by Macho et al. (1982): their study also demonstrated a phase shift in corticosterone periodicity (in control and experimental animals), moving gradually from a peak just after "lights-off" to a peak just before "lights-off". While this change occured over days 30-120, a trend was apparent in weanlings. It may that in the work presented here, the rhythm onset may be advanced in experimental animals, or else (or perhaps, as well) the phase shift is accelerated in these animals. If the phasing of the rhythm of control animals is dissimilar to that of experimentals the blood-sampling times (intended to assess corticosterone levels at peak and trough times) may be appropriate for one group but not the other. A more detailed study is needed here to assess the phase of the diurnal rhythm in control and experimental animals at different ages.

If prenatal stress effects are mediated by chronic stress, resulting in chronic corticosterone elevation during pregnancy, then

one would predict reduced pituitary-adrenocortical development in the offspring (after Milković et al., 1973). One might further predict that this would be shown either in low basal corticosterone (peak or trough) levels, or in lower stress responses. Neither effect is seen here. This could imply that crowding during gestation does not result in chronic elevation of corticosterone levels. Harvey (Doctoral thesis, in preparation) demonstrated an elevation of corticosterone levels only during the first 48hrs of crowding. Lau et al. (1971) have demonstrated that adrenalectomy and ACTH do not necessarily interfere with the development of the adrenal cortex of the offspring "at least in terms of its basal resting secretory function". Joffe (1978) points out that prenatal pituitary-adrenal activation could produce effects on mechanisms controlling systems other than the pituitary-adrenal system, and that, conversely, mechanisms controlling the pituitary-adrenal systems of offspring could be altered by prenatal changes in other hormonal systems. Furthermore, in the rat the foetal adrenal becomes functional at around day 16-17 of gestation (Michaud and Burton, 1977), while foetal ACTH action is initiated at around day 17-18 (Milković et al., 1973). As stress in the crowding procedure employed here is poorly controlled, it may be that the pregnant females are "coping" by the time these systems become functional, or at least by the time at which these systems become susceptible to exogenous corticosterone influences. Neonatal corticosterone treatment and handling can lead to reduced corticosterone levels, either in terms of basal levels or in terms of the stress response (eg. Nyakas and Endroczi, 1972; Grota, 1976; Erskine et al., 1981), and it is thought that this is due to reduced adrenal responsiveness to ACTH, and to reduced ACTH production. With regard to the lack of differences in the stress response here, it should be noted that Taylor et al. (1982), in examining the effects

of foetal ethanol exposure, demonstrated a larger stress response by experimental subjects to cardiac puncture, and to noise and shaking, but not to novel environments, cold or fasting. It may be that the "stress" applied here was too mild. The lack of differences in this stress response of control and prenatally stressed subjects may well indicate that emotionality differences (see Chapter 4) in prenatally stressed animals are unlikely to depend on corticosterone differences. Indeed, Stein et al. (1973) argue that open-field behaviour and pituitary-adrenal function are not causally related. However, adrenal responsiveness to ACTH, or ACTH output per se., may still be important.

Rhythm and stress responses of the adrenal cortex appear to have independently-developing mechanisms. The stress response matures far earlier - day 14 in the rat (eg. Guillet and Michaelson, 1978). Krieger (1972, 1974) and Turner and Taylor (1976) found that early postnatal corticosterone administration had varying effects on basal levels and on rhythms of corticosterone, depending on the time of administration.

Work by Nichols and Chevins (1981_a) indicated that peak plasma corticosterone levels are elevated at proestrus and oestrus in mice. This was not confirmed here, most probably because of the small sample size of proestrous controls at the peak period since there does appear to be a slight (though not significant) difference in the expected direction in this group. No such trend is apparent in experimental animals, and the difference, though again not significant, is in the opposite direction. The lack of proestrus-dioestrus differences is interesting and would repay further study. This would almost certainly relate to minor oestrous cycle changes in prenatally stressed females. Turner and Taylor (1976) related differences in oestrus cycling (caused by early

corticosterone treatment administered at different ages) to corticosterone levels. Animals treated early (day 3 or 6) in life showed normal puberty and had cycles showing some persistent oestrus, the day 6-treated animals also showing some long dioestrous periods. This Turner and Taylor related to lowered adult corticosterone levels. Animals treated later in life (day 12 or 18) showed advanced puberty and cycles with an increasing trend to long dioestrous periods. The authors related this to attenuated diurnal corticosterone fluctuations. A rhythm attenuation, in terms of the expected proestrous elevation, was seen here in experimental females (although the expected elevation missed significance in controls). Oestrogen may be the key to the (expected) increase in corticosterone at pro-oestrus. ACTH (Coyne and Kitay, 1969) and corticosterone (Kitay, 1963) are depressed by ovariectomy and restored by oestradiol-17-β in rats. Oestrogen induces increased TSH and thyroxine secretion, which in turn raise levels of corticosterone-binding globulin (CBG) (Gala and Westphal, 1966). As the corticosterone-ACTH feedback operates only on unbound corticosterone (Fortier et al., 1970) such a rise in CBG can lead to increased ACTH secretion, elevating plasma corticosterone. Oestradiol is high during the morning of proestrus in the 4-day cycling rat (Butcher et al., 1974), and TSH and thyroxine levels rise in the late morning and early afternoon (Buckingham et al., 1978; Brown-Grant et al., 1977). Hence, if prenatally-stressed females have lowered oestrogen levels compared with controls, they may show a reduced proestrous vs. dioestrous corticosterone difference. Altered oestrogen levels could also explain oestrous cycle differences in prenatally-stressed females. Politch and Herrenkohl (1984) have reported longer oestrous cycles in prenatally-stressed female mice, and effects on sexual receptivity have also been found (Allen and

Haggett, 1977; Politch and Herrenkohl, 1984). Effects have also been found in this study (see Chapter 6), which although being not totally clear and having a strong maternal component, indicate a reduced phermonal (and hence sexual) responsiveness in experimentally-treated females. Since the proestrous-dioestrous corticosterone level difference was not observed here (although admittedly there was only a trend towards this in controls), it may be that the proestrous oestrogen peak is reduced in experimental animals - this would help to explain the longer oestrous cycles (with higher incidence of dioestrous) sometimes seen in prenatally-stressed females. The fact that differences in oestrous cycles have not always been found (eg. Beckhardt and Ward, 1983) may be due to the high variability of the oestrous cycle in mice (Bingel and Schwartz, 1969): problems associated with examining female reproductive functioning are discussed in Chapter 6. The fact remains that an examination of oestrogen levels and, indeed, ACTH levels - is needed to clarify the effects on female reproductive functioning a point brought out by potential differences between control and experimental females in plasma corticosterone fluctuations during the oestrous cycle.

Hence this work has served to make four points. Firstly, that the development of adrenal rhythmicity is advanced by prenatal stress in (male) mice, but that this does not apparently affect the amplitude of the adult rhythm. Secondly, it is unlikely that behavioural differences between control and experimental animals are due to altered corticosterone responsiveness in prenatally-stressed subjects, since no differences were observed in the hormonal responses to novel environments. Hence there is no evidence here for reduced responsiveness and hence, as argued in Chapter 4, altered analgesia, in experimental animals. Finally, there is an indication that females do differ in terms of corticosterone rhythmicity, but

only with respect to the expected elevation at proestrus: this would appear to be primarily a function of altered oestrogen levels, though whether this is due to altered ovarian function, or to reduced pituitary activity (reducing LH, prolactin or even ACTH secretion) must await further research.

Chapter 6: Aspects of female reproductive functioning

There is a considerable body of evidence to support the theory that prenatal crowding, and other forms of prenatal stress, may affect the reproductive potential of the adult offspring. Much of the work carried out has been concerned with effects on males, and only limited research has been directed towards the effects of prenatal stress on females. This work will be discussed later in this section. Preliminary results from this laboratory (Harvey, Doctoral thesis, in preparation) have emphasised effects of prenatal crowding on female puberty, and other literature, on population regulation, has emphasised effects of high densities on reproduction in general.

The adverse effects of reproductive functioning of individuals in high-density populations have been mentioned in Chapter 1: high densities may lead to a reduction in the number of pregnancies (eg. Southwick, 1958), and inhibition to maturation and reproduction (eg. Chitty, 1952; Kalela, 1957). Housing density effects can also be demonstrated in the laboratory on mice. In males, group-housing may lead to a decline in circulating plasma gonadotrophin levels (Bronson, 1973), and also to impaired testicular function and spermatogenesis (Lloyd, 1971; Gartner et al., 1973). Group-housing female mice can result in altered oestrous cycles (eg. van der Lee & Boot, 1955) and delayed implantation, increased intrauterine mortality and poor lactation (Christian & Lemunyan, 1958). Crowding pregnant females has also been shown to increase abortion or resorption of embryos (Christian, 1959) and to reduce litter birth weights (eg. Harvey, 1984). This general decline in reproductive efficiency with increasing population size is seen to be adaptive in terms of population control, the various effects acting in combination to decrease reproduction or reproductive sucess within

the population, via cessation of reproduction in adults, increased mortality in the very young, and inhibition of maturation in juveniles. The eventual result is a decrease in population size. Another factor to be taken into consideration in this model for population regulation is the possibility that the crowding of females before and during pregnancy may itself prenatally affect aspects of reproductive functioning in the adult offspring, as distinct from changes in behaviour induced postnatally by the crowded environment. Before discussing the evidence for this, a brief summary of the aspects of reproduction in question is necessary: these include puberty, oestrous cyclicity, sexual behaviour, and responsiveness to pheromonal cues. More attention will be paid to the female in this respect, not so much to reflect differences in importance of the male and female roles as because sexual behaviour in males has already been investigated and discussed in this laboratory by Harvey (1984, Doctoral thesis, in preparation), and also because the emphasis in this work is on the female.

As puberty will be studied here, and in order to formulate possible hypotheses regarding the mode of action of prenatal stress, factors influencing puberty must be discussed. Puberty is a period of secondary sexual development that culminates in fertility and appropriate expression of sexual behaviour. Although this period in fact represents a sequence of events, indicators of puberty are hard to observe with any degree of precision in the living animal. In female rats and mice vaginal opening is taken as an indicator of this secondary sexual development, an event which is closely followed by first ovulation, usually taken to be assessable by vaginal smears. In males there is no outwardly visible sign of puberty other than testicular enlargement. These indicators of puberty are not seen until some time after weaning, with fertility occurring from around

five weeks in females and seven weeks in males, but it is thought that the process of secondary sexual development in fact begins much earlier, at about 15-20 days of age in both sexes (rats) with the onset of a mature rate of spermatogenesis and the onset of follicular development. To avoid confusion, I shall refer hereafter to puberty (in females) being the age at which vaginal opening occurs. The precise timing of puberty may vary between strains (Mandl & Zuckerman, 1952) so is presumably to some extent under genetic control. There can be a seasonal variation in the age at puberty, vaginal opening occuring approximately six days earlier in summer than in winter (Ramalay & Bunn, 1972). Animals housed under constant lighting conditions reach puberty earlier than do animals housed under a alternating light-dark cycle (eg. Ramalay & Bartosik, 1975). These effects of season and constant lighting apply to both intact and adrenalectomised females. Adrenalectomised females attain puberty at a slightly later date than intact females (Ramalay & Bunn, 1972; Gorski & Lawton, 1973), and the date of first oestrus may also be delayed (Meijs-Roelofs & Kramer, 1977). It has been suggested (Meijs-Roelofs & Kramer, 1977) that the effects of adrenalectomy on both gonadotrophin release and puberty onset are primarily due to effects on general body development (as characterised by body weight). However while direct correlations between puberty onset and body weight (as opposed to age) have been seen (eg. Kennedy & Mitra, 1963), the relationship does not always appear consistent (eg. Colby & Vandenberg, 1974). Crane et al. (1972) found that the relative age or weight dependency of vaginal opening depended in turn on whether mice were maintained on high or low energy diets, although Mandl and Zuckerman (1952) found no effect of a high protein diet on puberty onset in rats.

The presence of the adrenals is necessary for the development of

corticosterone and progesterone rhythms in the prepubertal rat (Ramalay & Bartosik, 1975; Cost & Mann, 1976), rhythm onset being delayed by a single neonatal injection of cortisol acetate, dexamethasone acetate or progesterone. Retardation of adrenal rhythmicity may alter the normal process of sexual maturation (Cost & Mann, 1976) in that females neonatally treated with cortisol acetate or dexamethasone acetate (but not progesterone) display desynchronised puberty events, vaginal opening being delayed and dissociated from first oestrous. In this study, neonatal cortisol also led to lengthened oestrous cycles in adulthood.

Ramaley (1979) has proposed a model for puberty onset based on the development of adult rhythmicity. Certain adult features of gonadal function can be demonstrated several days before puberty onset, and so puberty cannot be attributed directly to their maturation. These features include the generation of a LH surge in response to oestradiol (Puig-Duran & MacKinnon, 1978), the production of daily prolactin surges in response to cervical stimulation (Smith & Ramaley, 1978), and the production of a secondary FSH surge after PMS-induced ovulation (Sasamoto & Johke, 1975). Ramaley has observed that a population as a whole seems to set its biological clocks at the onset of the peripubertal period, indicated by peripheral corticosterone rhythms that bear adult phase relationships to photoperiod (1973, 1975). Ramaley argues that the emergence of these functional properties of the gonadal and adrenal axis depends neither on steroid feedback nor on photoperiod, but rather represents an unfolding endogenous program of events which can be followed by monitoring hormonal patterns and responses, none of which by themselves trigger puberty. She further suggests that the population synchrony that develops in the peripubertal period relates to the appearance of the capacity to link endogenous rhythms that already

exist to environmental cues, especially to the light cycle. Although light-dark periodicity is not essential for puberty onset in rats, it may enable a rhythm to be seen in the population as a whole that would otherwise be free running and out of synchrony in individual rats. This rhythm might initiate puberty as it sharpens and shifts in timing.

Various elements of the social environment are also known to influence sexual maturation. Female mice reach puberty sooner if they are housed with a male than if they are kept in an all-female group and solitary animals mature at an intermediate rate (eg. Vandenberg, 1967; Vandenberg et al., 1972). This effect, known as the "Vandenberg effect", can be mimicked using male urine rather than male pesence. Cowley and Wise (1972) showed that exposure of female mice to adult urine during the first three weeks of life accelerated sexual maturation, while, in contrast, exposure to urine of adult virgin females led to a retardation of sexual maturation. The effect may also operate after weaning: Colby and Vandenberg (1974) showed that exposure to male urine for three successive days between days 21-29 led to an acceleration of first oestrous by 4-6 days. The older the mouse at the time of exposure, the faster was its sexual development. Exposure to male urine also tended to shorten the interval between vaginal opening and first oestrous, indicating an acceleration of maturation rather than merely a "triggering" of puberty. Colby and Vandenberg (1974) further showed that urine from preputialectomised mice, adult male rats and adult male mice were all effective in accelerating puberty in this case, whereas urine from grouped females led to a retarded sexual maturation. Drickamer (1983) has further demonstrated that urine from dominant males has a greater accelerating effect on puberty than does urine from subordinate males, although grouping of males does not affect the urinary

chemosignal that accelerates female puberty, and neither does varying the daily source of male urine. Urine (or presence) of close male relatives neither accelerates nor delays puberty. Wilson et al. (1980), using uterine weight as a bioassay for puberty-acceleration by urine, demonstrated a dose-response pattern with a maximal response at a dilution of 10^{-2}, and no response at dilutions greater than 10^{-3}. Dilute urine was equally effective as the presence of an intact male in eliciting the uterine weight response. The vomeronasal organs are receptors for the chemosignals in male urine (Kaneko et al., 1980): olfactory bulbectomy leads to an abolition of the uterine weight response to males.

Chemosignals, or pheromones, are important in many aspects of reproductive physiology. This is certainly true of oestrous cycles. Laboratory mice and rats typically have regular four or five-day cycles, one day each spent in proestrous and oestrous, perhaps a day (or occasionally two) in metoestrous, and the rest in dioestrous. Two phenomena have been described in which social factors alter this cyclicity, and are known as the "Lee-Boot effect" and the "Whitten effect". Van der Lee and Boot (1955) found increased cycle length among female mice housed in groups of four. These long cycles "interrupted" short cycles, and the authors described this phenomenon as "spontaneous pseudopregnancy". Whitten (1959) increased the number of animals per cage while maintaining the same floor area per animal, and while he also found lengthened cycles the interval between ovulations was in this case a true dioestrous and not pseudopregnancy. Pseudopregnancy in mice was thought, perhaps erroneously (see later) to result from a depression of FSH secretion leading to an increase in prolactin: Whitten (1959) suggested that the effect of grouping seen in Lee and Boot's study was due to suppressed FSH secretion, and that by intensifying the social

conditions leading to this effect further depression of FSH might be expected and might lead to the anoestrous he observed. The "Whitten effect" consists of the tendency of group-housed female mice to display synchronised cycles when a male is introduced (Whitten, 1956). Short oestrous cycles may be induced by male urine instead of male presence, but this is less effective (Nichols & Chevins, 1981_c).

The relationship between housing effects on oestrous cyclicity and adrenal function has also been investigated. Brain and Nowell (1971) showed that isolation led to increased adrenal weight, with elevated pituitary-gonadal function. The increase in adrenal weight isolates in Brain and Nowell's work was not reflected in basal corticosterone levels, which were higher in grouped animals: no differences were seen in stress levels of corticosterone. Nichols and Chevins (1981_b), on the other hand, found no effects of housing on basal corticosterone levels. Nichols and Chevins (1981_b) showed that male urine applied daily to the bedding of singly-housed females led to an increase in basal corticosterone levels above that of group-housed females similarly treated, or that of females housed singly or in groups with males. A single application of male urine to the bedding led to a rise in corticosterone levels 30 minutes later, irrespective of housing conditions. Ramalay (1975) demonstrated that rats having no detectable diurnal corticosterone rhythm following ovariectomy and hormone replacement therapy tended to have irregular vaginal cycles, and she concluded that the presence of an adrenal rhythm was usually associated with regular cycles. Nichols and Chevins (1981_a) further showed that the circadian rhythm of corticosterone persists throughout the oestrous cycle, but that the levels are elevated at proestrous and oestrous. Dominguez et al., (1982) confirmed that there was a circadian rhythm of gonadotrophin secretion throughout the oestrous cycle, and furthermore that there

was a close relationship between that rhythm and the cholinergic system, in that the ability of atropine required to block ovulation has a circadian rhythm and the dosage required depends on the stage of the oestrous cycle.

A female's attractiveness towards males (in hamsters) also alters across the oestrous cycle (Steel, 1980), increasing just prior to oestrus - as does her proceptivity. Also in the hamster (Steel, 1979), receptivity increases throughout the day of oestrus, reaching a peak at about 5 hours after lights-off, and over this period the female is more likely to stay in proximity to the male.

Another well-defined phenomenon in which the female's reproductive state is affected by social factors is the "Bruce effect". This refers to the fact that either pregnancy or pseudopregnancy may be "blocked" at an early stage by replacing the stud male (the male to which the female had been mated) with a strange male, either of the same or a different strain. Females exposed to strange males within four days of mating with the stud male experience neither pregnancy nor pseudopregnancy, and return to oestrous within 3-4 days (Bruce, 1960). The incidence of the pregnancy block is not augmented by increasing the number of strange males to which the female is exposed, but is reduced by the presence of other females, in proportion to their numbers, (Bruce, 1963). The incidence of this effect may also depend to some extent on the strain of mouse: pregnancy was less easily disturbed among TO females exposed to males, either singly or in groups, than among P, CBA and Dutch strains (Bruce, 1963).

The influences of males on female puberty, oestrous cyclicity and pregnancy have until fairly recently been thought to be due to a variety of primer and releaser pheromones. It was thought that primer pheromones initiated a chain of physiological events in the

recipient, whereas releaser pheromones elicited a more or less immediate response which is reversible (eg. Whitten, 1966). These pheromones may serve as sex-attractants, or may suppress (or stimulate) reproductive function. The full scope of pheromonal action is of course far wider, and varies between species, but discussion is confined here to the subject in question - namely, female reproduction function. Recently (1983), Keverne proposed an alternative theory for pheromonal influences on endocrine regulation of reproduction. He showed that all pheromones are effective via the accessory olfactory system (which has receptors in the vomeronasal organ), and that all have a common neuroendocrine mechanism in that they lead to a decrease in prolactin secretion. Keverne argues that there is thus no need for several types of pheromone (eg. one to block pregnancy, one to suppress maturation, etc.), just a "complex" representing maleness. This "complex" would have to differ slightly in structure between strains however, in order to account for differential responses to animals of different strains or species. In addition, some form of olfactory imprinting would be necessary to prevent a stud male blocking pregnancy: Keverne suggests that this could be dependent on noradrenergic mechanisms, possibly activated by coitus. Anything that interfered with olfactory reception or prolactin levels might therefore be expected to alter pheromonal responsiveness.

It should perhaps be noted here that the term "pheromone" is nowadays considered a somewhat controversial one when one is discussing mammals. The term was originally used in terms of insect responses to chemical signals, and whereas insect responses rigidly follow a certain pattern mammalian responses are much more flexible. Mammals are able to "learn" about pheromones (eg. Keverne & de la Riva, 1982), to some extent at least, whereas insect responses would

appear to be more innate. It occurred to us during the course of study that many of the effects of prenatal stress on male mice (see next paragraph) which appeared to be demasculinisation might in fact be poor responsiveness to odour signals. This would explain altered aggressive and sexual behaviour, both odour-mediated patterns of behaviour in prenatally stressed males. This reduced responsiveness might also relate to the changed responsiveness in open-field and other emotionality tests.

Prenatal crowding has been shown by some (eg. Dahlöf, Hård & Larsson, 1977) to increase the incidence of lordosis in male rats ("feminisation"), and by others (eg. Harvey & Chevins, 1984) to reduce male sexual behaviour ("demasculinisation"). Other prenatal treatments have also demonstrated effects on males: prenatal heat and restraint can increase feminine receptivity quotients (Politch & Herrenkohl, 1984_a) in mice, and prenatal ACTH or corticosterone treatment can reduce masculine copulatory behaviour although not affecting feminine copulatory behaviour (Politch & Herrenkohl, 1984_b). Results tend to vary from one laboratory to another, some workers finding a demasculinisation and a feminisation of male rats as a result of prenatal treatment (eg. Ward, 1972), others finding either one or the other, but not both. Differences in results may however be due to differences in strains of animal, housing or stress procedures. What work there is on females shows similar inconsistencies in results. Allen and Haggett (1977) found that prenatal crowding led to reduced receptivity in female mice (following ovariectomy and hormone replacement therapy). Dahlöf et al. (1977), while demonstrating effects on male sexual behaviour in rats, failed to demonstrate differences in the oestrous cycles of prenatally-crowded females. Herrenkohl (1979), using prenatal heat and restraint, described several reproductive deficiencies in adult

female rats (oestrous cycle disorders, spontaneous abortions and vaginal haemorrhaging during pregnancy, stillbirths, neonatal mortality and low birth-weight young). Beckhardt and Ward (1982) found no effects of the same stress on ovarian cyclicity, sexual behaviour, pregnancy parturition, pup survival, or maternal behaviour in rats although male offspring showed both demasculinisation and feminisation of sexual behaviour. Politch and Herrenkohl (1984_a) demonstrated retarded vaginal opening, longer oestrous cycles and higher "median quality receptivity scores" in prenatally-stressed (heat/restraint) female mice, although no effects were seen on pregnancy, parturition or pup survival. They had earlier (1979) demonstrated a reduction of maternal aggression in rats by prenatally-stressed offspring. Differences in results could again be due to species/strain differences or stress procedures, or indeed to different fostering techniques. Some authors allowed prenatally-treated litters to be reared by their natural mothers (eg. Dahlöf et al., 1977; Beckhardt & Ward, 1982; Politch & Herrenkohl, 1984_a, 1984_b) while others either foster all litters to control dams (eg. Harvey & Chevins, 1984) or experimental dams (eg. Herrenkohl & Politch, 1978), or use a cross-fostering technique (eg. Allen & Haggett, 1977). Opinions differ as to the importance of the postnatal maternal environment: some (eg. Politch & Herrenkohl, 1984_a, 1984_b) state that it has no effect on the offspring's sexual development, whilst others have found significant maternal effects - Allen and Haggett (1977) found that fostering and cross-fostering procedures increased the frequency of abnormal scrotal sacs, and also influenced both male and female sexual behaviour.

Hence, while the results of previous work are somewhat confused, there is evidence that prenatal stress can affect female sexual behaviour, and also that postnatal mothering may play a role in this

respect. There is other, if oblique, evidence to indicate that maternal behaviour might affect puberty in that studies of infantile handling almost certainly have effects via maternal attention towards the offspring following manipulation (eg. Morton et al., 1963).

To summarise, three major factors are known to influence puberty - body weight (i.e. physical development), pituitary-gonadal function, and adrenal rhythms. Prenatal stress effects on sexuality have been demonstrated in some cases (principally in mice rather than in rat studies), and there is scope for maternal mediation of these effects. One aim of this study was to try and determine the relative importance of the three factors mentioned above.

Since preliminary experiments in this laboratory (Harvey, Doctoral thesis, in preparation), have indicated that prenatal crowding may retard puberty in female mice, one aim of this study was to see whether this effect could be confirmed, and if so, whether pre- or postnatal influences could be distinguished. Another aim was to examine the possibility that the female's pheromonal responsiveness was diminished or in any way altered by prenatal stress, and hence housing effects on puberty (the "Vandenberg effect") and on oestrous cycles (the "Lee-Boot effect") were assessed. In addition a comparison was made of receptivity in control and prenatally stressed females.

Materials and Methods

Experiment 1:

This experiment examined the age and weight at puberty of CC, CE, EE and EC females. These groups were further separated into singly-housed and group-housed animals. The animals were weighed at weaning, and then transferred to one of the two housing conditions.

In the group-housing conditions there were ten animals in each cage: no litter was represented more than once in each housing condition. Animals were examined daily for vaginal opening: on the second consecutive day this was seen (sometimes the aperture may close on the day following that on which opening is first observed) the animal was weighed and a vaginal smear was taken. Smears were thereafter taken and examined daily. All examinations and smearing were carried out between 09.00 and 10.00 hrs. On the first day an oestrous smear was observed the animal was again weighed. Birth weights are included in the results for comparison with weaning weights.

Experiment 2:

This experiment also examined the age and weight at puberty. In this case however, due to lack of housing space, only CC and EE animals were studied, and the effect of introducing a male to the group-housing condition was assessed. The procedure was as in Experiment 1, except that weaning weights were not recorded, and that there were three housing conditions - single-housing (SH), group-housing (GH), and group-housing with a male ($\sigma^{\prime}$GH). The male was housed in a small (30 x 13 x 11 cm) cage with a wire grid base that was placed on top of the cage housing the females: this permitted animals to see and smell each other but prevented most physical contact.

Experiment 3:

This experiment examined the effects of housing conditions on the oestrous cycle in CC, CE, EE, and EC females. The females had been housed either singly or in groups of twelve from weaning, and testing commenced at 7-10 weeds of age. Vaginal smears were taken daily in

three conditions. Animals were initially housed in a room containing males (RM), and were smeared for 13 days before being transferred to another room where no males were present (ROM). After a "break" of 10 days, smearing was continued for 15 or 20 days (singly and group-housed animals respectively). Male bedding was then added to all cages daily (RMB) and after seven days smearing recommenced, continuing for a further 15 or 20 days. Smearing was performed between 09.00 and 12.00 hrs. Smears were examined for incidence of each of the four stages, for cycle length, and for the incidence of two or more consecutive oestrous days or five or more consecutive dioestrous days.

Experiment 4:

This experiment examined receptivity of CC and EE females. Males used in these tests were singly-housed sexually competent animals with prior mating experience. Females used were aged approximately ten weeks, were sexually naive, and had been in proestrus on the morning of the day of testing. Testing took place between 14.00 and 15.30 hrs (lights-off at 13.00 hrs). The male to be used was placed in a large (42 x 25 x 11 cm) cage for three minutes before introducing the female, this serving to reduce exploratory behaviour in the presence of the female. Each pair of animals was then observed over a 90-minute period, and latencies of the male to mount, intromit and ejaculate were recorded, in addition to the frequencies of these behaviours. Since lordosis was not often observed, intromission was taken as an indicator of receptivity instead.

All experimental animals had been maternally crowded over days 12-17 of gestation.

Summary of Experiments

Expt. 1: Age and Weight at Puberty:

CC, CE, EE, EC - Singly-housed

Group-housed

Expt. 2: Age and Weight at Puberty:

CC, EE - Singly-housed

Group-housed

Group-housed with a male

Expt. 3: Oestrous Cycles:

CC, CE, EE, EC - Singly- or Group-housed,

a) In the presence of males

b) In the absence of males

c) In the absence of males,but with presence of male bedding.

Expt. 4: Receptivity:

CC, EE - Singly-housed

All animals had been maternally stressed over days 12-17.

Results

Experiment 1.

Table 6.1.: Mean (±S.E.) litter birth weights (g):

Control	Crowded	
1.64	1.49	Litter n = 17 in both groups
(±0.02)	(±0.03)	

On a Student's t-test the experimental group had significantly lower birth weights ($p < 0.001$).

Table 6.2.: Mean (±S.E.) litter weaning weights (females only):

CC	CE	EE	EC
12.74g	13.08g	12.66g	11.85g
(±0.5)	(±0.5)	(±0.3)	(±0.9)
n = 9	n = 9	n = 10	n = 8

No significant differences were seen between these groups on Student's t-tests.

Table 6.3.: Mean age and weight at vaginal opening and first oestrus (±S.E.).

		Age (days)		Weight (g)		n
		Vaginal opening	First oestrus	Vaginal opening	First oestrus	
SINGLY HOUSED	CC	29.7 (±0.9)	31.7 (±1.1)	22.6 (±0.5)	23.0 (±0.5)	10
	CE	27.8 (±0.6)	28.7 (±0.6)	20.6 (±0.5)	20.8 (±0.6)	12
	EE	27.8 (±0.4)	28.8 (±0.5)	20.1 (±0.5)	20.8 (±0.6)	13
	EC	31.7 (±1.4)	32.4 (±1.5)	21.5 (±0.8)	22.2 (±0.9)	10
GROUP HOUSED	CC	32.0 (±1.0)	33.0 (±0.9)	23.1 (±0.5)	23.1 (±0.5)	11
	CE	29.0 (±1.0)	31.6 (±1.0)	20.4 (±0.8)	22.1 (±0.5)	11
	EE	29.5 (±1.2)	30.8 (±1.4)	21.9 (±0.8)	22.6 (±0.7)	12
	EC	27.7 (±0.9)	30.5 (±1.4)	20.5 (±0.3)	21.9 (±0.5)	10

Those groups bracketed together showed significant differences on Student's t-tests. ($p < 0.05$; $^{*}p < 0.01$).

To summarise the results of Experiment 1, differences in age at vaginal opening were observed between CC and other groups, the group difference depending on housing conditions. Among singly-housed females, both CC and EC animals reached vaginal opening and first oestrus later than EE animals. Among group-housed females CC animals reached vaginal opening later than either CE or EC animals. No clear maternal or pup effect emerges from results on vaginal opening in group-housed conditions, but it is possible that a pup effect exists to some extent among singly-housed groups. Similar differences were observed in ages at first oestrus. Among singly-housed animals the CE/EE and EC/EE differences were again seen, but with the addition of an age difference between CC and CE groups, CC animals again being the eldest. Among group-housed animals only the age difference between CC and EC animals is repeated. The differences observed among singly-housed groups would seem to suggest that there is indeed a pup effect here, with experimental females generally being younger at puberty than control females. This again does not seem to hold for group-housed females, where the only difference observed appears to be maternally-based. The only consistency between housing conditions is that the experimental treatment tends to accelerate, comparatively speaking, puberty events.

Differences were also observed in weights at both vaginal opening and first oestrus, and these fairly closely followed age differences. Hence among singly-housed animals differences in weight at vaginal opening were observed between CC and EE groups , and also between CC and CE groups. In both cases CC animals were heavier. Exactly the same differences were seen in weights at first oestrus. Among group-housed animals weight differences were only observed at vaginal opening, and these mirrored the age differences: CC animals were heavier at vaginal opening than either CE or EC animals. Again, thereseems to be a pup

effect in singly-housed conditions, control animals being heavier, but no clearly-based maternal or pup effect when animals are group-housed.

Further analysis indicated that EC animals were older at vaginal opening if housed singly rather than in groups (Student's t-test, $p<0.05$). CE animals were both older and heavier at first oestrus when housed in groups ($p<0.05$).

Finally, the association between ages at vaginal opening and first oestrus was assessed, again using Student's t-tests. The age difference was found to be greater in CC than CE groups under single-housing conditions, and greater in EC than CC groups under group-housing conditions (both $p<0.05$). This again would suggest pup effects in singly-housed animals, and possible maternal effects among group-housed animals. Comparing singly-housed and group-housed animals, both CE and EC females show a greater dissociation of the two puberty events if group-housed rather than singly-housed ($p<0.05$).

Experiment 2:

Table 6.4.: Mean (±S.E.) age and weight at vaginal opening and first oestrus for females housed singly (SH), in groups (GH) or in groups with a male present (♂GH).

		Age (days)		Weight (g)		
		Vaginal opening	First oestrus	Vaginal opening	First oestrus	n
SH	C	39.13 (±2.3)	42.38 (±2.6)	23.16 (±0.8)	24.29 (±0.6)	8
	E	39.63 (±1.8)	44.13 (±3.4)	20.37 (±0.7)	21.74 (±0.6)	8
GH	C	36.67 (±1.2)	41.89 (±1.4)	21.89 (±0.6)	23.07 (±0.9)	9
	E	36.33 (±1.6)	42.56 (±2.4)	21.47 (±0.8)	23.65 (±0.7)	9
♂GH	C	36.00 (±0.9)	39.60 (±0.8)	22.08 (±0.5)	23.09 (±0.4)	10
	E	37.56 (±1.6)	42.11 (±1.1)	22.83 (±0.6)	24.50 (±0.6)	9

On Student's t-tests:

Singly-housed females: weight at vaginal opening: C>E, $p<0.05$.

weight at first oestrus: C>E, $p<0.02$.

Experimental females: weight at vaginal opening: ♂GH>SH, $p<0.05$.

weight at first oestrus: ♂GH>SH, $p<0.05$.

The results for singly-housed females confirm the results of Experiment 1, in that CC females are heavier at vaginal opening and at first oestrus than EE females, although the age differences are not seen here. No differences were seen among group-housed females. Comparison of housing conditions revealed a weight difference at both vaginal opening and first oestrus between experimental females housed singly and those housed in

groups with a male. The male's presence would seem to have increased body weight at puberty, although not accelerating it.

No differences were observed here in the degree of association between vaginal opening and first oestrus, either between groups within each housing condition, or within groups between housing conditions.

Experiment 3:

Table 6.5.: Mean (±S.E.) number of days spent by singly-housed females in each stage of the oestrous cycle over the testing period.

		Stage of cycle				
		Proestrus	Oestrus	Metoestrus	Dioestrus	Proestrus + oestrus
RM (13 days)	CC	2.2 (±0.3)	4.4 (±0.5)	4.8 (±0.4)	2.3 (±0.3)	5.9 (±0.5)
	CE	3.5 (±0.3)	4.1 (±0.3)	4.6 (±0.3)	2.5 (±0.3)	5.8 (±0.3)
	EE	2.4 (±0.3)	4.5 (±0.5)	4.9 (±0.3)	2.3 (±0.2)	5.9 (±0.3)
	EC	2.1 (±0.2)	4.1 (±0.5)	5.1 (±0.5)	2.3 (±0.5)	5.5 (±0.3)
ROM (15 days)	CC	2.9 (±0.2)	4.1 (±0.4)	6.0 (±0.3)	2.6 (±0.2)	6.5 (±0.3)
	CE	2.9 (±0.3)	3.7 (±0.4)	5.6 (±0.3)	3.4 (±0.4)	6.1 (±0.3)
	EE	2.4 (±0.2)	3.9 (±0.3)	5.9 (±0.2)	3.1 (±0.3)	6.4 (±0.2)
	EC	2.3 (±0.3)	3.1 (±0.5)	6.1 (±0.4)	4.1 (±0.4)	4.9 (±0.6)
RMB (15 days)	CC	3.0 (±0.2)	4.5 (±0.4)	5.7 (±0.2)	3.0 (±0.3)	6.5 (±0.4)
	CE	2.5 (±0.3)	3.8 (±0.4)	5.2 (±0.3)	3.8 (±0.3)	6.4 (±0.2)
	EE	2.5 (±0.2)	4.0 (±0.3)	5.7 (±0.3)	3.8 (±0.4)	5.9 (±0.2)
	EC	2.5 (±0.3)	3.7 (±0.7)	6.1 (±0.6)	3.6 (±0.7)	5.4 (±0.6)

CC n = 11
CE n = 11
EE n = 13
EC n = 11

N.B. Ambiguous proestrus/oestrus smears have been included in both Proestrus and Oestrus columns, but are counted only once in the Proestrus + Oestrus column.

Table 6.6.: Percentage of singly-housed females tending towards persistent oestrus (≥ 2 consecutive days) or dioestrus (≥ 5 consecutive days), and percentages showing short (≤5 days), extended (6-9 days) and long (≥10 days) oestrous cycles.

		≥2 days in oestrus	≥5 days in dioestrus	Cycle length ≤5 days	6-9 days	≥10 days
RM	CC	72.7	-	72.7	9.1	18.2
	CE	63.6	-	54.6	45.5	-
	EE	69.2	-	69.2	23.1	7.7
	EC	90.9	9.1	45.5	27.3	27.3
ROM	CC	54.6	-	36.4	63.6	-
	CE	27.3	9.1	90.9	-	9.1
	EE	38.5	-	69.2	30.8	-
	EC	45.5	-	45.5	36.4	18.2
RMB	CC	45.5	-	72.7	18.2	-
	CE	27.3	-	90.9	9.1	-
	EE	30.8	9.1	84.6	15.4	-
	EC	45.5	-	63.6	27.3	9.1

CC n = 11

CE n = 11

EE n = 13

EC n = 11

Table 6.7.: Mean (±S.E.) number of days spent by group-housed females in each stage of the oestrous cycle over the testing periods.

		Proestrus	Oestrus	Metoestrus	Dioestrus	Proestrus + oestrus
RM (13 days)	CC	1.8	3.3	5.3	3.2	4.5
		(±0.2)	(±0.4)	(±0.3)	(±0.6)	(±0.4)
	CE	1.8	3.0	4.8	4.0	4.2
		(±0.3)	(±0.4)	(±0.4)	(±0.8)	(±0.5)
	EE	1.5	3.3	4.5	3.8	4.6
		(±0.3)	(±0.4)	(±0.2)	(±0.5)	(±0.5)
	EC	1.1	2.9	4.2	4.9	3.9
		(±0.2)	(±0.8)	(±0.5)	(±1.1)	(±0.8)
ROM (20 days)	CC	2.2	3.1	5.6	9.8	4.8
		(±0.3)	(±0.2)	(±0.7)	(±0.9)	(±0.5)
	CE	2.0	3.2	5.6	9.7	4.8
		(±0.3)	(±0.4)	(±0.5)	(±0.7)	(±0.4)
	EE	1.8	2.9	6.9	8.7	4.5
		(±0.2)	(±0.4)	(±0.5)	(±0.7)	(±0.5)
	EC	1.8	3.2	5.4	10.1	4.5
		(±0.3)	(±0.4)	(±0.6)	(±0.8)	(±0.5)
RMB (20 days)	CC	2.9	3.3	5.8	8.8	5.5
		(±0.4)	(±0.5)	(±0.5)	(±0.9)	(±0.6)
	CE	1.8	2.8	5.9	10.0	4.1
		(±0.5)	(±0.5)	(±0.05)	(±1.12)	(±0.8)
	EE	1.9	2.8	5.9	9.9	4.2
		(±0.3)	(±0.6)	(±0.6)	(±1.0)	(±0.6)
	EC	2.3	2.9	5.1	10.7	4.3
		(±0.3)	(±0.5)	(±0.7)	(±1.1)	(±0.6)

n = 12 in all groups

N.B. Ambiguous proestrous/oestrous smears were included in both oestrus and proestrus columns, but are only counted once in the P+O column.

Table 6.8.: Percentage of group-housed females tending to persistent oestrus (≥2 consecutive days) or dioestrus (≥5 consecutive days), and percentages showing short (≤5 days), extended (6-9 days) or long (≥10 days) oestrous cycles.

	≥2 days in oestrus	≥5 days in dioestrus	Cycle length		
			≤5 days	6-9 days	≥10 days
RM	50.0	16.7	58.3	16.7	25.0
	33.3	16.7	66.7	-	33.3
	66.7	25.0	50.0	8.3	41.7
	41.7	41.7	33.3	-	66.7
ROM	-	75.0	25.0	-	75.0
	16.7	91.7	25.0	8.3	66.7
	-	75.0	25.0	16.7	58.3
	33.3	83.3	-	16.7	83.3
RMB	-	50.0	83.3	-	16.7
	8.3	66.7	50.0	-	50.0
	-	66.7	58.3	-	41.7
	-	83.3	66.7	8.3	25.0

n = 12 in all groups

Data in Tables 6.5 and 6.7 were analysed using Student's t-tests; data from Tables 6.6 and 6.8 were analysed using Chi-square contingency tables or Fisher's exact-probability tests.

The results were considered in terms of maternal and offspring treatment. Data were hence combined for offspring with similar prenatal treatment, or for offspring with similarly-treated mothers. Differences were observed as follows:

1. Maternal effects:

Group-housed females: RM: No. of days in proestrus: C>E, $p<0.05$.

Singly-housed females: RM: Incidence of 5^{+} days' dioestrus:
E C, $p=0.05$ (Fisher's test).

2. Pup effects:

Singly-housed females: ROM: Cycle length: C>E, $p=0.009$ (Fisher's test).

Individual groups were also compared:

Singly-housed females: ROM: No. of days in dioestrus: EC>EE, $p<0.05$

No. of days in proestrus + oestrus: CC>EC, $p<0.001$

No. of days in proestrus + oestrus: EE>EC, $p<0.02$

RM: Incidence of 5^{+} dioestrus: CC<EE, $p=0.02$ (Fisher's test)

Incidence of 5^{+} dioestrus: CE<EE, $p=0.02$ (Fisher's test)

Cycle length: CC<EC, $p=0.05$ (Fisher's test)

Group-housed females: RM: No. of days in proestrus: CC>EC, $p<0.05$

No. of days in metoestrus: CC>EC, $p<0.01$

No. of days in Metoestrus: CC>EE, $p<0.05$

Comparing the incidence of proestrus+oestrus and dioestrus between singly-housed and group-housed females, a greater incidence of proestrus + oestrus and a reduced incidence of dioestrus was seen under singly-housed conditions in all groups, with the following exceptions (where no differences existed):

RM: No. of days in dioestrus: CC, CE

ROM: No. of days in proestrus+oestrus: EC

RMB: No. of days in proestrus+oestrus: CC, EC

A chi-square analysis of cycle length reveaked that in nearly all groups cycles were longer when animals were group-housed rather than singly-housed (at least at the 5% level). The two exceptions to this, where cycle length did not significantly differ, were CC and EE groups when housed in a room with males (RM).

Effects of male absence were only observed in singly-housed animals, whereas effects of male presence were only observed in group-housed animals. Male bedding did not have any observable effect on cyclicity.

In singly-housed animals, both maternal and pup effects were seen, whereas among group-housed animals the effects would seem to have been influenced mostly by maternal treatment. Control animals had longer cycles, in part due to an increased incidence of dioestrus, in singly-housed conditions. In group-housed conditions the tendency was for control females to increase the number of days spent in proestrus, a "reproductive" phase of the cycle.

Experiment 4.

Table 6.9: Median scores (with 95% confidence limits) on receptivity tests:

	CC	EE
Latency to 1st mount (s)	723 (550-936)	764 (206-930)
Latency to 1st intromission (s)	5400^{+} ($1336-5400^{+}$)	2868 ($1181-5400^{+}$)
Number of mounts	19 (3-60)	34 (12-47)
Number of intromissions	0 (0-14)	2 (0-24)
n	9	9

No significant differences were seen between the two groups of females on Mann-Whitney 'U' tests. It is possible that the parameters measured are not really indicative of female attractiveness (latency to mount) or receptivity (latency to intromit).

N.B. "5400^{+}" indicates that the median value lay outside the test duration (= 5400 seconds). An animal that showed a latency of 5400^{+} seconds would thus have failed to display the behaviour in question by the end of the test.

Discussion

Effects of prenatal stress have been demonstrated here, both on puberty and on the ovarian cycle, although not on sexual behaviour. Hence these results do not confirm the findings of some researchers (eg. Dahlöf et al., 1977; Beckhardt & Ward, 1982) who report that prenatal stress has no efect on reproductive functioning. On the other hand, nor do they entirely support the findings of those researchers (eg. Allen & Haggett, 1977; Politch & Herrenkohl, 1984) who do report effects, typically effects of delayed puberty and reduced sexual receptivity.

The results from the puberty experiments did not confirm previous results from this laboratory (Harvey & Chevins, in press; Harvey, Doctoral thesis, in preparation) which demonstrated that prenatal stress delays puberty in female TO mice. However several variables may account for these differences. The preweaning environment differed: animals from the earlier studies were reared in small (30 x 13 x 11 cm) cages, while animals in this study were reared in large (42 x 25 x 11 cm) cages. The post-weaning groups differed in size: whereas in this study animals were either housed individually or in groups of 10, animals in the earlier studies were housed in intermediate-sized groups of 4 or 5. In addition different animal houses were used, and temperatures therein may have varied. Season is also thought to influence puberty onset (Ramalay & Bunn, 1972) and this may also have varied between studies. Season may also in part explain the disparity of the results given here for Experiments 1 and 2, since Experiment 1 was carried out during the summer, and Experiment 2 at the end of the year. The ages given for vaginal opening and first opening are very different in these two studies. While season may be a contributory factor it should also be

pointed out that the animals used in Experiment 2 appeared underdeveloped, though otherwise healthy, at weaning. Delayed physical growth may account for the later ages at puberty onset. This might in turn be due to environmental temperature (or other factors) during lactation, or even perhaps to lower body weight of the mothers at the start of the experiment.

One framework within which prenatal stress effects on reproductive functioning could be discussed is in terms of reduced responsiveness to odours or pheromones, or even in terms of a general reduced responsiveness to environmental stimuli. This could be considered in terms of olfactory responsiveness or general adrenal responsiveness. Prenatally-stressed females can be less sexually responsive (eg. Allen & Haggett, 1977), even though this effect was not seen here, and reduced olfactory responsiveness is known to reduce the increased uterine weight response in immature female mice to the presence of males (Kaneko et al., 1980). Hence reduced olfactory responsiveness could influence the typical pheromonal effects of housing density on puberty and ovarian cyclicity. It is possible that stress could interfere with early developmental stages of the olfactory system, although most of the olfactory bulb development occurs after birth (Rodier, 1980). On the other hand, reduced adrenal responsiveness could also influence olfactory responses, and is more susceptible to both prenatal and postnatal influences. Hence an altered general responsiveness due to altered adrenal activity seems a reasonable working hypothesis.

No real confirmation of this hypothesis is seen in the results on puberty in that one might expect differential responses in single and group housed conditions. This was only seen in EC animals at vaginal opening and in CE animals at first oestrous, both groups being older at puberty in group-housed conditions. The example of the

EC group is interesting in that differences between CC and EC groups only exist under group-housed conditions, implying that group-housing accelerates vaginal opening in EC females but not in CC females. It is possible that this is due to adrenal rhythmicity: adrenal rhythms at weaning may be established to differing degrees in these groups, due to altered mothering (see Chapter 5). It is possible that weak rhythms may be better enforced in grouped situations than when an animal is isolated and hence if rhymicity of adrenal function is better established at weaning in EC animals, this might account for differences in the effects of housing conditions on puberty among CC and EC animals. The expected trend is for animals to show delayed puberty in group-housing conditions, but this is only seen in CE females at first oestrous. In this comparison the situation is rather different: it is only in singly-housed conditions that CE females differ from CC females on this measure. Group-housing appears to suppress the acceleration over CC animals seen among singly-housed females.

As already stated, contrary to earlier results from this laboratory, the findings here indicate that the experimental treatment tends to accelerate puberty rather than delay it. This tends to apply, where differences occur, whether the treatment is of the mother or of the pup. This may again be due to differences in adrenal rhythmicity: data from Chapter 5 suggest that experimental pups develop an adrenal rhythmicity earlier than do control pups, and as there is evidence (eg. Ader, 1969) to indicate that early handling accelerates the maturation of circadian periodicity in corticosterone secretion, it is possible that altered behaviour of the mother towards her litter, or indeed altered periodicity of the same also influences rhythm development.

Another question to be considered is the degree of association

between vaginal opening and first oestrous. Cost and Mann (1976) demonstrated that early hormone treatment could desynchronise puberty events. Among singly housed animals in this work, a greater degree of dissociation was seen in CC than in CE females. Among group-housed animals the dissociation is greater in EC than CC females. The implications of these results are unclear, but they do imply separate determinants of the two puberty events. This is perhaps further supported in that sometimes differences were observed between specific groups on one of the two measures but not the other. This could be due to different oestrogen thresholds needed for the two events. Oestrogen levels in this context might be used to explain the greater dissociation of vaginal opening and first oestrous seen in Experiment 2 when compared to Experiment 1. If the animals in Experiment 2 were indeed developing more slowly it is possible that at vaginal opening the ovaries were producing only a smaller number of follicles, and hence less oestrogen, until the animals were further developed.

Body weights at puberty were measured with the aim of eliminating weight as a causal factor, and to try and establish the cause of group differences as being due to altered adrenal rhythm or pituitary-gonadal development. Differences were observed in weights at puberty that were consistent with the trend towards accelerated puberty in experimentally-treated females, eg. singly-housed CC females were both older and heavier at first oestrous than CE females. On only one occasion did a weight difference occur independently of an age difference. However, occasional (three) age differences were seen that had no corresponding weight differences. For example, singly-housed EC females were older than EE females at both vaginal opening and first oestrous, but no differences were seen in body weight at these events. Likewise, group-housed CC females

were older, but not heavier, than EC females at first oestrous. In these cases weight would seem to be a determinant of puberty, but the fact remains that most differences in age at puberty occured concurrently with weight differences. It is thus unlikely that physical development was the overiding determinant of puberty in these animals, although a minimal body weight must be required. This is supported by the results of Experiment 2, where although the ages at puberty were greater, presumably due to delayed development, the weights at puberty were similar to those seen in Experiment 1.

The results from Experiment 3 do give some support to the hypothesis of reduced responsiveness in experimental animals. Both maternal and pup effects were demonstrated, but maternal influences predominated. When housed in a room devoid of males effects were only observed among singly-housed females. In these conditions pup effects were strongest, with control pups having longer cycles due to increased evidence of dioestrous and reduced proestrous and oestrous periods. When housed in a room where males were present the effects were principally maternal in origin and were observed in both singly- and group-housed animals. In these cases animals reared by experimental dams showed a higher incidence of dioestrous and less proestrous. In other words, experimental animals had shorter cycles than did controls when males were absent, but tended towards longer cycles than did controls when males were present. This could imply a reduced responsiveness in experimentally treated animals. What is interesting here is the importance of maternal treatment over pup treatment. One could perhaps argue a case for reduced responsiveness at puberty within each housing condition. Taking the results from CC females to be the "norm", and then taking into account that where differences occur it is the experimentally-influenced animal that is, comparatively-speaking, accelerated, one could argue that these

experimental animals are less influenced by whatever delays puberty in their control counterparts.

However, a clear model of what controls puberty, and how environmental influences act at the physiological level, is lacking. It is hence hardly surprising that effects of prenatal stress on puberty cannot be clearly interpreted. Puberty is influenced by several factors, for example weight, adrenal rhythm, environmental factors and season; it is also not a clear, single event, but a continuous process whose true end-point - ovulation of fertile ova coupled with the ability to show appropriate sexual behaviour - cannot be measured in the living animal. Different parts of the process, including those observable in living animals (vaginal opening and first oestrous) may well be separately controlled and may therefore respond independently of one another to experimental manipulation.

Prenatal stress, interfering as it does with developmental processes that are themselves both diverse and complex (ranging from "simple" weight gain to mother-infant interactions), is also a multi-factorial phenomenon. With the benefit of hindsight it now seems obvious that no clear-cut effect on puberty could be produced. It does not even seem surprising that results are not always easily replicable, since so many of the processes which are interacting seem extremely sensitive to variations outside our control. Of these the most important may be the stress procedure itself, which while having the merit of being (comparatively) naturalistic, suffers from poor control of, for example, the behaviour of the crowded animals. A second important variable of which we lately became aware is the phenomenon described by Vom Saal (1980), Clemens et al. (1978), and others, known as intrauterine position. It now seems certain that amongst female offspring of rodents and other species with multiple

offspring there exist several phenotypes, dependent on intrauterine positioning between males and/or females. As we are here dealing with the development of female sexuality the experiment ought to have taken account of this phenomenon. However although we became aware of this part-way through the study, the addition of yet another layer of variables seemed an impractical experimental strategy to adopt and it was decided to proceed as originally planned.

Much the same lines of argument apply to the oestrous cycle of adult females and its variations. Although the oestrous cycle is better understood than puberty, and the mode of action of odour signals is at least partially understood, the intrauterine position phenomenon undoubtedly adds to the variance in the data, and may well be instrumental in obscuring some of the effects of prenatal stress.

Hence further experiments are needed in this area, with meticulous control of caging before and after weaning, and records of maternal as well as pup weights. In addition environmental variables such as temperature and season must be controlled for, as well as intrauterine position. Three major factors are known to affect puberty - weight, pituitary-gonadal function, and adrenal rhythms. Any one of these, if underdeveloped, seems capable of delaying puberty. Definitive experiments would still be difficult despite the above controls, and would have to further include measures of gonadal function (oestrogen production), pituitary-gonadal function (LH and FSH production) and adrenal function.

Chapter 7: General Discussion

The original aims of this project were given in Chapter 1. These included an examination of the effects of prenatal stress on female sexuality and an examination of effects on emotionality with a view to distinguishing between prenatally and postnatally (maternally) caused alterations. Another aim was to examine adrenocortical function in terms of rhythm development and in terms of mild stress responses as at the outset these factors were thought of as possible mediators of emotionality and sexuality differences. A final aim was to make a detailed study of maternal behaviour of prenatally stressed and control dams rearing both types of litter, to examine for differences that might later be related to differences between offspring.

Effects of prenatal stress were observed on both female sexuality and emotionality. The outcome of the work on female sexuality was somewhat confusing with both maternal and pup factors as influences, although there was a general indication that prenatally-treated animals were less responsive to pheromonal stimuli. Emotionality tests tended to support the generally-held view that prenatal stress increases emotionality in the adult male offspring. Emotionality in female offspring seemed to be decreased by prenatal stress however, and the relative importance of prenatal and postnatal factors in the outcome of the stress effects differed between males and females. No differences were observed in adrenal responsiveness to mild stress as a consequence of prenatal treatment, nor in basal corticosterone levels of females. Basal "peak" corticosterone levels of males were increased by the prenatal treatment, however, and corticosterone rhythm onset was advanced. Examination of maternal behaviour revealed no gross quantitative

differences in caretaking behaviour, but instead differences in the time courses of changing behaviour frequencies and hence changing rhythmicities.

Throughout the work three themes have provided frameworks within which to consider the causation of prenatal stress effects. These provide explanations in terms of (a) effects on maternal behaviour and mother-infant interactions, (b) effects on rhythmicity, and (c) altered sexual differentiation. While none of these themes can be independent of each other, I propose to discuss each separately before showing how they may interrelate.

The first of these three explanations is that the effects of prenatal stress are at least in part mediated by maternal behaviour, and that alterations in maternal care may occur as a result of altered developmental rates of the experimental litters. Data have been presented here which indicate that although experimental animals may be developmentally retarded (in terms of body weight) at birth, this difference is not apparent at 11 days of age or at weaning. Reflex development is also delayed in prenatally crowded pups (Harvey, Doctoral thesis, in preparation), and preliminary assessments of pup vocalisation frequency carried out here showed a similar retardation early in postnatal life. However, no differences were observed in ages at eye-opening. The general indication is that for a while at least experimental pups, initially physically retarded, actually develop faster than control pups, and thus "catch up". This alteration in the developmental rate of experimental litters may well influence maternal behaviour, in that the nature of the relationship between mother and offspring is based on a reciprocal responsiveness of one to cues from the other. In this work, delays in the physiological development of experimental pups are reflected in early tests of retrieval latency and in assessments

of nest quality during the first half of lactation. Other researchers have demonstrated that mothers respond differentially to pups that are underdeveloped due to prenatal maternal adrenalectomy (eg. Hennessy et al., 1978) or pup undernutrition (eg. Fleischer & Turkewitz, 1981).

It was also suggested (Chapter 3) that the effect of prenatal stress on the mother herself might affect her behaviour towards her offspring, and hence their physiological and/or behavioural development. That postnatal maternal factors can influence the offspring's behavioural development has already been demonstrated by several workers. Harlow (1962), in a series of experiments on maternal deprivation in rhesus monkeys, demonstrated the importance of the mother, or a surrogate substitute, in the normal social and sexual development of the offspring. Since then Ottinger et al., (1963) have shown that emotionality in rats is positively associated with the mother's level of emotionality (as rated prior to pregnancy), and using a series of cross-fostering studies further demonstrated that offspring emotionality can be independently related to both prenatal and postnatal emotionality of the mother. Southwick (1968) demonstrated that aggression in the normally passive A/J strain of mice may be significantly increased if the offspring are reared by females of the (comparatively aggressive) CFW strain. Cross breeding studies here also suggested strong maternal influences. Fostering mouse pups to rat "aunts" (Denenberg et al., 1969) leads to decreased open-field activity and a decreased corticosterone response to novel stimuli. Denenberg et al. argue that this is due to behavioural mechanisms involved in mother-young interactions rather than to biochemical differences between rat and mouse milk: the mouse mother was present in the cage with the rat "aunt" and the litter, and was the sole source of milk while the "aunt" performed other

caretaking activities. In the work presented here there is an indication that experimental mothers, as well as mothers rearing experimental litters, show some disruption of the normal behaviour patterns: when examining the overall time course of behaviour patterns (see Chapter 3) it was stated that for groups in which control mothers were rearing control litters the morning (light phase) was the most stable phase, whereas for groups with an "experimental element" it was the afternoon (dark) phase. Maternal effects were also demonstrated on frequency changes in the amount of time spent on the nest, nursing, and engaged in non-pup directed activity. Since pup effects were also seen, it must be argued that both pre- and postnatal maternal factors may be important, perhaps in different ways.

One way in which maternal behaviour can be thought of as influencing offspring development is in terms of infantile stimulation. The mother is a primary source of stimulation in early postnatal life, and the quantity and quality of her interactions with the litter will determine the form of stimulation received. Strictly speaking, effects of infantile stimulation can only really be shown if mothering is rigidly controlled - for example, by rearing without mothers. All existing evidence on this point must hence be a bit conjectural, but be that as it may, one should not ignore research in this area. Infantile stimulation, usually given in the form of handling or electric shock has been shown to have far-reaching effects on offspring development. Levine et al. (1967) demonstrated that rats handled in early life were more active in an open-field and defecated less, and also showed a lesser corticosterone response to open-field exposure than did non-handled controls. Here, early stimulation acted to reduce responsiveness to novel stimuli at both a physiological and behavioural level. DeNelsky and Denenberg (1967)

further showed that an increased degree of stimulus variation resulted in increased exploratory behaviour in handled subjects but decreased exploratory behaviour in non-handled subjects. Defecation was also higher in control animals. Levine (1957) examined the effects of handling or shock in infancy on consummatory behaviour, and found that whereas no prior differences existed, after 18 hrs water deprivation unmanipulated controls drank less than did shocked or handled animals. Levine argued that the increased upset experienced by control animals interfered with their normal responses. More recent work (Weiner et al., 1983) suggests that the ability to use a consummatory behaviour to modulate the adrenal response to novelty is also reduced by perinatal malnutrition. Sexual development may also be modified by stimulation in infancy (Morton et al., 1963). Among group-housed (though not singly-housed) females, vaginal opening has been shown to occur earlier in handled animals than non-handled controls. In males, weights of testes, prostates and seminal vesicles may be higher (at 35, 41 or 47 days) in handled animals. Earlier vaginal opening in experimental animals was demonstrated in the work presented in Chapter 5.

Early stimulation effects are not confined to rodents: Solkoff et al. (1969), examined the effects of extra stimulation (hourly five-minute stroking for ten days) on the development of low birth weight human infants. Stimulated infants were more active, regained their initial birth weights faster and were described as physically healthier in terms of growth and motor development than infants given standard nursing care. The general effects of infantile handling as outlined above, would seem to be to accelerate development, while at the same time modifying "emotional" responsiveness. This latter effect may in part be due to the accelerated development, particularly in the case of prenatal stress studies. If pups are born

underdeveloped and then show accelerated maturation, it may well be that some components of, for example, the hypothalamo-pituitary-adrenal system develops out of synchrony, so permanently affecting the animal's stress responses. Ward and Weisz (1980) demonstrated demasculinization and feminisation in prenatally-stressed male rats which they associated with a premature (in terms of CNS development) testosterone surge during gestation. Such desynchronisation of developmental events could operate at both prenatal and postnatal levels. No effects were demonstrated on stress responses, nor on basal corticosterone levels either in males or females. However an acceleration of the adrenal rhythm onset was demonstrated in experimental litters.

The second of the three themes suggests that the effects of prenatal stress are due to alterations in circadian rhythms, both hormonal and behavioural. Again, rhythms may be influenced both prenatally and postnatally by the mother. The biological clock may be set *in utero* (Reppert & Schwartz, 1983), as determined by synchronised maternal and foetal periodicity of SCN activity. Given this, the finding by Grota and Ader (1970) that peak corticosterone levels may be sustained throughout most of the dark period in handled mothers is of importance: the close relationship between maternal and foetal pituitary-adrenal systems indicates that the altered corticosterone periodicity of the mother will influence that of the foetus. Likewise any alteration in maternal behavioural periodicity is likely to influence the foetus. There is evidence to suggest that in man cortisol production is diminished in chronic fatigue, being produced during paradoxical sleep (A. Poteliakhoff, personal communication): should the same hold true for rodents, and then the increased stress and activity associated with crowding during gestation could result in a temporary alteration of maternal

corticosterone levels. Work by Harvey (Doctoral thesis, in preparation) has indicated an increased corticosterone production over the first 48 hours of crowding, followed by a decline to normal gestational levels on the third day. The final two days of the crowding period were not examined, however, and it is extremely likely that corticosterone levels will rise on the day prior to posturition when the female is unable to build a nest or prepare in any way for parturition undisturbed. In a number of ways, then, alterations in adrenal periodicity may occur. Rhythms in behaviour may also be altered during prenatal crowding by, for example, restricted access to food, and by constant disturbance within the cage from inter-male fighting and attempts to mount the females.

Adrenal rhythms may also be set postnatally. Both adults (eg. Krieger, 1979) and infants (eg. Miyabo et al., 1980) will entrain to feeding times if placed on a restricted feeding regime. That rhythmicity in maternal behaviour exists has been shown here, and also in work by Ader and Grota (1970): maternal feeding rhythms have also been demonstrated (Levin & Stern, 1975; Stern & Levin, 1975). Maternal feeding rhythms may be to some extent disrupted in early stages of lactation if access to food was restricted during the crowding phase of gestation. If nursing periodicity was to be altered in gestationally stressed females, this would provide another means by which early influences on clock-setting could be affected. However this entrainment by feeding regimes may not persist if the animal is allowed to feed *ad lib* (eg. Krieger, 1974, 1979; Miyabo et al., 1980; Hiroshige et al., 1982), and hence any differences in rhythmicity due to altered nursing periodicity may disappear at around weaning age. Altered rhythmic influences during early postnatal life might nevertheless still have an effect on the organisation and/or maturation of rhythmic processes in the offspring.

Changes in adrenal status have been shown to alter the onset of puberty not only in terms of vaginal opening and first oestrous (Ramalay, 1974), but also in terms of ovarian weight (Ramalay, 1973) and the number of ova shed at first ovulation (Gorski-Firlit & Lawton, 1974). The absence of regular adrenal rhythms has been associated with irregular ovarian cycles (Ramalay, 1975). In addition several neonatal treatments have been shown to affect both adrenal rhythmicity and puberty. Neonatal hydrocortisone or cortisol has been shown to delay the onset of circadian periodicity of corticosterone (Krieger, 1974; Cost & Mann, 1976), and to desynchronise puberty events (Cost & Mann, 1976). Hence endogenous corticosterone levels may be considered important in influencing later sexual development. The mother may also influence endogenous corticosterone levels in the neonate: corticosterone may pass into the infant through the mother's milk (Zarrow et al., 1970), albeit only in small quantities. Evidence (Denenberg, 1970) suggests that in fact the mother affects her offspring more by means of her behavioural interactions with them rather than through her milk supply, but if her adrenal rhythmicity was altered as a consequence of gestational stress then this could provide another means by which some influence could be had on the offspring. Offspring that are underdeveloped due to undernourishment (eg. Adlard & Smart, 1972) or malnourishment (eg. Wiener & Levine, 1977) have been shown to have higher basal corticosterone levels than normally-fed control offspring, at least prior to weaning. Undernourishment would appear to depress the stress response, malnourishment having no apparent effect in this particular study other than to accelerate the development of the stress response. Infantile handling has been shown to accelerate development of circadian periodicity in corticosterone secretion (Ader, 1969), and also to accelerate puberty (Morton et al., 1963) although the latter

study failed to control for inter-litter variation and so may be unreliable.

Support for this hypothesis that alterations in rhythmicity result in the observed effects of prenatal stress can be found among the results presented in this work. Maternal behaviour was shown to be affected by prenatal stress inasmuch as frequencies of the components of maternal care varied in their change over lactation and hence in rhythmicity over lactation. The corticosterone rhythm onset was brought forward in experimental animals in both studies of this phenomenon. Puberty was likewise accelerated in experimentally treated animals. However, there did not appear to be any differences between control and experimental animals in the corticosterone response to graded novelty. Neither were there differences in corticosterone rhythm between adult control and experimental females, either at proestrous or at dioestrous.

The third explanation to be considered here is that prenatal stress exerts its effects through alterations to the process of sexual differentiation. In many animals species-specific periods exist for sexual differentiation of the central nervous system, during which time exposure to sex hormones may irreversibly alter the animal's brain and subsequent sexual behaviour. The effects of early gonadal hormone secretions are diverse, and in rats include effects on activity, maze learning, active avoidance conditioning, circadian rhythms, reproductive behaviour and regulation of gonadotropin secretion (eg. Gorski, 1971; Gerall et al., 1972; Beatty & Beatty, 1970; Scouten et al., 1975; Stewart et al., 1975; ter Haar et al., 1974; Beatty, 1979: all cited in MacLusky & Naftolin, 1981). In mammals masculine CNS differentiation includes firstly the suppression of the behavioural and neuroendocrine patterns of the female ("defeminisation"), and secondly enhancement of the

characteristic patterns of the male ("masculinisation"). Sexually dimorphic areas of the brain have been identified, mainly in the preoptic area, amygdala, ventromedial hypothalamus and arcuate area. The differences exist in terms of neuronal nuclear and nucleolar size (eg. Pfaff, 1966; Dorner & Staudt, 1968), synaptic vesicles and terminals (eg. Ratner & Adamo, 1971), synaptic organisation (eg. Raisman & Field, 1973) and dendritic branching patterns (eg. Meyer et al., 1978). A major difference in the preoptic area is visible to the naked eye - the so-called dimorphic nucleus is five times larger in the male than in the female. The two key sex hormones involved in sexual differentiation are testosterone and oestradiol. Raisman and Field showed that when newborn rats are castrated during the critical period they develop a female pattern of synaptic connections, and when newborn females are given testosterone during the critical period, they develop a male pattern of synaptic connections. If males are castrated during the critical period their sexual dimorphic nuclei are much smaller, while those of females neonatally treated with testosterone increase in volume (Christiansen & Gorski, 1978). The secretory activity of the testes during late prenatal and early postnatal life is critical for masculine differentiation of both internal organs and external genitalia. The importance of oestrogen becomes apparent when one considers that when testosterone reaches the brain cells of newborn males it is metabolised to dihydrotestosterone and oestradiol (Ball & Knuppen, 1980). Early exposure to oestrogen affects mammalian feminine sexual development in much the same way as early exposure to testosterone: female rats perinatally treated with oestrogen show a pattern of anovulatory sterility in adulthood that closely resembles the pattern following early testosterone treatment. Dihydrotestosterone and other reduced androgens are far less effective than either oestradiol or

testosterone at inducing feminisation of the rat brain (eg. Whalen & Rezek, 1974). It follows that if oestrogen formation in the brain plays a vital role in sexual differentiation that the fetus must be protected from the effects of circulating oestrogen. In rats and mice the developing yolk sac and fetal liver synthesise an oestrogen-binding protein (fetoneonatal oestrogen binding protein, or FEBP) which circulates at high levels during the latter part of gestation and then gradually declines over the first three weeks of postnatal life (eg. Aussel et al., 1973). This protein does not bind testosterone, and hence testosterone is free to enter the brain where it can be converted to oestrogen and act at cellular oestrogen receptors in areas of the brain that are devoid of α-fetoprotein (a plasma α-globulin immunochemically indistinguishable from FEBP) - regions of the hypothalamus, preoptic area and amygdala.

Recently there has been some suggestion that oestrogens play a slightly different role in female sexual differentiation. Dohler and Gorski (1981) have demonstrated that postnatal treatment with tamoxifen (an oestrogen antagonist) can significantly reduce the size of the sexually dimorphic nucleus in both males and females. In female rats this treatment also reduced sexual receptivity without increasing male copulatory patterns, and all females developed permanent anovulatory sterility. Work by Toran-Allerand (1981) further suggests that the α-fetoprotein-oestrogen conjugate may be important in this respect. Further confirming work on this is, however, limited.

Effects of early treatment with sex hormones have been demonstrated not only on brain morphology but also on gender-specific behaviour. Male rats deprived of androgen prenatally, either by injection of cryptoterone acetate (an anti-androgen) or by neonatal castration, display less male copulatory behaviour and more feminine

lordotic behaviour than do normal males (eg. Grady et al., 1965; Gerall et al., 1967; Neumann et al., 1967; Ward, 1972). Female rats exposed to exogenous androgen during the critical period show male-like copulatory behaviour while female receptivity is partially or totally impaired (eg. Gerall & Ward, 1966; Gorski & Barraclough, 1961). Prenatal stress is also known to reduce male copulatory behaviour (eg. Ward, 1972; Masterpasqua et al., (1976) and Ward and Weisz (1980) have associated this phenomenon with altered plasma testosterone in foetal males. Ward and Weisz demonstrated that circulating testosterone levels in foetuses of stressed mothers were highest on day 17, declining on days 18 and 19, whereas testosterone levels in control foetuses increased from a fairly low level on day 17 to the highest amounts on days 18 and 19, and then declined. They suggested that stress increases circulating androgens (largely adrenal), causing a desynchronisation between CNS maturation and patterns of testosterone secretion by foetuses at a critical stage in development. Increasing circulating androgen levels would also facilitate "defeminisation" of female animals.

If altered sexual differentiation was the causal factor in prenatal stress effects, one would expect to see a reduction in sex differences, both in terms of behaviour and adrenal function, in experimental animals. No such erosion of sex differences was seen, either in the results of the behaviour tests discussed in Chapter 4, or in basal corticosterone levels as reported in Chapter 5.

However, differences were observed in puberty onset and oestrous cycles of prenatally-treated females. The accelerated puberty onset may well be related to the advance in adrenal rhythm onset seen in prenatally stressed males. That the effects on oestrous cycles may be due to alterations to the function of the pituitary-gonadal axis is indicated by the elimination of the proestrous elevation of plasma

corticosterone levels.

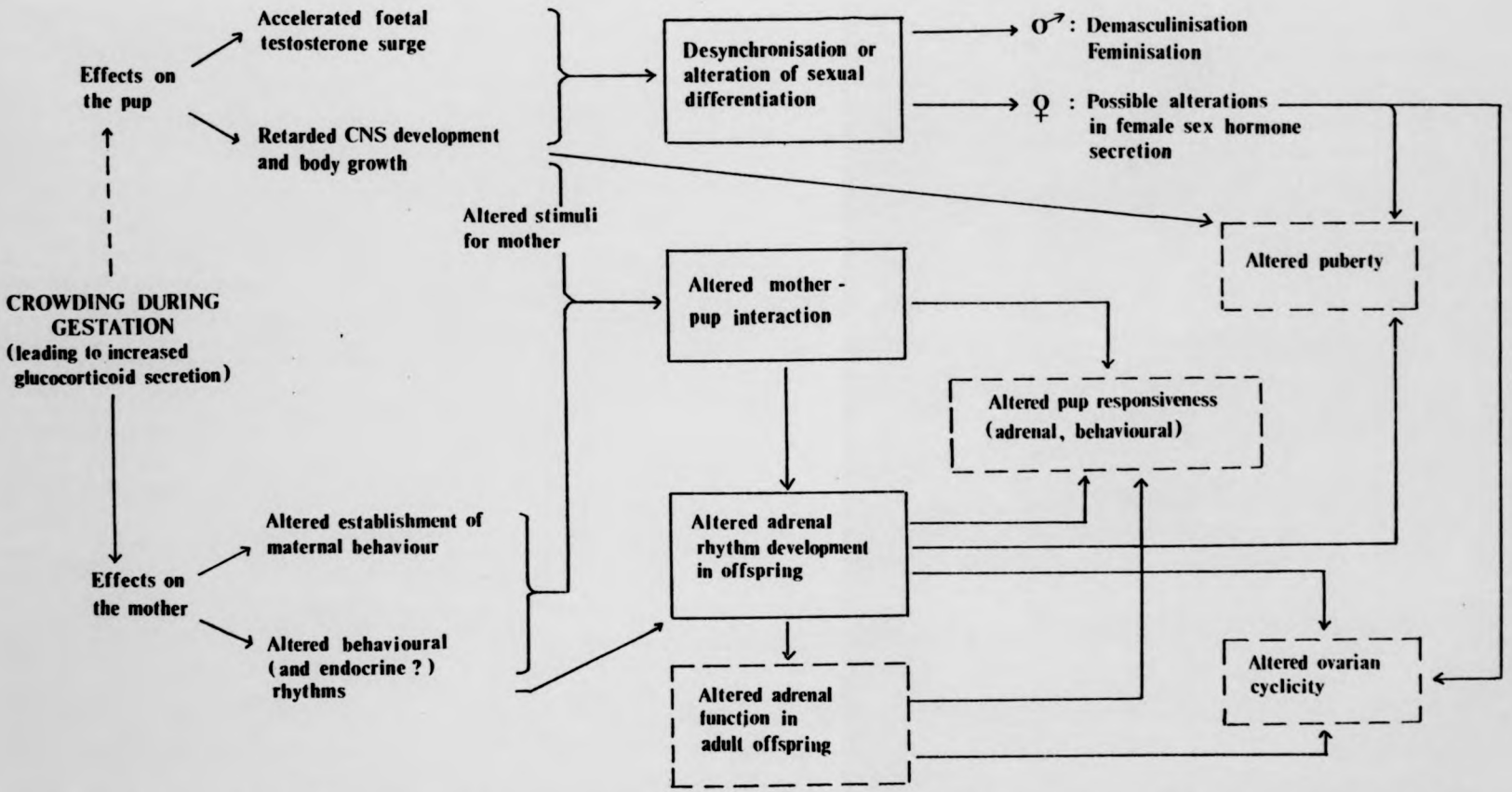
In the light of this summary it becomes clear that prenatal stress has no single, concise causal mechanism. It remains to discuss what can be said about its causation. The following represents a theoretical model for the mediation of prenatal stress effects, based on a complex interaction of events.

The effects of prenatal stress can be summarized as retarded development, altered emotionality, altered aspects of adrenocortical function, changes in the timing of puberty in females, and changes in both male and female sexuality. Since the development of an animal relies on a complex series of interlocking events following on each other (both in a temporal and causal sense) some of the "effects" given above are also causal factors of some of the other consequences of prenatal stress. Underlying these however, are two more fundamental factors, resulting from different components of the mother's responses to environmental stress - the endocrine response and the behavioural response. During the crowding phase of gestation, the pregnant female is likely to be harassed by males attempting to mount her, who furthermore constantly fight amongst themselves. She probably also has to compete with them for access to food. During the latter stages of gestation when she would normally be preparing for parturition - building a nest, etc., her normal activity and activity patterns are being disrupted. Her endocrine status may be altered by presumed increased "emotional upset", so influencing the normal establishment of maternal behaviour. The upset to behavioural rhythms, and indeed to the animal's emotional state "carry over" to influence the mother's behaviour in the early postpartal period. The endocrine stress response is usually considered in terms of the pituitary-adrenocortical response, but the effects of the other steroids should not be ignored. Neither should the possible

activation of the sympathetico-adrenal medullary system, which could decrease blood flow across the placenta to the uterus and so subject the foetuses to hypoxia. Whatever the endocrine response in detail, it appears capable of retarding CNS development and body growth, and at the same time leading to desynchronised sexual differentiation (as, for example, in the premature testosterone surge observed by Ward and Weisz in 1980). The altered maternal behaviour, compled with developmental alterations in the offspring, leads to altered mother-litter interactions. Altered maternal behaviour in terms of, for example, nursing periodicity, would influence the adrenal rhythm and its onset in the offspring, as too could altered mother-litter interaction. Both altered adrenal rhythm and altered mother-pup interactions would influence pup responsiveness: this altered responsiveness can be thought of in terms of emotional responsiveness and/or adrenal responsiveness to novel or stressful situations, or even in terms of olfactory responsiveness to pheromonal cues. Altered sexual differentiation also acts on responsiveness, in that sex differences exist in responses to novelty and to specific scent signals. In addition, interference with sexual differentiation processes, known to demasculinise and/or feminise male rodents, would influence puberty and ovarian cyclicity in females. This could be achieved through altered female sex hormone secretions resulting from desynchronised sexual differentiation processes, although this aspect was not studied here. On the other hand, alterations in puberty and ovarian cyclicity can result either from altered responsiveness (eg. to pheromones) or from altered adrenal rhythm and/or function. This theoretical model is summarised in Fig. 7.1.

To expect a complete clarification of the effects of prenatal stress effects and their mediation within the scope of this project is naive. Nevertheless, this work has established three important

Fig. 7.1: Theoretical model proposed for the mediation of prenatal stress effects.



"Boxes": Solid outlines distinguish major causation themes discussed in the text, while dashed outlines distinguish effects on the offspring examined in this work.

points for future research in this field. Firstly, it has indicated that maternal behaviour may indeed vary as a consequence of prenatal stress; not in the gross quantitative terms that others (Herrenkohl & Whitney, 1976) have considered but in more subtle ways that may well have a bearing on the offspring's development. These differences may result directly from the treatment itself, or else from an interaction between this and altered pup development. Since maternal influences are apparent on both offspring emotionality and female sexuality, the maternal variable should be taken into account in future work, especially where an explanation of the causation of prenatal stress effects is sought.

A second point made here is that given the number of factors that influence the development of the offspring and the complexity of their potential interaction, it is hardly surprising that studies of prenatal stress have to date produced such a conflicting body of results. This is especially true when, in addition to the factors just discussed, one adds variables such as species and strain differences, seasonal fluctuations and intra-uterine positioning effects. This point leads to the third: namely, that in all prenatal stress studies far stricter controls must be applied.

To conclude, a statement about the standing of prenatal stress studies, and the possibilities for future research, seems appropriate. The fact that prenatal stress retards early development is well established, as is the "demasculinisation" and/or "feminisation" in male offspring. Effects on emotionality tend to vary from one study to another (as detailed in Chapter 4), although the majority are in favour of an increased emotionality in prenatally stressed subjects. The work presented here tends to support this view. Up till now little work has been carried out on the effects of prenatal stress on adrenocortical function: this study shows for the

first time that the onset of the diurnal corticosterone rhythm can be accelerated by prenatal stress. To date, most information on adrenocortical function has been derived from other perinatal treatments (eg. early hormone treatment or malnutrition). A more extensive study is now indicated, both of rhythm onset and on rhythm amplitude and phasing. Other hormonal rhythms (eg. progesterone) could also usefully be studied when considering the role of circadian rhythms not only in activity levels, but also as influences on puberty and ovarian cyclicity. Examination of the diurnal corticosterone rhythm in females has also revealed that pituitary-gonadal function may be altered by prenatal stress: this, together with the fact that female puberty and oestrous cycles were altered by prenatal stress indicate that the area of female sexuality needs to be more extensively researched than heretofore. Future work should include studies of oestrogen, FSH and LH levels, as well as measures of attractivity, proceptivity and receptivity. An examination of the effects of intra-uterine positioning, not only on sexuality, but also on emotionality and adrenal function is also needed. In addition, given their possible role in the mediation of "emotional" behaviour (Roth & Katz, 1979), an assessment of endorphin and enkaphalin production is required. Finally, in support of the approach in this project, as wide an examination as possible of all known effects of prenatal stress should be made within individual strains of any given species. The cross- referral from one strain or even species to another must in some part contribute to the characteristic variation in the results of these studies. Only by building up a large body of information for individual strains, and then perhaps comparing the data as whole units rather than in terms of details thereof, can one hope to achieve a real understanding of the mediation of the effects of stress before birth.

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