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"Social Stress in Female Mice: Effects of Differential Housing on Adrenocortical Activity and the Oestrous Cycle."

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Thesis for degree of Ph.D.

TITLE "SOCIAL STRESS IN FEMALE MICE: EFFECTS OF DIFFERENTIAL HOUSING ON ADRENOCORTICAL ACTIVITY AND THE OESTROUS CYCLE."

I certify:

(i) That the greater portion of the work submitted in the above thesis has been done subsequent to my registration for the degree of Ph.D.

(ii) That the above thesis is my own account of my own research.

(iii) That no part of the work incorporated in the above thesis has previously been incorporated in a thesis submitted by me for a Higher Degree at any university.

Date 1st September 1980 Signature
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Abstract

The general aim of this study has been to examine some physiological and behavioural aspects of crowding in mice. The notion of stress has been central to this study. Using Selye's General Adaptation Syndrome hypothesis as a framework, modern ideas of stress have been re-examined. Social stress and population regulation in the house mouse have been reviewed. It has been concluded that social factors can regulate mouse populations and that it can be female reproductive productivity that is limiting. Little is known about the social behaviour of females and, in particular, social stress.

Plasma corticosterone levels in female mice were measured, using radioimmunoassay, as an indicator of stress. Basal plasma corticosterone levels were unaffected by housing density, social stability and some methodological procedures. Small differences in plasma corticosterone between grouped and individually housed mice were found when values of mice sampled at pro-oestrus were excluded. Pro-oestrus values were almost twice those at other stages of the oestrous cycle.

The circadian rhythm of plasma corticosterone was not different in grouped and individually housed female mice but stress response levels were higher in the grouped mice after blood sampling and ether but not after ether treatment only. It was concluded that whilst some differences in adrenocortical functioning occur between differentially housed
female mice these are not indicative of social stress. Further, the use of adrenocortical activity as an indicator of stress was examined critically.

It was proposed that the failure to replicate the results of "semi-natural" studies in laboratory experiments was due to either genetic strain differences or to the presence of males. Preliminary studies showed that male urine can activate the adrenal cortex in females.

The suppression of oestrus found in grouped females was due to pseudopregnancy and not anoestrus.
CHAPTER 1

Concept of Stress

It is an everyday belief that crowding animals has deleterious effects on health and that it is in some way "stressful". Similarly, overcrowding people is believed to generate emotional and physical imbalance resulting in social disruption as evidenced by the "abnormal" behaviour of people in cities (Milgram, 1970). With the ever increasing human population size and consequent increase in intensity of agriculture, the understanding of the nature and effects of crowding and overcrowding in both people and animals seems important. It is within this context that this study has been carried out. The general aim has been to study some physiological and behavioural aspects of crowding and overcrowding.

The term crowding is usually used rather loosely to mean a large number of animals or things massed together and overcrowding is too many people or things massed together. Both terms also imply a limitation of space. Clearly the notion of 'too many' implies that there must be a normal, natural number. With humans or animals it is not easy to state what are normal, natural numbers and on what criteria this is decided. It cannot be on a strict numerical basis since it is obvious that different species of animal have different requirements for space. The space requirement for any particular species will depend on a variety of factors. Amongst the higher vertebrates social organisation is a prominent factor.
There are two components of density; numbers and space. Because social organisation is important in determining the space requirement in higher vertebrates attention will be paid mostly to numbers of animals since this, largely determines density effects. It is however recognised that physical space is a component of density and may in some circumstances operate independently of numbers (Brittain, 1973).

Attempts to define what are normal, natural numbers of animals objectively must include some consideration of the selective forces operating. It is recognised that in human or "artificial" animal populations (laboratory or farm animals) survival of the species (or genes) may not be the prime selective force. Thus assessment of normal numbers and overcrowding of these populations is a subjective value judgement. The term grouping does not involve the emotiveness of the notion of overcrowding and can be considered as an assembly of animals (or people) which can be regarded as a collective unit.

House mice (*Mus musculus L*) have been chosen as an experimental model in this study for a variety of reasons. They are readily available, cheap, easily kept, easier to experiment with than primates (or humans) and are frequently used in similar studies so that there is now a considerable volume of literature on most aspects of mouse biology. Despite this knowledge there are substantial gaps in understanding of social behaviour and the physiology of behaviour in mice.

One of the central paradigms drawing together work
on overcrowding and grouping was a hypothesis formulated by Christian (1950) in which overcrowding led to social stress, reproductive failure and hence regulation of population numbers, (Chapter 2). This hypothesis was based on Selye's notion of a General Adaptation Syndrome (GAS), postulated first in 1936. This hypothesis stated that an animal, as a result of noxious stimuli responded in a both specific and general fashion. The general response was seen in three phases; an activation of adaptation mechanisms, notably the adrenal cortex, then a settling to a new level of adaptation if the stimulus persisted and finally an exhaustion of the "adaptation organs" leading to death. The adrenal cortex was considered the main organ of adaptation although it was recognised that the response was co-ordinated by the central nervous system (C.N.S.). It was possible to recognise the three phases of the G.A.S., alarm, resistance, and exhaustion by changes in the size and weight of the adrenal glands. Thus in the initial alarm stage the adrenal would increase in size, indicating an increase in corticosteroid output, then throughout the period of resistance the adrenal gland would remain in an enlarged state, indicating continued hyperactivity. Finally the adrenal would decrease in size as its secretory capacity was exhausted.

The adaptation response was seen as a result of a variety of different stimuli eg. trauma, burns, surgery, haemorrhage, temperature, X-rays, electric shock, emotional disturbance. Apart from the specific action of these stimuli (eg. local skin damage in the case of burns) there was a general activation of adaptation mechanisms. The stimuli were termed stressors and the response, stress. The degree of
stress was dependent on the severity of the stressors and the length of time they affected the animal. It was thus only through prolonged stress that the full G.A.S. would be shown.

The ability to adapt to various stressors was vital to an animal's survival. Without the organs of adaptation eg. adrenal glands, animals die. This could be prevented in adrenalectomised animals by the replacement therapy of corticosteroids. It was presumed that the corticosteroids aided adaptation, by means of a release of metabolic reserves, but that continued secretion of corticosteroids was maladaptive. The G.A.S. was seen as leading or giving rise to diseases of adaptation.

Since the crystallization of Selye's hypothesis in 1950 there has been considerable attention devoted to the concept of stress. An attempt is made here to identify some of the major strands in the understanding of stress. This has not been performed comprehensively.

The layman's meaning of stress is that of a person being under pressure psychologically and finding it difficult to cope with the situations faced. The results of stress are psychological breakdown and physical disorders such as stomach ulcers. This understanding of stress is however largely intuitive and it has been the attempt of biologists to define stress more objectively (Table 1). Some workers have defined stress with respect to the changes that occur in an organism eg. Selye (1950), whilst others, as the stimuli that elicit such changes eg. Coffey (1971). In this study the term stressor will be used to indicate the stimulus and stress as the changes. It should be noted however that it is difficult to identify those stimuli that are stressors (without
TABLE 1
DEFINITIONS OF STRESS

"Systemic stress"...."a condition in which, due to function or damage, extensive regions of the body deviate from their normal resting state".
"Stressor"...."any stimuli that causes stress".

( Selye, 1950 )

Stress...."the force producing or tending to produce deformation in a body, as distinct from strain which is deformation resulting from a stress".

( Lee, 1966 )

Stress...."any aversive stimulus for the animal".

( Coffey, 1971 )

Stress...."some divergence from the normal which through homeostatic mechanisms, will eventually be brought back to the original level".

( Davis, 1971 )

Stressor...."any input, external or internal, which is potentially capable of driving a variable, whose stability is essential to life, outside of its permissible bounds; stressors, in addition to eliciting specific compensatory responses in a homeostatic organism, also usually serve as inputs which increase ACTH secretion".

( Rodgers et al, 1974 )
TABLE 1 continued

Stress...."factors, usually considered noxious in some way, that activate the pituitary-adrenocortical axis and promote glucocorticoid secretion".

( Brain, 1975 )

Stress...."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 )
resorting to anthropomorphism) and this can only be achieved by measuring some physiological or behavioural variable in the animal that is deemed an indicator of stress, ie, a stressor is any stimulus that causes stress.

There is a general agreement that stress is a perturbation of normal homeostatic mechanisms of an animal. It is implicit in most definitions that physiological changes that occur in a stressed animal affect the body generally. It is also accepted that the physiological and behavioural changes that accompany stress are systems of adaptation that become inefficient or mal-adaptive. At present, it is not possible to measure stress directly but one can measure some physiological and behavioural consequences of stress. The net result of stressors are physical and psychological disorder and a lack of ability to cope with environmental demands. Some of the functional changes are characteristic of stress eg. adrenal activation. It does not necessarily follow that a change in one indicator system's activity implies stress.

What is meant by adaptation? "It implies an active change (in physiology and behaviour) initiated by contact with the environment. This change can result in the maintenance of a steady state at a higher or lower than normal level as caused by activation of adjustive responses or it can involve the adoption of a new pattern and/or intensity of bodily reactions conforming to the new demands", (Bajusz, 1969). We must again tackle the question of what is "normal". If an animal is placed in an abnormal environment, various mechanisms operate to adjust to the new setting, and the animal survives. There may come a point however where
this adjustment prevents successful reproduction, although the individual survives. In physiological terms the animal has adapted successfully. In evolutionary terms it is not successful, if it is assumed that natural selection operates on the individual.

In attempting to ascribe behaviour (or physiology) as adaptive or mal-adaptive, two separate theoretical viewpoints have been taken: cf. Daly (1973); Levine (1971). The ethologists argue that adaptiveness must refer to the particular species natural habitat whereas the behaviourists argue that adaptiveness refers to the setting to which the animals are exposed. For example, in explaining early handling effects of rats on subsequent behaviour the high "emotionality" and hesitancy of approach in an "open field" in non-handled rats is seen as mal-adaptive by the behaviourists, because it is inappropriate to a relatively innocuous situation like the "open field". However, these responses are seen as normal by the ethologists since in the wild a wariness of being preyed upon would be highly adaptive and that the "open field", which is a brightly lit novel arena, is a highly noxious stimulus to a small rodent. At the root of these differences lies the importance attached to either genetically fixed, species specific behaviour or environmentally determined behaviour characteristic of a variety of species. The function of adaptive behaviour and physiology in a natural setting is ultimately survival. There may be no function in an unnatural setting.

Selye's G.A.S. hypothesis emphasised physical factors as causing stress. More recently it has become clear that "emotional disturbance" may be a more important
component of stress than physical damage (Mason, 1968). Thus a physical stressor eg. burn, has a psychological component eg. fear, pain and that it is the latter which activates systems of adaptation. Stress is then dependent not only on a particular external stimulus but also on how that stimulus is perceived by the individual. This perception will depend upon an individual's past experience, psychological conditioning and upon any fixed inherent needs.

The sort of factors which can act as stressors include extreme sensory stimulation, pain, novelty, frustration and a variety of social stimuli which can all bring about emotional arousal and stress. In fact stress has been seen as a non-specific or general "emotional arousal". This generality of response is in accord with Selye's G.A.S.

One important aspect of stress is the notion of prolonged exposure to a stressor (Archer, 1979; Selye, 1950). An animal may become "emotionally aroused" due to some stimulus. This only becomes a damaging stress when the animal continues to be "emotionally aroused" and the normal homeostatic mechanisms are over-ridden by the continuing responses to the stressor. This distinguishes what is an adaptive process and what is a mal-adaptive one. The adaptive value of emotional arousal can be seen as the preparation for a "fight or flight" response. When an animal is faced with a situation that is perceived as a potential danger, systems are activated in preparation for coping with that danger. On the behavioural side these may be, for example, flight, fighting, "freezing" or submissive signalling as appropriate to the circumstance and species.
What are the physiological systems involved in this "emotional arousal"? At the outset it must be realised that the CNS plays a major role in co-ordinating the responses to a stressor. Since body functions are ultimately regulated by nervous and/or hormonal systems it is not surprising that the CNS plays a major role although this has frequently been forgotten in the enthusiasm for a particular physiological system.

Historically, the sympathetic nervous system was first seen as the physiological system accompanying stress (Cannon, 1929). Adrenaline and nor-adrenaline are secreted by the system, particularly the adrenal medulla, in response to emotional arousal, (Smith, 1973). The catecholamines have a variety of functions but specifically increase heart output, divert blood to the skeletal muscles from the viscera and increase blood glucose. The changes can be interpreted as a mobilization of resources for a "fight or flight" response.

With Selye's G.A.S. hypothesis attention was turned to the adrenal cortex as a physiological system of adaptation. The adrenal cortex secretes a variety of steroid hormones, the main ones being mineralocorticoids, which regulate mineral and water balance and the glucocorticoids. The glucocorticoids eg. corticosterone, cortisol have a variety of actions. In fact glucocorticoid receptors have been found in almost every tissue of the body that has been examined for their presence. The glucocorticoids affect carbohydrate, lipid and protein metabolism, generally increasing liver glycogen deposition and glucose production at the expense of protein production (gluconeogenesis), (eg. Schulster et al, 1976).

The conventional view of the function of the adrenal cortex is that it is the main organ of adaptation, perhaps operating in conjunction with the adrenal medulla. Overall, the peripheral actions of the hormones of the adrenal are seen as elevating blood glucose levels and acutely maintaining blood pressure, these actions are seen as crucial in mobilization of resources for coping with danger. Certainly if animals are adrenalectomised and given salt to restore the mineral balance they can survive, but only under "ideal" conditions. Any rapid change in these conditions leads to death since these animals are unadaptable to environmental conditions. The prolonged action of high levels of glucocorticoids is also detrimental to the health of the animal and the manifestations are similar to those encountered in stress. It is because the adrenal cortex is consistently activated when an animal is stressed (as judged by other criteria) and that the adrenal corticoids show adaptive and mal-adaptive actions that adrenocortical activity and stress have become almost synonymous. In the literature, the secretion of glucocorticoids from the adrenal cortex in response to any stimuli has been called the stress response.

The adrenocortical activity has consistently been used as a measurement of stress. However, definitions of stress as activation of the adrenal cortex (eg. Brain, 1975) whilst being satisfactorily specific either do not embody the notion
of adaptability or assume that this system is the main mechanism of adaptation. There is also the danger of circular reasoning: a stimulus is stressful if it activates the adrenal cortex and stimulation of the adrenal cortex is stress.

The level of adrenocortical activity has been measured in a variety of ways. Firstly there is the quantitative assessment of adrenocortical functions in terms of hyper- and hypo-secretion of hormones. Adrenal weight has been used most frequently in the past; the larger the adrenal the more active the secretion of hormone (Selye, 1950). More recently direct measurements of plasma concentrations of hormone have been used. These include measurements of basal levels, stress response levels and circadian rhythm phase. The basal levels are the normal, unstressed concentrations, usually the control values in an experiment used as a comparison for a treatment effect. Basal levels vary with a large variety of factors, including species, age and developmental stage, genetic strain and environmental factors. The stress response levels are measured after acute activation of the adrenal cortex by any stimulus, external or internal. Sometimes these levels are related to a known and usually standardised external stressor eg. electric shock, ether exposure. The time period for the maximum stress response in mice and rats is characteristically fifteen to sixty minutes. However, persistent stimulation can prolong the activation.

There is a characteristic daily variation in pituitary-adrenal activity which is expressed in glucocorticoid levels which approximately fit a cosinor wave pattern. It is possible to assess the phase of the wave pattern, in
terms of peak or trough or amplitudes etc, usually in relation to the light dark cycle of twenty four hours. There are rhythms in both basal and stress response levels. The timing of the rhythm can have effects independently of basal or stress response levels per se. Other quantitative methods of assessment have included adrenocortical histology using cell numbers and zone widths, in vitro glucocorticoid output and measurement of the pituitary or hypothalamic factors regulating adrenal activity.

In addition to quantitative output the qualitative output has been assessed in vivo and in vitro. The main glucocorticoid secretion is species specific; in rats and mice this is corticosterone whereas in man and dogs this is cortisol. Since cortisol and corticosterone have different physiological activities (or strength of activities) the ratio of these has been measured as a function of a stressor. Attention has also been paid to supplementary adrenal steroid output eg. adrenal androgens, progesterone, oestrogens.

Factors frequently ignored are the dynamics of glucocorticoid secretion, the metabolism and plasma binding. Measuring a plasma level of glucocorticoid at one time point conveys no information about the effects on any rate sensitive receptors. Measurement of glucocorticoids has, with few exceptions, been made of total plasma corticosterone. However, over ninety per cent of glucocorticoids in plasma are bound to a specific binding protein, corticosteroid-binding-globulin (CBG) and the physiological characteristics of bound and free corticoid are different.

With this array of different methods of measuring adrenal activity a serious problem in the relationship
between adrenocortical function and stress can be identified. What parameter or parameters of adrenocortical function accurately indicate stress (in terms of adaptive and maladaptive functioning)?

One novel approach to this whole problem has been to search for the design goal, for the adrenal cortex. As stated, the conventional supposed design goal has been viewed as the peripheral actions of glucocorticoids on metabolic function, mobilising energy reserves for a fight or flight reaction. This assumption has been challenged (Yates & Maran, 1974).

Peripheral metabolic effects of high doses of glucocorticoid usually require two to four hours to appear yet the stress response of the adrenal cortex, which can produce glucocorticoid levels twice or three times as high as the circadian peak of basal levels, operates within minutes of stimulation, reaches a peak within fifteen to sixty minutes and can decline to basal levels within four hours if the stimulation is removed. Further, it has never been convincingly proven that changes in glucocorticoid during stress responses have beneficial or significant effects on peripheral target tissues. All the peripheral metabolic actions of glucocorticoids can be explained by a permissive action of the hormone that requires their presence above a certain level but not a change in concentration. Whilst it is true that it is difficult to see the function of a single stress response in terms of peripheral metabolism, prolonged stress responses would have cumulative effects.

Yates and Maran proposed that the target organ for the stress response of the adrenocortical system was the brain
and that the design goal was the preservation of the stability of information processing and coping capacity of the brain. Thus under normal conditions when an animal can cope there is a "euphoric state" characterised by a "concordance between an ideal image of conditions defining a comfortable niche and the interpretation of a daily, averaged estimate from sensory inputs of the reality conditions provided by the environment". Stressors bring about a lack of concordance and a loss of ability to cope ("a dysphoric state"). The euphoric and dysphoric states correspond to levels of general emotional arousal. The emotion generating components of the brain (perhaps situated in the limbic region) are seen as the stress response targets of glucocorticoids. Glucocorticoid output during the stress response is then an attempt to restore the euphoric state.

These notions are based on the facts, (a) that glucocorticoids are consistently released in response to emotional disturbance (although not to any specific emotion and not sensitively to the intensity of emotion), (Smith, 1973), (b) glucocorticoids are taken up by various regions of the brain (Yates & Moran, 19742), (c) glucocorticoids can affect various behaviours (de Wied, 1974; Levine, 1968). Whilst much of the hypothesis is highly speculative and difficult to test experimentally it does illustrate the weaknesses of conventional explanations of adrenocortical function. Further it recognises and distinguishes between general adrenal activity and the stress response of adrenal activity.

In this discussion of the adrenal cortex no mention has yet been made of the regulation of adrenocortical
activity. Since this is reviewed frequently, (e.g. Dallman & Yates, 1968; Dallman & Jones, 1973; Yates & Maran, 1974; Krieger, 1979; Wilkinson et al., 1979; Ottenweller et al., 1978; Jones, 1978) it will not be dealt with in detail.

The adrenal glucocorticoid production is mediated by the hypothalamic-hypophyseal axis. Stimulation from higher brain centres causes release of a neurotransmitter, as yet uncharacterised, Corticotrophin Releasing Factor (CRF) from the hypothalamus. This passes to the anterior pituitary via vascular portal vessels causing the release of adrenocorticotropic hormone (ACTH). The released ACTH is carried by the blood circulation to the adrenal where it stimulates glucocorticoid production and release. Glucocorticoids can prevent ACTH release acting in a negative feedback system that can self-regulate normal levels. The site or sites of feedback receptors may be at the pituitary, hypothalamus and higher brain centres. During the stress response the negative feedback system is partly or wholly overridden.

Of importance are the findings that ACTH has extra adrenal effects on both physiology, (Berliner et al., 1969) and behaviour, (de Wied, 1974; Brain, 1978). A hypothesis has been generated, dissociating the effects of ACTH and glucocorticoids on some behaviour, (Weiss et al., 1970).

The hypotheses illustrated so far have dealt with the adaptive function of the adrenal cortex. They do not deal with maladaptive functioning brought about by prolonged exposure to danger. Selye, (1950) and others, (e.g. David et al., 1970) have documented some of the pathological effects of prolonged corticoid treatment and, although it must be
stated that most of Selye's work utilised deoxycorticosterone, a relatively minor adrenal product, the overall conclusions do not differ substantially. Some of the pathological effects include arteriosclerosis, increased blood pressure (with subsequent damage to the kidneys), peptic ulcers and reduced disease resistance.

An integral part of Selye's GAS hypothesis was the notion that an animal under stress ceases reproductive activity. Thus, apart from the effects of other pathological disorders, prolonged stress is maladaptive because reproductive activity is curtailed. The mechanisms for this cessation of gonadal activity was a supposed shift in anterior pituitary activity. Increased ACTH production leads to decreased gonadotrophin production, in order to mobilise energy for survival. The evidence in favour of the simplistic notion is overwhelmed by more modern studies, which indicate a much more complex relationship between the pituitary hormones, (see Chapter 2).

To conclude, there is substantial evidence that the adrenal gland (medulla and cortex) is important in adaptive physiology and that it is instrumental in maladaptive functioning brought about by prolonged activation (stress). Smith, (1973) wrote "as a respondent to emotional arousal, glucocorticoid levels are reliable, non-specific and insensitive". The hypothesis that emotional arousal is a respondent to glucocorticoid level (eg. stress response) is speculative and as yet unproven. The specific design goals of the different aspects of adrenal function eg. basal levels, stress response etc. remain elusive.

Some investigators have criticised the importance
placed upon the adrenal in adaptation. Mason, (1968) proposed that rather than a single endocrine response to stress there is an overall response of all the different endocrine glands i.e. thyroid, pituitary, gonads, adrenals. This conclusion was based on extensive data from experiments subjecting monkeys to avoidance learning for several days and measuring a wide variety of hormones throughout the procedure. Some hormone levels increased during the period of learning i.e. adrenaline, noradrenaline, glucocorticoids, growth hormone and thyroxine, all of which have a catabolic function in metabolism. The other hormones, insulin, oestrogen and testosterone which have an anabolic function, decreased.

However, subsequent work (Mason, 1975) indicated that the view of two groups of hormones with antagonistic actions operating during stress was simplistic and that with different stimuli, there was a different overall hormonal response. This notion is supported by many modern endocrinological studies which indicate that endocrine systems interact synergistically and antagonistically, (eg. Ogle & Kitay, 1979). However, it does tend to break down the long established view that stress is a general or unitary response, with some specific physiological changes brought about by non-specific stimuli.

To summarise: stress is a theoretical concept; it is not possible to measure it directly; it does not correspond to any one physiological system. Stress refers to the failure of normal physiological and psychological homeostatic systems to cope with environmental demands. The initial response to these environmental demands is adaptive; prolonged
exposure leads to maladaptation and pathological changes. An essential part of the need for adaptation is the psychological perception of stimuli. The response almost always involves a general emotional arousal. Both physical and psychological stimuli can activate the adaptation systems. The adaptation systems are regulated by the CNS. Current opinion still favours the adrenal (both medulla and cortex) as the main endocrine organ of adaptation. However, it is recognised that other hormone systems interact to modulate the effects of the adrenal. No distinction is made when considering adrenocortical activation between the different possible mechanisms of glucocorticoid action; level sensitive actions (basal levels), acute rapid changes in level or rate sensitive actions (stress response) and temporal actions (circadian rhythm phase).
Social Stress and Population Regulation

Christian's hypothesis concerning the regulation of animal populations was first formulated in 1950 to explain the cyclic fluctuations frequently found in rodent populations (Elton, 1942). It has been extended and developed since, (Christian, 1955₁, 1955₂, 1956, 1959, 1960, 1961, 1963₁, 1963₂, 1968, 1970, 1971₁, 1971₂, 1975; Christian & Davis, 1964; Christian, Lloyd and Davis, 1965). Briefly, the hypothesis said that as a population increased in numbers, social interactions between individuals increased and intensified leading to"social stress", the activation of the adrenal cortex and other organs of adaptation, increased mortality, dispersion and decreased reproduction. Thus behavioural and endocrine mechanisms were seen as instrumental in regulating the population numbers.

In its original form, the hypothesis was a logical extension of Selye's GAS hypothesis: excessive social interaction acting as a stressor inducing the GAS. The responses to social stress would be to escape from it (leading to dispersion perhaps to less favourable habitats), or eventually to the pathological symptoms of stress, including reproductive failure. Over the years the emphasis has shifted with respect to the specific mechanisms believed to be involved and it has become apparent that in different species there may be different social mechanisms. Christian's hypothesis does not suffer from the theoretical difficulty encountered in other notions of population regulation by social mechanisms (eg. Wynne-Edwards, 1962) of having to assume group selection.
The evidence for Christian's hypothesis comes from three types of study. Firstly there is the study of natural populations, where animals are trapped, counted and various physiological parameters measured eg. adrenal weight, reproductive condition. The physiological parameters are then correlated with the estimate of population density. For example at high density there may be large adrenal weights and small gonadal weights. A few studies have utilised the method of artificially manipulating the population size and measuring the physiological characteristics (eg. Christian and Davis, 1955).

The second approach has been to set up "semi-natural" populations in an enclosure. Usually a few animals are placed in an enclosure supplied with adequate food, water, bedding material etc and the population allowed to grow freely. At periodic intervals the population size is assessed and various parameters measured. In addition to physiological and reproductive measures, it is possible to record behavioural influences and follow individual animals success or failure.

The third method of study has been the purely laboratory technique of grouping animals together, recording the various parameters and comparing these with a control situation. Most of the studies investigating specific physiological mechanisms have involved laboratory experiments.

Evidence from these three approaches to the problem have collected together to support Christian's hypothesis. In view of the vast literature and the breadth of the hypothesis no positive or negative conclusions have been reached. The hypothesis is not amenable to
falsification and critical papers (e.g., Negus et al., 1961) have concentrated on specific aspects. Apart from Christian's papers the influence of population density on behaviour and physiology has been reviewed by others (Anderson, 1961; Bashenina, 1963; Calhoun, 1962; Thiessen, 1964; Snyder, 1968; Myers, 1966; Bronson, 1967; Archer, 1970; Brain, 1971; Valzelli, 1973; de Feudis, 1975; Brain, 1975; Brain and Benton, 1979).

Some studies on natural populations of a variety of different species have revealed correlations between population density and reproductive efficiency and an adrenal measure in the apparent absence of conventional limiting factors. Food, weather, predation, disease did not have a primary role in regulating numbers. These studies are summarised on Tables 1 and 2. At higher densities there was an increase in adrenal weight and a decline in reproduction. Whilst this does not unequivocally prove or disprove the idea of social stress regulating population size it lends some support to the notion that the effects observed in semi-natural and laboratory studies may operate in the wild. There is a growing feeling amongst ecologists that in small mammal populations at least, social behaviour can operate to regulate numbers (e.g., Krebs, 1978).

Attention will be paid mostly to the House Mouse (Mus musculus L.) because it is used in this study. House mice have a world wide distribution and are highly adaptable, flourishing in a wide diversity of habitats, including cold stores (Laurie, 1946), open fields (Linduska, 1950) heathland (Berry, 1968), grassland (Lidicker, 1965) salt marsh (Breakey, 1963), urban areas including warehouses,
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<th>SPECIES</th>
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<td>Dicrostony groenlandicus</td>
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<tr>
<td>Mice</td>
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<tr>
<td>Peromyscus maniculatus bairdi</td>
<td>(1) Inhibited reproduction</td>
<td>Christian, 1971</td>
</tr>
<tr>
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<td></td>
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<tr>
<td>Peromyscus leucopus</td>
<td>(2) Inhibited maturation</td>
<td></td>
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<tr>
<td>Mus musculus</td>
<td>(1) Number of pregnancies</td>
<td>Southwick, 1958</td>
</tr>
<tr>
<td></td>
<td>(2) Number of fetal resorptions</td>
<td>Evans, 1959</td>
</tr>
<tr>
<td>SPECIES</td>
<td>ADRENAL MEASURE</td>
<td>REFERENCE</td>
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<tr>
<td>Rats</td>
<td>Adrenal weight</td>
<td>Christian, 1959</td>
</tr>
<tr>
<td>Rattus norvegicus</td>
<td></td>
<td>Wodzicki and Roberts, 1960</td>
</tr>
<tr>
<td>Rabbits</td>
<td>&quot;</td>
<td></td>
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<tr>
<td>Oryctolagus cuniculus L.</td>
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<td></td>
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<tr>
<td>Voles</td>
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<tr>
<td>Microtus montanus</td>
<td>&quot;</td>
<td>Adams et al, 1959</td>
</tr>
<tr>
<td>Microtus pennsylvanicus</td>
<td>&quot;</td>
<td>Louch, 1956</td>
</tr>
<tr>
<td>Microtus agrestis ♀</td>
<td>&quot;</td>
<td>Chitty, 1960</td>
</tr>
<tr>
<td>Lemmings</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lemmus trimicronatus</td>
<td>(1)Adrenal weight</td>
<td>Andrews, 1968</td>
</tr>
<tr>
<td></td>
<td>(2)Adrenal secretory function</td>
<td></td>
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<tr>
<td>Mice</td>
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<tr>
<td>Peromyscus maniculatus</td>
<td>Adrenal weight</td>
<td>Christian, 1971</td>
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<td>bairdii ♀</td>
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<tr>
<td>Peromyscus leucopus ♀</td>
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shops, restaurants and dwelling houses (Laurie, 1946), flour stores (Laurie, 1946), farm buildings (Brown, 1953) and corn ricks (Southwick, 1958). Because of this diversity of habitats it is clear that regulatory factors will vary from population to population. Some factors identified as important, include inter-specific competition (Lidicker, 1965) and climatic conditions, notably temperature (Berry, 1968).

Under laboratory conditions of controlled light and temperature, mice will breed throughout the year. Pregnant females have been found throughout the year in some studies of natural populations (Laurie, 1946; Smith, 1954). However, various studies clearly indicate a cessation of breeding throughout the winter months (Parkes, 1924; Kalabukhov, 1937; Southern and Laurie, 1946; Lidicker, 1965; Berry, 1968; Brown, 1953). The reasons for this have been attributed to low temperature (Berry, 1968), a combination of low temperature and absence of food (Parkes and Brambell, 1927-8) and photoperiod (Pennyciuk, 1972).

The characteristic feature of "semi-natural" populations of house mice has been the failure of reproduction as numbers increase, in the absence of uncontrolled temperatures and photoperiod and with provision of bedding material, nest boxes, food and water (Crowcroft and Rowe, 1957, 1958; Christian, 1956, 1961; Lloyd and Christian, 1969; Snyder, 1968; Brown, 1953; Southwick, 1955, 19552; Calhoun, 1956). In confined populations, low temperature (Southwick, 1955) and limited food (Streckler and Emlen, 1953) can inhibit reproductive efficiency but social factors clearly play a role in the other studies. Density per se is not the factor in limiting numbers, since absolute numbers of different static
populations given the same area varied (eg. Southwick, 1955). It must be concluded that it is the social inter-
actions among individuals that limit growth (Petrusewicz, 1957). Laboratory studies grouping males and females to-
gether reveal the same picture of lack of reproductive
success at high densities (as well as increased mortality),
(Crew and Mirskaia, 1931; Retzlaff, 1938; Christian and
Lemunyan, 1958).

It is necessary then to examine more closely the
social behaviour of house mice. A characteristic feature is
fighting between adult males (Ulrich, 1937; Calhoun, 1956;
Crew and Mirskaia, 1931; Retzlaff, 1938; Brown, 1953). This
is believed to be related to the establishment of territo-
rial behaviour amongst males (Crowcroft, 1955; Crowcroft
and Rowe, 1963; Anderson and Hill, 1965; Mackintosh, 1970).
Male mice, given sufficient area, will form distinct defen-
ded territories. In smaller areas, one male will dominate all
the male mice in that area, forming a dominance hierarchy
with dominant, subdominant and subordinate males. Territories
and dominance are established in male mice by fighting
(Anderson and Hill, 1965).

Under most circumstances there is no fighting bet-
ween male and female mice (eg. Crowcroft and Rowe, 1958;
Ulrich, 1937). However lactating or pregnant females have
been observed attacking males (eg. Brown, 1953). It has been
presumed that this is defensive behaviour rather than social
hierarchical behaviour.

The question of social structure amongst female
mice remains unclear. It has been shown that in a crowded
group only a few females are reproductively active (eg.
Retzlaff, 1938; Lewontin and Dunn, 1960; Lloyd and Christian, 1969; Snyder, 1968) and that these are socially dominant, in terms of activity and distances moved (Lloyd and Christian, 1969). It has also been shown that these reproductively active females are predominantly the oldest (and largest), best established and cohabit with males of high social rank (Snyder, 1968).

Retzlaff, (1938) reported prolonged fighting amongst female mice leading, in some cases, to death. Other reports mention the existence of aggressiveness amongst females (Weltman et al, 1968; White, Mayo and Edwards, 1969; Schartz et al, 1974). Fredericson, (1951) found fighting amongst females but only when they were subjected to competition for food. There is a large body of evidence supporting the view that females are largely unaggressive to each other (eg. Bronson, 1967; Anderson, 1961; Calhoun, 1956; Ulrich, 1937; Crowcroft and Rowe, 1957, 1958; Brown, 1953).

Mice are believed to form breeding units or demes (families), (Lewontin and Dunn, 1960) which are socially stable. Individuals are recognised (Rowe and Redfern, 1969) and both males and females are said to defend the home area of the deme against strangers (Anderson, 1961; Reimer and Petras, 1967).

Christian's hypothesis predicts that at increasing population density there is an increase in the number and/or intensity of social interactions causing social stress and leading to increased emigration, reproductive failure and mortality. With the knowledge of territorial or dominant behaviour of male mice and the fact that mice form stable breeding groups it could be predicted that dispersion might
be a prominent factor in population regulation. In fact, it has been proposed that reproductive failure is of little importance in natural populations of house mice (Crowcroft and Rowe, 1958; Anderson, 1961). This was because densities used in semi-natural studies exceed any density found in the wild and that the first response to crowding would be emigration. Crowcroft and Rowe, (1958) showed that when a confined static population was given extra room, the new area was rapidly colonised, reproduction increased and the numbers went up. Streckler, (1954) showed that emigration prevented a decline in fertility usually found in a confined population with limited food. The emigrants are predominantly subordinate males (Rowe et al, 1964; Butler, 1980; Calhoun, 1956).

Christian, (1961) has countered this reasoning so that whilst accepting dispersion as one factor, there is a limit to the number of suitable habitats. The emigrants would be expected to find marginal habitats since it has been shown that strange males are unable to join breeding demes and it is only rarely that females succeed (Reimer and Petras, 1967). Also, there is evidence that high densities of mice do occur in the wild and that reproduction is inhibited (eg. Evans, 1959; Young, Streckler and Emlen, 1950).

The failure in reproduction operates at different levels (see Table 3). In house mice it is generally agreed that there are usually sufficient fertile males to impregnate any oestrus females (Snyder, 1968; Christian, 1971). Southwick, (1955) observed a high incidence of abnormal male sexual behaviour, termed copulation pressure (Calhoun, 1949), whereby because a number of males were attempting copulation, no one male could successfully achieve
TABLE 3

Summary of Endocrine and Reproductive changes found within confined, freely growing populations of *Mus musculus: Effects of increasing density.*

<table>
<thead>
<tr>
<th>REPRODUCTION</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Reduced female fertility</td>
<td>Southwick, 1955&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>(i) inhibition of gonadal function</td>
<td>Christian, 1956, 1959, 1961, 1963&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>(ii) inhibition of sexual maturation</td>
<td>Lloyd &amp; Christian, 1969</td>
</tr>
<tr>
<td>(iii) increased time between ovulation</td>
<td>Lloyd &amp; Christian, 1969</td>
</tr>
<tr>
<td>(b) Reduced number of pregnancies</td>
<td></td>
</tr>
<tr>
<td>(i) male sexual behaviour abnormal</td>
<td>Southwick, 1955&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>(c) Reduced litter size</td>
<td></td>
</tr>
<tr>
<td>(i) abortion or resorption of embryos</td>
<td>Christian, 1959</td>
</tr>
<tr>
<td>(ii) reduced number of ova</td>
<td></td>
</tr>
<tr>
<td>(d) Increased infant mortality</td>
<td></td>
</tr>
<tr>
<td>(i) nest disturbance</td>
<td>Christian, 1956</td>
</tr>
<tr>
<td>(ii) suppressed lactation</td>
<td>Brown, 1953</td>
</tr>
<tr>
<td>ENDOCRINE</td>
<td></td>
</tr>
<tr>
<td>(a) Increased adrenal weight</td>
<td>Christian, 1956, 1961, 1963&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>(b) Increased zone widths of adrenal cortex</td>
<td></td>
</tr>
<tr>
<td>(c) Decreased ovary weight</td>
<td></td>
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<tr>
<td>(d) Decreased testis weight</td>
<td></td>
</tr>
<tr>
<td>(e) Decreased seminal vesicle weight</td>
<td></td>
</tr>
<tr>
<td>(f) Decreased thymus weight</td>
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</tbody>
</table>
ejaculation. Snyder, (1966) showed a decline in capacity to impregnate females in grouped males. Also grouped males produced fewer spermatozoa than controls. Dominant males, in a freely grouping population had normal sperm counts whereas subordinates had lower sperm counts and collection of sperm by electrically induced ejaculation was less successful.

Several studies have identified the primary cause of reproductive failure in house mice as an increase in infant mortality (Christian, 1959; Brown, 1953; Southwick, 1955). This was due to a failure of females to build a successful nest, cannibalism of the young by mothers, abandonment of pups by mothers (Brown, 1953) and a failure in lactation (Christian, 1959). Brown, (1953) observed that attempts to build nests were thwarted in pregnant females by disturbance from other mice. The failure to build a successful nest and the disturbance during parturition led directly to abandonment of pups or cannibalism. Brown, (1953) proposed that the key to success in reproduction was nest defence by females and it is plausible that dominance in females confers success in defending the nest.

Christian, (1959) in a study of freely growing populations also identified litter survival as an important factor in reproductive failure at high densities but concluded that this was due to suppressed lactation. Nests were built and undisturbed by other mice yet the stomachs of the nestlings contained no milk. Christian and Leminyan, (1958) also found suppressed lactation in grouped females.

Crowcroft and Rowe, (1958) found in their populations reduced fertility of female mice and no evidence of significant infant mortality. They proposed that infant mortality
would only be important where there were inadequate physical conditions for nest building, eg. small nest boxes with only one opening. Accepting this and the notion that it is female productivity that limits reproduction in mice the mechanisms of reproductive failure could be (a) inhibition of ovulation (b) inhibition of sexual maturation (c) increased abortion or resorption of embryos (d) reduced number of ova. Evidence has been found supporting all these factors and this will be dealt with later.

Central to the social stress hypothesis is the idea of adrenocortical activation. Christian (1956, 1961, 1963) found increases in adrenal weight in both males and females and decreases in ovary weight and testis weight from confined populations at high density. It should be noted that some similar studies failed to reveal any differences in adrenal weight (Southwick and Bland, 1959; Southwick, 1958). Most of the evidence for social stress comes from studies of the male mouse where increased density is associated with adrenal weight increases (eg. Bronson and Eleftheriou, 1963) and increases in plasma corticosterone (eg. Brain and Nowell, 1971). Other studies have indicated lower adrenal weight (Davis and Christian, 1957) and lower basal corticosterone (Louch and Higginbothan, 1967) in dominant males than subordinates. It is noted that other reports find no difference in plasma corticosterone levels between isolates and groups and between dominant and subordinate (eg. Benton et al. 1978). Adrenal weight is increased in males exposed to a trained fighter mouse and those subjected to defeat (Bronson, 1967; Bronson and Eleftheriou, 1965, 1965, 1965, 1965). There is also evidence that social disruption
through emigration and immigration of male mice can lead to an increase in adrenal weight (Bailey, 1966, 1969).

It is generally accepted that fighting amongst males constitutes a stress. There is however no evidence of what specific social behaviour constitutes a stress in female mice. The assumptions are that inter-male fighting stresses females or that females are accidentally attacked by fighting males (eg. Bronson, 1967; Calhoun, 1956). The role of female aggression and a dominance hierarchy may be important but this remains unproven.

One alternative mechanism of altering behaviour by overcrowding has been proposed - through prenatal or pre-weaning effects. It has long been recognised that young animals are very sensitive to environmental disturbances. Similarly if pregnant or lactating females are stressed it is conceivable that there are permanent changes in the behaviour and physiology of the young which could alter future reproductive success or at least determine dominance/subdominance positions.

Differences in exploratory behaviour have been demonstrated in the offspring of mothers grouped during the last week of pregnancy compared to individually housed controls (Keeley, 1962). Others have shown prenatal effects on growth, white blood cell count and "vasiveness" (Sackler et al, 1969), brain protein, RNA and DNA (Petropoulos et al, 1972), "emotionality", corticosterone levels and susceptibility to gastric ulcers (Ader and Plaut, 1968) and sexual behaviour (Dahlof et al, 1977). It has been presumed that these effects are caused by stress operating through the adrenal cortex although this has not been proven. Christian
and Lemunyan, (1958) demonstrated persistent stunting in offspring of crowded mothers which was presumed to be due to deficient lactation.

Post-natal effects of various sorts of stimulation have been investigated and reviewed (Daly, 1973) and will not be dealt with here. This does not necessarily reflect their importance.

Crowcroft and Rowe's studies on confined populations of house mice indicated inhibition of female gonadal function and cessation of the oestrus and ovulation at high density. In particular females had closed or partly closed vulvae, inactive ovaries and thread-like uteri indicating anoestrus. There are however varying degrees of inhibition of gonadal function in female mice. It has long been recognised that ovulation in the mouse is spontaneous, occurring at regular intervals, mostly between four to six days (Allen, 1922; Parkes, 1926, 1928). In all-female groups, ovulation is suppressed and pseudopregnant cycles of ten to twelve days occur (eg. Lee and Boot, 1955). However the ovaries and uteri remain functional in contrast to true anoestrus where the organs atrophy.

The understanding of the physiology of the oestrous cycle and ovulation has mostly been from work carried out on the rat, and it is necessary in attempting to deal with inhibition of ovulation in the mouse to draw information from these studies. Whilst there are similarities in the mechanisms of the oestrous cycle in the rat and mouse (eg. Michael, 1976) it is clear that there are also substantial differences in some aspects of the cycle (eg. Cambell et al, 1976).

A variety of factors have been shown to cause an-
oestrus in rats and mice. Nutritional deficiency in rats (Evans and Bishop, 1922) and mice (Halberg and Visscher, 1952), low temperature in rats (Lee, 1926) and mice (Barnett, 1965) can cause anoestrus or prolonged dioestrus. Photoperiod can affect productivity in mice (Pennycuik, 1972) and alter cycle length in rats (Hoffmann, 1968). Continuous lighting disrupts regular oestrous cycles in rats (Everett, 1961) causing prolonged anovulatory oestrus but in mice, whilst the oestrous cycle is lengthened it does not become anovulatory as in rats (Cambell et al, 1976). Grouping female mice in the absence of males, is said to produce lengthened cycles (Lamond, 1958) and anoestrus (Whitten, 1959). However since the return of a male brings on a rapid oestrus and mating it is not clear what part these effects may have in a natural population.

The endocrine mechanisms behind anoestrus remain obscure. A model for the regulation of seasonal anoestrus by photoperiod has been developed whereby daylength alters the negative feedback characteristics of estradiol on luteinising hormone (LH), such that anoestrus occurs when the feedback of estradiol is high and cycling when this is low (Legan and Karsch, 1979).

Constant light-induced anoestrus is believed to be a result of either desynchronisation of endocrine rhythms (Cambell and Schwartz, 1974) perhaps due to the absence of a 'zeitgeber' (Hoffmann, 1978) or prolonged secretion of oestradiol. However, this induction of anoestrus is not believed to be related to photoperiodic seasonal anoestrus (Turek and Cambell, 1979).

The notion that anoestrus can be caused by stress
stems back to Selye's GAS (Selye, 1950). ACTH and gonado-
trophin secretion were believed to be in opposition. An
increase in ACTH led to decreased gonadotrophin (GTH) and
vice versa. The release of GTH during stress has been re-
viewed (Giuliani, 1969) and it is clear that some noxious
stimuli increase gonadotrophin secretion along with ACTH.

It has nevertheless been shown that stressors can
disrupt the oestrous cycle in rats, eg. noise and formalin
odour (Hagino, 1968), and unpredictable shock (Pollard et al,
1975). In the latter study, disruption of the oestrous cycle
by unpredictable shock was shown to be accompanied by high
levels of corticosterone. Jarrett, (1965) injected ACTH into
mice and found suppressed oestrus and lowered ovary, vaginal
and uterus weights compared to controls. These effects were
abolished in adrenalectomised mice. In rats it has been
shown that ACTH can inhibit PMS-induced ovulation but that
corticosterone is ineffective (Hagino et al, 1969). ACTH
treatment at met-oestrus in rats blocked ovulation (Feder et
al, 1971). Thus, whilst the adrenal is required for ACTH-
induced suppression of ovulation it would seem that cortico-
sterone was not the critical factor.

Progesterone is secreted from the adrenal gland
(Short, 1960) and is under the control of ACTH (Fajer et al,
1971). The amount of progesterone secreted from the adrenal
can exceed the amount from the ovary (Shaikh and Shaikh,
1975). Stress stimulates adrenal progesterone secretion
(Ogle and Kitay, 1977). Surgical stress can block ovulation
(Schwartz, 1964) as can progesterone administration (given
at the correct time), (Lisk, 1969). It is possible that
stress-induced adrenal progesterone can block ovulation per-
sistently and lead to anoestrus (Ogle and Kitay, 1977). The findings that irregular oestrous cycles in mice and rats (Paris and Ramalay, 1974; Ramalay, 1975) are correlated with the absence of adrenal corticosterone rhythms (or free running rhythms) would also fit into this explanation, since adrenal progesterone also follows a circadian rhythm (Mann and Barraclough, 1973).

Christian, (1956, 1961, 1959) demonstrated that female sexual maturation was inhibited in confined but freely growing populations of mice. The endocrine regulation of sexual maturation has been reviewed recently (Ramalay, 1979). Mechanisms of inhibition of maturation remain speculative.

ACTH inhibits sexual maturation in both intact and adrenalectomised immature female mice, indicating a direct effect of ACTH (Christian, 19641, 19642). The effects were however greater in intact mice. It has been shown that low doses of weak androgens of the type secreted by the adrenal, inhibited maturation for considerable periods (Varon and Christian, 1963; Duckett, Varon and Christian, 1963) and it was proposed that the dual effects of ACTH and adrenal androgens inhibited maturation (Christian et al, 1965). However, Christian (19712) noted that the inhibition of sexual maturation found in freely growing populations involved no ovarian, uterine or vaginal development in contrast to the laboratory manipulations and concluded that other mechanisms must be operational.

Other factors involved in reproductive failure, implantation of the embryo, intrauterine mortality and reduction in number of ova, are believed to be of less importance in population regulation although they have been observed in
confined populations of mice (Christian, 1959). Failure of implantation was observed in the laboratory where a newly pregnant female was exposed to a strange male (Bruce, 1959). This factor is pheromonal (Dominic, 1965) and the effect is due to failure of prolactin secretion (Bruce and Parkes, 1960). Chipman and Fox, (1966) demonstrated this effect in wild mice and also showed that it could be induced by a change in physical environment. It has also been shown that stressors eg. noise, enforced swimming, handling and "open field exposure" can block pregnancy (Weir and Defries, 1963; Runner, 1959).

Christian, (1956) reported an increase of fifty eight per cent in the number of resorbing embryos in mice at high density. Females mated to grouped males produced significantly smaller litters than those bred to males that had been caged alone, and resorption of embryos was higher in the former (Snyder, 1966) Injection of cortisol, cortisone or ACTH in intact rats increases intrauterine mortality (Seifter et al, 1951; Davis and Plotz, 1954; Velardo, 1957). It has been presumed that the increase in resorption of embryos at high density is a result of social stress and adrenal activation (Christian, 1971).

To summarise: Christian's hypothesis concerning the regulation of population size by social stress is an extension of Selye's GAS hypothesis. Whilst the generality of the hypothesis has been questioned there is a growing volume of evidence supporting the notion that populations can be regulated by social behaviour. "Semi-natural" studies of house mice reveal a cessation of population growth in confined enclosures, in the absence of physical limiting factors,
attributable to social factors. The cessation of growth is due to reproductive inefficiency and/or an increase in infant mortality. It is generally believed that (in house mice) it is the females which are limiting in reproductive productivity. Lack of reproductive efficiency can operate at different levels; inhibition of sexual maturation, inhibition of the oestrous cycle, abortion or resorption of the embryos and reduced number of ova. Amongst males social stress can be attributed to fighting as a result of territorial or dominance behaviour. It is not known how or if social stress occurs in females. The mechanisms of reproductive inefficiency remain speculative. It is possible that stress-induced anoestrus in females is due to adrenal progesterone.
CHAPTER 3 Materials and Methods

Introduction

In Chapters 1 and 2 some areas of weakness in the notion of social stress have been identified. Having chosen the mouse as an experimental animal it has been necessary to understand some of the biology of the mouse which is species specific. Social stress in male mice can be explained by inter-male fighting and territoriality. However this would not seem to be important in population regulation, since it is female fertility which is the limiting factor, in house mice. Little is known of social stress in females but it appears that females can become anoestrus in response to social stimuli. It is also known that social stimuli can cause inhibition of regular oestrous cycles (the Lee-Boot Effect) although the cause of this is not fully understood.

The original aims of this study were to ascertain whether grouping female mice in different housing conditions constitutes a stress and leads to adrenal activation. Also, whether grouped females show true anoestrus or pseudopregnant or lengthened oestrous cycles and whether these cycles are caused by social stress and adrenal activation. At a more general level, an attempt has been made to identify which parameter of adrenocortical activity is an accurate measure of stress. In order to answer these questions it has been necessary to study some subsidiary problems, notably the effects of various methodological procedures and the oestrous cycle on adrenocortical activity.

Attention has been paid primarily to the all-female social environment but it is recognised that male/female social interactions may be of importance in social
stress. A few preliminary studies were attempted to examine the effect of presence of a male on the oestrous cycle and adrenocortical functioning in the female.
Materials and Methods

Animals

Two strains of mice have been used. Most of the experiments have utilised TO strain (Tuck and Sons Ltd.), an outbred Swiss albino line. C57 B1/10 inbred black mice were used in one experiment (Chapter 4).

First generation virgin females aged between twelve and twenty weeks old were used except for one experiment (Chapter 7), where females direct from the supplier were used. Original males and females from the supplier were allowed at least two weeks to settle into the animal houses before mating. Males and females were then paired in small cages. The male was removed before birth of the litter. At birth, litters were randomly culled to eight pups and the mothers and litters undisturbed until weaning at 18-22 days. At weaning, the sexes were separated and females were either randomly assigned to experimental groups or housed in fours in large cages.

Individually housed animals were kept in opaque plastic cages 30 x 13 x 11cm and grouped animals in similar cages sized 42 x 26 x 11cm. Cages had wire tops with ad libitum supply of Oakes P.M.D. pellets and clean water. Sterile sawdust bedding was provided and the cages were routinely cleaned out weekly except during pregnancy and lactation when they were undisturbed. The cages were checked daily when fresh water was provided.

Several animal houses were used all of which were relatively quiet, had controlled lighting and temperature and air extraction. These rooms were used exclusively for keeping the animals in and no experimental manipulations
such as blood sampling were carried out in them, (excluding vaginal smearing). With the exception of experiments utilising male mice, all female mice were used in a "male smell free" room. No male mice or old male bedding was allowed in these rooms. All cages used by male mice were thoroughly washed out before housing females.

Temperature was controlled at between 18°C- 23°C. In individual experiments this range was considerably smaller. Reversed lighting was used mostly with fourteen hours light, ten hours dim red light. Lights were then on at 8 or 10pm and off at 10 or 12am.

The decisions of these experimental conditions is somewhat arbitrary but have been based on an attempt to reduce the variability between animals of adrenal or gonadal function. It is recognised that the mouse strain, age, conditions of rearing, cage size, external disturbance, presence or absence of males, temperature and photoperiod can all influence adrenal and/or reproductive physiology in mice.

**Ovarian Function**

The object of assessing ovarian function has been identification of ovulation. Various methods are available including direct hormone measurements, histological examination of the ovaries, microscopic examination of fallopian tubes, housing with a male and vaginal smearing. Vaginal smearing has the advantage that it is easy, quick and can be carried out over long periods. Disruption of the oestrous cycle in mice by smearing has been reported (Lamond, 1958; Bingel and Schwartz, 1969) but was not observed in this study.

Smears were taken daily by the lavage method,
(Snell, 1941). They were dried, then stained with Giemsa stain for thirty minutes, washed in water and redried. Smears were routinely taken daily, 1-3 hours before lights on, except when blood sampling took place, when smears were taken after the blood sample.

The smears were staged after the description given by Bingel and Schwartz, (1969). In addition some variations are noted in this study.

Six day cycles had longer dioestrus, oestrus, pro-oestrus or metoestrus stages. Pseudopregnant cycles could be identified. These were characterised by five or more days of dioestrus. The usual cycle length in pseudopregnancy was eleven days, often with a characteristic mucification and the appearance of small, nucleated cell types mid-cycle. Towards the end of the cycle the smear was thin and had isolated cornified cells. Occasionally the pro-oestrus/oestrus smear following pseudopregnancy lasted only one day and the cells never became fully cornified. Abnormal cycles were observed with four or more days of heavy cornified cells or with over fifteen days of dioestrus smears.

Whilst it has been shown that in the mouse there is a correlation between the appearance of a pro-oestrus smear and ovulation (Bingel and Schwartz, 1969; Wallace, 1965, 1965; Snell et al, 1940) it is not known whether this is so during pseudopregnant or abnormal cycles. Oestrus cycles in mice are notoriously variable compared with rats (Allen, 1922; Parkes, 1926, 1928; Burns et al, 1936; Suntzeff et al, 1936; Brown-Grant, 1966; Bingel and Schwartz, 1969).
Adrenocortical Function

The different types of adrenal measure have been discussed in Chapter 1. Adrenal weight is not a valid measure of stress unless certain precautions are observed (eg. Christian, 1963). Even then there are studies which fail to correlate adrenal weight with plasma corticosterone (eg. Brain and Nowell, 1972; Andrews, 1968; Sung et al, 1977). Further, adrenal weight cannot provide accurate information about adrenocortical periodicity. Adrenal weight may reflect the recent history of secretion of glucocorticoids (Bronson, 1967).

Two main methods have been developed for the measurement of total plasma concentrations of corticosterone in mice and rats. The fluorometric method, without a chromatographic step, has been used commonly: the most popular method being that of Glick et al, (1964) which is based on methods of Guillemen et al, (1958, 1959). More recently saturation analysis methods have been utilised based on either competitive protein binding assay (CPB) after Murphy, (1967) or radioimmunoassay (RIA) after Yalow and Berson, (1959).

Fluorometric methods without chromatographic purification have been shown to be highly non-specific (Frankel et al, 1967). Further studies on the rat and mouse have indicated that the interfering non-specific fluorescence is non-steroidal, that it varies with age (Diez et al, 1976) and physiological stress (Butte et al, 1978).

Two saturation analysis methods have been developed and used in this study. A CPB assay using dog plasma, validated in the mouse by Grad and Khalid, (1969), with some

**Materials**

Ethyl acetate (Analar) and 2,2,4 Trimethyl pentane (spectrograde) were supplied by BDH.

Ethyl alcohol (absolute) was supplied by Burroughs.

Phosphate buffer (BDH) 0.04m, pH 7.4 and Borate buffer (Analar, BDH) pH 8.0, 0.01m containing 0.1% bovine serum albumen (RIA grade, Sigma) and 0.1% bovine gamma globulin (Sigma) were prepared monthly and stored at 4°C.

Florisil 60/100 mesh (BDH) treated to remove "fines" after Ito, Woo, Haning and Horton, (1972) and stored in sealed containers.

Dextran coated charcoal prepared by adding 0.05% dextran T 70 (Pharmacia) and 0.5% charcoal (Norit X, BDH) to borate buffer (without BSA or BGG) was prepared monthly and stored at 4°C.

Unlabelled corticosterone (Sigma) was prepared in two stock solutions in ethanol of 100μg/ml and 1μg/ml and stored at -20°C. Working solution of 10ng/ml in ethyl acetate was prepared fortnightly and stored at -20°C.

Labelled corticosterone (1,2 ³H corticosterone, specific activity approximately 50Ci/mmol, Radiochemical Centre) was diluted with ethanol on arrival to 4μCi/ml and stored in 5ml aliquots at -20°C. It was replaced yearly.

Lumagel (Lumac) was used for liquid scintillation counting.

Binding protein was prepared using fresh dog plasma. It was shaken gently for 1 hour at 45°C with Florisil (1g Florisil/
10ml plasma) to remove steroids (Diez et al. 1976). Plasma was decanted and frozen in 2ml aliquots at -20°C until used. Antiserum (rabbit anti-corticosterone-21-thyroglobulin, Miles/Yeda) was obtained in freeze dried form and was stored at 4°C and reconstituted as needed. All glassware was soaked in Decon (BDH) and cleaned ultrasonically. Glassware was then rinsed in deionised water, soaked in 5% HCl, rinsed in deionised and then distilled water and dried. Disposable glass tubes were used in RIA method and these were washed only in ethanol, deionised and distilled water before used.

Methods
Extraction: CPB
Either 50 or 100µl plasma was pipetted into ground glass stoppered extraction tubes (50µl plasma was made up to 100µl with distilled water). 1ml Ethyl Acetate was added, the tubes were stoppered and sealed with a drop of water and then shaken vigorously for 3 minutes. Layers were separated by a brief centrifugation. A 40-400µl aliquot was transferred to the assay tubes in duplicate and dried either overnight in a fume cupboard or under nitrogen. Standards were pipetted into assay tubes in duplicate, triplicate or quadruplicate from the working solution as 0.25, 0.5, 0.75, 1.0, 1.5, 2.0, 3.0ng. Four tubes had no steroid added but had solvent (zero controls), another 4 tubes were prepared for the charcoal blank and two tubes for total counts.

Extraction; RIA
Extraction was identical except plasma was washed with 2,2,4 trimethyl pentane before extraction. One ml solvent
was added to the tubes and the tubes shaken vigorously for 1 minute. The aqueous phase was then frozen in a freezing mix (dry ice and acetone) and the solvent discarded. Assays routinely had 100 tubes for each set of standards. The standards were in duplicate 0.1, 0.25, 0.5, 0.75, 1.0, 1.5, 2.0ng. Two tubes were used for zero, two for the charcoal blank and two for total counts.

Assay: CPB

1 ml binding solution (prepared with 2μCi \(^{3}\)H-corticosterone, 3.5 ml stripped dog plasma made up to 100 ml with phosphate buffer) was dispensed into 50 assay tubes. Two tubes had only buffer and \(^{3}\)H-corticosterone made up to the same radioactive concentration as binding solution. Tubes were held at 37°C for 30 minutes and shaken twice. They were then placed in an ice bath (3.5°C) for 1 hour and tubes shaken once. Approximately 40 mg Florisil (one trial n=50, av. weight \(\pm\) S.D. = 44.5 ± 1.3 mg) was dispensed into all tubes except the two tubes for total counts. The tubes were shaken together for 2 minutes, the Florisil allowed to settle for 5 minutes, then 0.5 ml pipetted in liquid scintillation minivial with 5 ml Lumagel.

Assay: RIA

0.7 ml binding solution (prepared with one 100 tube vial antibody and 1μCi \(^{3}\)H-corticosterone made up to 70 ml with borate buffer) was dispensed to all tubes except charcoal blanks, which had 0.7 ml \(^{3}\)H-corticosterone and borate buffer. After 25 minutes at room temperature tubes were vortexed. Tubes were placed in a water bath at 37°C for 1 hour and vortexed. Then they were placed in an ice bath for at least 30 minutes. 200 μl dextran coated charcoal, which was stirred continuously, and kept at 4°C, was dispensed
into 50 tubes using a Computet pipetting machine in less than 45 seconds. The tubes were vortexed and left for 10 minutes, then centrifuged at 3000 rpm for 15 minutes at 4°C. 0.5 ml supernatant was pipetted into 5ml Lumagel in liquid scintillation minivials. Two tubes in every 100 had no dextran coated charcoal added. To these 200ul buffer was added.

**Counting**

Tubes were counted for 10 minutes (after shaking and two hours equilibrium) on a Packard Tricarb. Counting error was less than 2%. Counting efficiency was between 31-34%, and was monitored by automatic external standardisation.

**Calculation of Results: CPB**

Results were calculated using a hand drawn graph of percentage binding against concentration.

**RIA**

Results were calculated by computer using logit-log transformation of Rodbard et al., (1970). Printout included slope, intercept, correlation coefficient and test of linearity of standard curve.
Results

Specificity: CPB

Murphy, (1967) found significant cross-reaction with cortisol, 11-deoxycortisol, progesterone, 17 hydroxy progesterone and cortisone in dog plasma. Similarly Grad and Khalid, (1969) found cross-reaction with cortisol, 17 hydroxy-11-deoxy-corticosterone and 17 hydroxy progesterone, (see Table 1). Using male human plasma as the binding source Clark and Nowell, (1978), found that 95% of total CBG bound steroid is corticosterone in TO strain male mice. This is in agreement with others (Bloch and Cohen, 1960; Halberg et al, 1959; Hoffmann, 1956; Triller and Birmingham, 1965; Ertel and Ungar, 1968; Nandi et al, 1967; Wilson et al, 1958) who found little or no evidence of 17-hydroxylated corticosteroids in mice. There is evidence that some strains have these steroids, particularly cortisol and 11-deoxy cortisol (Varon, Touchstone and Christian, 1966; Badr and Spickett, 1965 and Badr, 1971).

In females interference with progesterone is a possibility and this was assessed. A pool of plasma was assayed with and without added progesterone (100ng/ml) with and without 2,2,4-trimethyl pentane wash. Twelve plasma samples were also assayed with and without a solvent wash (Table 2). These results indicate that progesterone can cause significant cross-reaction but that the effect is minimal in plasma samples presumably because progesterone is at too low a concentration normally to interfere. The 2,2,4 trimethyl pentane wash is effective at removing progesterone interference without markedly lowering recovery of corticosterone.
### TABLE 1

**Specificity of Corticosteroid Binding Protein in Dog Plasma.**

<table>
<thead>
<tr>
<th>Steroids tested</th>
<th>% Cross reaction from (Murphy, 1967)</th>
<th><em>Time taken to count 5000 CPM (Grad and Khalid, 1969)</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>100</td>
<td>2.18</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>67</td>
<td>1.79</td>
</tr>
<tr>
<td>11 deoxy cortisol</td>
<td>75</td>
<td>-</td>
</tr>
<tr>
<td>Progesterone</td>
<td>40</td>
<td>1.55</td>
</tr>
<tr>
<td>17 hydroxy progesterone</td>
<td>30</td>
<td>1.98</td>
</tr>
<tr>
<td>Cortisone</td>
<td>50</td>
<td>1.68</td>
</tr>
<tr>
<td>Testosterone</td>
<td>5</td>
<td>1.39</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>5</td>
<td>1.37</td>
</tr>
<tr>
<td>Estradiol-17-B</td>
<td>-</td>
<td>1.20</td>
</tr>
<tr>
<td>Estradiol</td>
<td>-</td>
<td>1.36</td>
</tr>
<tr>
<td>Estrone</td>
<td>-</td>
<td>1.28</td>
</tr>
<tr>
<td>17 hydroxy 11-deoxy corticosterone</td>
<td>-</td>
<td>1.93</td>
</tr>
<tr>
<td>None</td>
<td>1.26</td>
<td></td>
</tr>
</tbody>
</table>

* Time taken to count 5000 CPM of $^3$H-corticosterone bound to dog corticosteroid binding globulin following exposure to 1 ng of various steroids.
### TABLE 2
Assessment of interference in CPB assay by Progesterone.

<table>
<thead>
<tr>
<th></th>
<th>Est. Conc. Corticosterone with solvent wash (ng/ml)</th>
<th>Est. Conc. Corticosterone without solvent wash (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood samples n=12</td>
<td>49 ± 17</td>
<td>56 ± 19</td>
</tr>
<tr>
<td>Pool n=8</td>
<td>49.3 ± 1</td>
<td>52.5 ± 1.7</td>
</tr>
<tr>
<td>Pool + 100ng/ml progesterone n=8</td>
<td>52.3 ± 1</td>
<td>80.2 ± 2.3</td>
</tr>
</tbody>
</table>
**TABLE 3**
Specificity of rabbit anti-corticosterone-21-thyroglobulin (data from Miles/Yeda).

<table>
<thead>
<tr>
<th>Steroids Tested</th>
<th>% Cross reaction calculated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosterone</td>
<td>100</td>
</tr>
<tr>
<td>Progesterone</td>
<td>20.6</td>
</tr>
<tr>
<td>Deoxy corticosterone</td>
<td>10.9</td>
</tr>
<tr>
<td>20α hydroxy progesterone</td>
<td>7.6</td>
</tr>
<tr>
<td>Cortisol</td>
<td>5.8</td>
</tr>
<tr>
<td>20β hydroxy progesterone</td>
<td>5.7</td>
</tr>
<tr>
<td>Testosterone</td>
<td>3.8</td>
</tr>
<tr>
<td>17 hydroxy progesterone</td>
<td>3.5</td>
</tr>
<tr>
<td>Androstenedione</td>
<td>2.4</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>1.0</td>
</tr>
<tr>
<td>11 deoxy cortisol</td>
<td>11.5</td>
</tr>
<tr>
<td>5α dihydrotestosterone</td>
<td>0.5</td>
</tr>
<tr>
<td>Androsterone</td>
<td>0.4</td>
</tr>
<tr>
<td>Cortisone</td>
<td>0.3</td>
</tr>
<tr>
<td>Dihydroxyepiandrosterone</td>
<td>0.3</td>
</tr>
<tr>
<td>Estrone</td>
<td>0.1</td>
</tr>
<tr>
<td>Estradiol 17β</td>
<td>0.1</td>
</tr>
<tr>
<td>Estriol</td>
<td>0.1</td>
</tr>
</tbody>
</table>

* B = counts of bound fraction of unknown
  Bo= counts of bound fraction of zero
Specificity: RIA

The specificity of the RIA method for measurement of corticosterone in mice has been validated (Gross et al., 1972). The cross reactivity of the antibody was assessed by Miles/Yeda and is given in Table 3. The specificity is better than dog plasma, human plasma (Murphy, 1967) and the antisera used by Gross et al., (1972).

Standard Curve: CPB

A typical standard curve is shown in Figure 1. The maximum binding of the zero value in respect of the total radioactivity is 60%.

Standard Curve: RIA

A typical standard curve is shown in Figure 2. For fifty four assays the mean values for slope and intercept are:

Mean ± SD  
Slope = -1.0865 ± 0.054  
Intercept = -0.637 ± 0.102

The maximum binding of the zero value in respect of the total radioactivity is 65%.

Sensitivity and Blank: CPB

Sensitivity is two times the standard deviation of the zero values. A comparison of zero values with and without solvent indicated that the solvent blank did not deviate significantly from zero.

Sensitivity = 58 ± 36 pg for five assays
Water blank = 2.5 ng/ml, n=3 in three assays.

The effective least detectable limit is 2.9 ng/ml in assay range 0-100 ng/ml.

Sensitivity and Blank: RIA

Water blanks and solvent blanks were not
Fig. 1  Typical Standard Curve : CPB Method
Fig. 2  Typical Standard Curve : RIA Method

![Graph showing typical standard curve for RIA method. The graph plots CPM Standard x 100 on the y-axis against Corticosterone concentration (ng) on the x-axis. The curve is a decreasing function, indicating a decrease in CPM as the concentration of Corticosterone increases.]
significantly different from sensitivity. 
Sensitivity = 40 ± 34 pg for nine assays.

Plasma treated with charcoal to remove all steroids gave a concentration of 2.0 ng/ml ± 1.5, n=6. Since the plasma aliquot volume could be altered the sensitivity varies. The plasma blank was assessed using different aliquot sizes.

The effective least detectable limit is 2 ng/ml in the assay range 0-100 ng/ml rising to 8 ng/ml in assay range 100-300 ng/ml and 16 ng/ml in assay range 300-600 ng/ml. 

It was possible when assaying plasma samples, to accurately assess which expected range of concentrations the samples would fall into, and therefore decide which aliquot sizes to use.

Recovery:

$^3$H-corticosterone was added to plasma and the percentage recovery after extraction was estimated.
Without solvent wash 100.9 ± 3.4%, n=19 (3 experiments) 
With solvent wash 93.8 ± 4.8%, n=34 (4 experiments)

CPB

Corticosterone was added to a plasma pool, assayed and the percentage recovery estimated, (Table 4). Mean percentage recovery = 97.8%
The relationship of amount added to amount found is linear with slope 0.9985 and correlation coefficient, r = 0.997. 

RIA

The percentage recovery was estimated as for CPB method, (Table 5). Parallelism was further demonstrated in different dilutions of a plasma pool since the relationship was linear (slope = 1.007 correlation coefficient 0.972).
Mean percentage recovery = 94.6%

The relationship of amount added to amount found was linear with a slope 0.958 and correlation coefficient, $r = 0.982$.

**TABLE 4** Estimation of Recovery of Corticosterone: CPB

<table>
<thead>
<tr>
<th>Amount of Corticosterone added (ng/ml)</th>
<th>Amount Determined (ng/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22.1</td>
<td>3</td>
</tr>
<tr>
<td>50</td>
<td>61.8</td>
<td>3</td>
</tr>
<tr>
<td>100</td>
<td>115</td>
<td>3</td>
</tr>
<tr>
<td>150</td>
<td>160</td>
<td>4</td>
</tr>
<tr>
<td>200</td>
<td>215</td>
<td>4</td>
</tr>
<tr>
<td>300</td>
<td>318</td>
<td>4</td>
</tr>
</tbody>
</table>

**TABLE 5** Estimation of Recovery of Corticosterone: RIA

<table>
<thead>
<tr>
<th>Amount of Corticosterone added (ng/ml)</th>
<th>Amount Determined (ng/ml)</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>86.6</td>
<td>5</td>
</tr>
<tr>
<td>50</td>
<td>135</td>
<td>2</td>
</tr>
<tr>
<td>150</td>
<td>232</td>
<td>4</td>
</tr>
<tr>
<td>250</td>
<td>322</td>
<td>5</td>
</tr>
</tbody>
</table>

**Precision: CPB**

Within assay variation was assessed after the method of Braunsberg and James, (1960), (Table 6)

Between assay variation was assessed using pools of plasma, (Table 7).
**TABLE 6** Within Assay Variation: CPB

<table>
<thead>
<tr>
<th>Concentration Range (ng/ml)</th>
<th>Mean Concentration (ng/ml)</th>
<th>Standard Deviation</th>
<th>Coefficient of Variation %</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>24.1</td>
<td>± 1.86</td>
<td>± 7.7</td>
<td>117</td>
</tr>
<tr>
<td>50-100</td>
<td>68.6</td>
<td>± 4.8</td>
<td>± 7.0</td>
<td>33</td>
</tr>
<tr>
<td>100-150</td>
<td>121</td>
<td>± 6.1</td>
<td>± 5.1</td>
<td>62</td>
</tr>
<tr>
<td>150-200</td>
<td>173</td>
<td>± 8.0</td>
<td>± 4.6</td>
<td>34</td>
</tr>
<tr>
<td>200</td>
<td>281</td>
<td>± 9.8</td>
<td>± 3.5</td>
<td>46</td>
</tr>
</tbody>
</table>

**TABLE 7** Between Assay Variation: CPB

<table>
<thead>
<tr>
<th>Concentration of Corticosterone (ng/ml)</th>
<th>Standard Deviation</th>
<th>Coefficient of Variation %</th>
<th>Number of Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pool 1</td>
<td>63.3</td>
<td>± 12.0</td>
<td>± 19.0</td>
</tr>
<tr>
<td>Pool 2</td>
<td>120</td>
<td>± 15.5</td>
<td>± 12.9</td>
</tr>
<tr>
<td>Pool 3</td>
<td>199</td>
<td>± 13.3</td>
<td>± 6.7</td>
</tr>
<tr>
<td>Pool 4</td>
<td>250</td>
<td>± 24.2</td>
<td>± 9.7</td>
</tr>
</tbody>
</table>

**Precision: RIA**

Within and between assay variation was assessed in six buffer pools with known concentrations of corticosterone added. Two or three samples were included in over twenty assays spanning a year, (Table 8).
TABLE 8  Within and Between Assay Variation: RIA

<table>
<thead>
<tr>
<th>Conc. of Corticosterone in Pool (ng/ml)</th>
<th>Number of Samples</th>
<th>Conc. of Corticosterone (ng/ml)</th>
<th>Standard Deviation</th>
<th>Coefficient of Variation (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C₁ 12.5</td>
<td>8</td>
<td>10.7</td>
<td>± 1.3</td>
<td>± 12.1</td>
<td>85.6</td>
</tr>
<tr>
<td>C₂ 25</td>
<td>13</td>
<td>22.0</td>
<td>± 2.5</td>
<td>± 11.4</td>
<td>88</td>
</tr>
<tr>
<td>C₃ 50</td>
<td>16</td>
<td>45.8</td>
<td>± 2.7</td>
<td>± 5.9</td>
<td>91.6</td>
</tr>
<tr>
<td>C₄ 100</td>
<td>19</td>
<td>88.5</td>
<td>± 7.7</td>
<td>± 8.7</td>
<td>88.5</td>
</tr>
<tr>
<td>C₅ 200</td>
<td>13</td>
<td>183</td>
<td>± 17</td>
<td>± 9.3</td>
<td>91.5</td>
</tr>
<tr>
<td>C₆ 400</td>
<td>14</td>
<td>355</td>
<td>± 25.5</td>
<td>± 7.2</td>
<td>88.8</td>
</tr>
</tbody>
</table>

Mean recovery = 89 ± 2.3%
Mean Coefficient of Variation = ± 9.1%

Correlation of CPB and RIA methods:

Thirty plasma samples were assayed using both methods and the values correlated. There is a linear relationship with slope = 1.043, intercept 12.8 ng/ml and correlation coefficient, r = 0.989, (Figure 3).

Normal Values

In order to assess two different blood sampling procedures and to get an idea of normal values at the expected circadian peak and trough of adrenocortical activity a preliminary experiment was performed. Sixteen virgin female, TO mice, aged 120 days, individually housed, were randomly assigned to two experimental categories. The first group were blood sampled 1 hour before dark (at the expected circadian peak) by retro-orbital puncture after Riley, (1960) with small heparinised tubes, after rapid ether
Fig. 3  Correlation between CPB assay and RIA

Plasma corticosterone values measured by CPB assay (ng/ml)

Plasma corticosterone values measured by RIA (ng/ml)

$y = 12.8 + 1.04x$

$r = 0.989$
anesthesia. One hour after lights on they were re-sampled by decapitation after rapid ether anesthesia. The second group were sampled at the same time by orbital method and decapitated 1 hour before dark. All blood sampling was completed within 3 minutes of disturbance. Plasma samples were assayed by CPB assay.

<table>
<thead>
<tr>
<th>Group</th>
<th>Sampling Time</th>
<th>n</th>
<th>Mean ± SEM Corticosterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>Eye—sampled at peak</td>
<td>8</td>
<td>148 ± 36</td>
</tr>
<tr>
<td></td>
<td>Decapitated at trough</td>
<td>8</td>
<td>29.4 ± 5.7</td>
</tr>
<tr>
<td>Group B</td>
<td>Eye—sampled at trough</td>
<td>8</td>
<td>41.5 ± 11.4</td>
</tr>
<tr>
<td></td>
<td>Decapitated at peak</td>
<td>8</td>
<td>120 ± 22</td>
</tr>
</tbody>
</table>

Using Mann Whitney U test there are no significant differences between peak or trough values. These values are "basal" levels i.e. non-stressed. Great care was taken in all experiments to ensure no disturbance of animals prior to blood sampling. The animal house was entered up to five times on any one blood sampling period and cages were quietly removed. This experiment also indicates that blood samples can be taken from one mouse within 12 hours of each other, without the second value being affected.
Discussion

Two saturation analysis methods have been developed from existing methods for the measurement of plasma corticosterone in mice. The RIA method proved to be a better system for a variety of reasons.

Firstly, it was simpler to perform and routinely 160 plasma samples, assayed in duplicate, could be analysed in one week.

Secondly, the sensitivity of the RIA method was better than the CPB method. Both assays however were sensitive enough to allow the use of small volumes of plasma (50 ul or less); an essential requirement for work where repeated blood samples are to be taken from mice.

Thirdly, the precision was better in the RIA method although, again, both systems operate within the normally accepted range of variation and accuracy (Vecsei, 1974).

Fourthly, antibodies are believed to be more stable than natural binding proteins and consequently less likely to deteriorate in storage.

Finally, the RIA method is more specific than CPB method. Since it is believed that the primary corticosteroid in mice is corticosterone (eg. Clark and Nowell, 1978) and the antibody used here is more specific than binding protein in human or dog plasma (Murphy, 1967) or the antibody used by Gross et al, (1972) it is concluded that this RIA system genuinely measures corticosterone.

The "normal" values found here are in general agreement with those found by Grad and Khalid, (1969); Spackman and Riley, (1974) and Barlow et al, (1975) but are
lower than those found by Brain and Nowell, (19712); Solem, (1966); Hennessey et al, (1977) and Schwartz et al, (1974). Since these workers have utilised fluorometric methods it is possible that the differences are a result of known interference factors in plasma (eg. Diez et al, 1976).

Statistical Analysis of Results

A characteristic feature of plasma corticosterone values are their variability. Individual values can range from less than 10 ng/ml to over 2000 ng/ml in mice. Even apparently identical mice sampled at one point in time can have values ranging from 50-350 ng/ml despite rigidly controlling conditions. It is possible that this is a feature of the episodic release of corticosterone (eg. Yates and Maran, 1974).

Results in this study have mostly been expressed as means ± standard error. However, frequently the data do not conform to the necessary prerequisites of parametric statistical analysis. Notably, it cannot be assumed that the distribution of values follows a normal distribution nor that variances are equal. Because of this, most of the statistical analysis has been performed using non-parametric statistical tests (Siegel, 1956). p<0.05 is considered statistically significant.

To summarise: Methodology has been considered. Vaginal smearing has been chosen as the method for assessing ovarian function. Measurement of plasma levels of corticoid by saturation analysis has been chosen as the method of assessing adrenocortical function. Adrenal weights can be difficult to interpret. Fluorometric assay methods in the absence of a chromatographic step are unspecific for
measuring corticosterone. Two saturation analysis methods have been developed, a competitive protein binding assay and a radioimmunoassay, both of which have been validated. The RIA method is preferred because it is more specific, sensitive, precise and simpler to perform. Two types of blood sampling procedures; retro-orbital puncture and decapitation have been used to assess normal values of corticosterone in the mouse. Corticosterone levels do not differ with either method. The retro-orbital method allows repeated blood sampling from the same mouse.
CHAPTER 4
Effects of Social Environment, Genetic Strain and Reproductive Condition on Basal Plasma Corticosterone.

Introduction

Studies of density dependent population regulation in the house mouse in "semi-natural" environments have indicated that at increased density there is a decline in female fertility and an increase in adrenal weight (Chapter 2).

However, laboratory studies on crowding female mice have failed to reveal consistent relationships between housing density, social stress and pituitary-adrenal function (eg. Brain, 1975). Most work using adrenal weight as a measure of adrenocortical activity has shown that individually housed female mice have larger, heavier adrenals than group housed mice (Brain and Nowell, 1972; Bronson and Chapman, 1968; Sackler et al, 1969; Schwartz et al, 1974; Weltman et al, 1962, 1966). This has formed the basis of a concept of "isolation stress" where social deprivation is seen as a stressor (eg. Weltman et al, 1966). Others found no difference in adrenal weights of individually and group housed females but showed histologically that the zone widths of the glomerulosa and fasciculata of the adrenal cortex were greater in the latter, implying increased adrenocortical activity, (Christian, 1960; Mody and Christian, 1962).

Different investigators have shown that group housed females have higher basal plasma corticosterone levels than individually housed females (Brain and Nowell, 1972; Solem, 1966), that individually housed females have higher levels than pairs (Schwartz et al, 1974) and that
there is no difference between grouped and individually housed animals, (Champlin, 1969; Goldsmith et al., 1977).

In view of these variations in results, adrenocortical activity in female mice in relation to housing conditions has been re-examined.

Methodological factors can be of critical importance in studies of adrenal function. Novelty, daily exposure to a novel situation and daily handling can all lead to increased plasma corticosterone levels in mice (Hennessey and Levine, 1977; Smolensky et al., 1978) as can general disturbance in an animal house (Halberg et al., 1959; Riley and Spackman, 1977). Goldsmith and Brain, (1978) have shown that in male mice simply a change in housing can lead to increased corticosterone levels and they have suggested that social instability may account for some discrepancies in reported results. Group size has similarly been implicated as a source of variation in different studies with numbers of animals per cage differing markedly (Goldsmith and Brain, 1978).

Most studies on housing conditions of female mice have used albino mice (Brain and Nowell, 1971; Goldsmith et al., 1977; Champlin, 1969; Sackler et al., 1969; Schwartz et al., 1974; Weltman et al., 1962, 1966; Bronson and Chapman, 1968). However, Thiessen, (1963) using male mice was only able to show adrenal enlargement in two strains of mice out of six, C57 Black mice and C3H/2 in response to density. Further, Levine and Treiman, (1969) found that C57 mice had a higher stress response than three other strains tested.

In this chapter I have examined the effect on basal corticosterone levels of some methodological factors, group
size and genetic strain of mouse.

**Methods**

Animals were blood sampled at 11 am (1 hour before lights out, at the circadian peak of adrenocortical activity) by retro-orbital puncture (Riley, 1960) after rapid ether anesthesia. Less than 400 µl of blood was collected from any animal. Cages were quietly removed from the animal house to an adjacent room and blood sampling was completed within 3 minutes of entering the room. The animal room was not entered more than five times per sampling session.

Blood was centrifuged within 45 minutes of sampling and plasma frozen and stored at -20°C until assayed. In six samples out of 297 insufficient blood was collected for assay. Individual plasma samples were assayed for corticosterone by RIA, (Chapter 3).

Results are expressed as mean ± standard error. Friedman ANOVA and Wilcoxon matched pairs signed ranks test were used for statistical analysis of correlated samples i.e. samples from the same mouse. Kruskal Wallis ANOVA and Mann Whitney U test were used for uncorrelated sample analysis.
Experiment 1

The purpose of the first experiment was to assess the effect on plasma corticosterone of taking more than one blood sample from mice. Fifty two virgin female TO strain mice aged 18 weeks, housed in groups of four from weaning, were randomly assigned to four experimental groups: group of 10 (GR^10), n=10, individually housed (I), n=10, groups of four with unfamiliar cage mates (GR4 UNFAM), n=16 and groups of four with the same animals from weaning (GR4 FAM), n=16. All animals were then undisturbed for 28 days, then blood sampled. Seven days later a second blood sample was taken.

Results

The mean plasma corticosterone values for the four experimental categories at the two blood sampling times are shown in Table 1. Statistical analysis revealed no differences either between the first and second blood samples or between the different groups.
### TABLE 1

The Effect of taking a blood sample on Basal Plasma Corticosterone Levels in Differentially Housed Female mice.

<table>
<thead>
<tr>
<th>Group Description</th>
<th>Mean Plasma Corticosterone Level (ng/ml)</th>
<th>Wilcoxon Test*</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st Blood Sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group of 10</td>
<td>G10 n=10</td>
<td>163 ± 25</td>
</tr>
<tr>
<td>Individually housed</td>
<td>I n=10</td>
<td>139 ± 14</td>
</tr>
<tr>
<td>Groups of 4</td>
<td>GR4 UNFAM n=16</td>
<td>141 ± 12.3</td>
</tr>
<tr>
<td>Groups of 4</td>
<td>GR4 FAM n=15</td>
<td>171 ± 18</td>
</tr>
</tbody>
</table>

Kruskal Wallis ANOVA** N.S. N.S.

*Wilcoxon Sign Rank Test for differences between means (correlated data).

**Kruskal Wallis ANOVA testing first and second blood sample means independently (uncorrelated data). N.S. = not statistically significant.
Experiment 2

The purpose of this experiment was to assess the effect of changing housing conditions on plasma corticosterone levels. Twenty virgin TO strain females aged 18 weeks, housed in groups of four from weaning, were randomly assigned to two experimental groups: a group of 10 (GIG) and 10 individually housed (IGI). All animals were undisturbed for two weeks, blood sampled, then their housing conditions exchanged i.e. the group of ten were individually housed and the individually housed were grouped into ten. The animals were undisturbed for a further two weeks, blood sampled a second time and rehoused in their original conditions. Seven days later a third set of blood samples were taken.

Results

The mean plasma corticosterone values are shown in Table 2. There were no statistically significant differences.
<table>
<thead>
<tr>
<th></th>
<th>1st Blood sample</th>
<th>2nd Blood sample</th>
<th>3rd Blood sample</th>
<th>Friedman ANOVA*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>GIG</strong>*n=9</td>
<td>148 ± 22</td>
<td>102 ± 7</td>
<td>144 ± 23</td>
<td>N.S.</td>
</tr>
<tr>
<td><strong>IGI</strong>*</td>
<td>169 ± 13.7</td>
<td>124 ± 12</td>
<td>175 ± 20</td>
<td>N.S.</td>
</tr>
<tr>
<td>Mann Whitney U test**</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

*Friedman ANOVA for correlated data

**Mann Whitney U test for testing difference between means (uncorrelated data).

*** See text

N.S. = not statistically significant
Experiment 3

The purpose of this experiment was to assess the effect of group size on plasma corticosterone levels. Thirty TO virgin females aged 13 weeks, housed in groups of four from weaning, were grouped together. They were undisturbed for 26 days, then 21 animals were randomly selected and blood sampled (sampling over two days).

Results

The mean plasma corticosterone value was $136 \pm 10.7$ ng/ml. There was no statistically significant difference between this value and those in any of the groups in Experiment 1.
Experiment 4

In previous experiments animals were housed in groups of four from weaning, then assigned to experimental groups and housed with unfamiliar cage mates for less than a month. The purpose of this experiment was to assess the effect of a longer treatment of housing starting at weaning, on plasma corticosterone levels. Fifty TO virgin female mice were randomly assigned to either 3 groups of 10 animals per cage or individual housing at weaning. At 12 weeks of age (after 81 days of differential housing) all animals were blood sampled and vaginal smears taken.

Results

The mean plasma corticosterone values are shown in Figure 1. There were no statistically significant differences between individually or grouped housed mice. However, there was a statistically significant difference between plasma corticosterone levels of mice at pro-oestrus compared with mice at other stages of the oestrous cycle grouped (p<0.05) and individually housed (p<0.001) mice, (Figure 1). 37% of individually housed females were in pro-oestrus when blood sampled compared to 7% of group housed mice.
Fig. 1  Plasma Corticosterone levels in female TO mice after 81 days of differential housing

I - Individually housed
G10 - Groups of 10
Number of mice sampled is shown in columns
Experiment 5

The purpose of this experiment was to investigate the effect of differential housing on plasma corticosterone levels in an inbred strain of mice. Sixty two C57/B110 virgin female mice aged 18 weeks, housed in groups of four from weaning, were randomly assigned to three experimental conditions: individually housed (n=12), groups of ten (n=20) and a group of thirty (n=30). All animals were undisturbed for 34 days, then blood sampled. Only fifteen animals were blood sampled from the group of thirty. Blood sampling took place over three days.

Results

The mean plasma corticosterone values are presented in Figure 2. There was no significant difference between the categories, using KW ANOVA.
Fig. 2  Plasma Corticosterone levels in differentially housed C57Bl/10 female mice
I - Individually housed
G10 - Groups of 10
G30 - Group of 30
Number of mice sampled is shown in columns
KWANOVA  p > 0.3
Fig. 3 Summary of results of differential housing in female TO mice
I - Individually housed
G4 - Groups of 4
G10 - Groups of 10
G30 - Group of 30
Number of mice sampled is shown in columns
Data from Expt 1 are means of blood sample 1 and 2
KWANOVA \( p > 0.1 \)
Discussion

A characteristic feature of the results presented here and elsewhere (eg. Brain and Nowell, 1971; Bronson and Chapman, 1968; Champlin, 1969; Goldsmith et al., 1977; see Chapter 5) is their variability and inconsistency. Whilst it is recognised that the pituitary-adrenal system in mice is very sensitive to external stimuli and various methodological factors (Goldsmith et al., 1977; Halberg et al., 1959; Hennessey and Levine, 1977; Riley and Spackman, 1977; Smolensky et al., 1978) these fail to explain all the variation in results. Thus in experiments described here, mice have been reared from conception under controlled conditions with only minimal disturbance then blood sampled rapidly (ie. before the stress response). Previous blood sampling did not affect plasma corticosterone levels one week later. In contrast to male mice (Goldsmith and Brain, 1978) there was no elevation of plasma corticosterone in females as a result of changed housing conditions or social instability. The size of groups (individually housed, groups of four, ten and thirty) had no direct effect on plasma corticosterone. Again in contrast to males, duration of differential housing did not have a significant effect on plasma corticosterone. The difference between males and females is not that surprising if it is remembered that amongst males fighting is common in establishing dominance whereas in females fighting is rare.

If the effects of differential housing on plasma corticosterone in female mice were weak or indirect other factors could blur any difference that might exist. One major source of variability in the pituitary-adrenal system
is the activity of the reproductive system. Some workers (Brain and Nowell, 1971; Brain et al., 1971; Bronson and Chapman, 1968) have argued that adrenal weight increases found in individually housed females are due to gonadal activity; in particular increased oestrogen levels. It has been shown repeatedly that in all-female groups of mice, oestrous cycles become irregular and longer than in females individually housed (e.g. Whitten, 1966). Brain and Nowell, (1971) showed that the percentage of animals in pro-oestrus/oestrus is higher in individually housed animals than in group housed and the results presented here (Experiment 4) support that conclusion. Further, results here and elsewhere (Chapter 7) have shown that basal plasma corticosterone levels at pro-oestrus are almost twice as high as at other stages of the oestrous cycle. Re-analysis of the data from experiment 4 (Figure 1) to exclude pro-oestrus values reveals a higher mean value for the grouped than individually housed mice. Whilst this difference is not significant, collation of results from several experiments (Chapters 4, 5, 6) excluding pro-oestrus values revealed a significant difference. (Individually housed mean plasma corticosterone value = 128 ± 8.3 (S.E.M.), n=49; Groups of 10 mean = 162 ± 9.1, n=42. Significantly different at p<0.0005 level with Mann Whitney U test. Unpublished data.)

These results and others (Chapter 5) would imply that grouping does have some small effect on basal plasma corticosterone. However, it is not known whether this is related directly to social factors or mediated by, for example, reproductive function. Pseudopregnant oestrous cycles are characteristic of all-female groups (e.g. Whitten, 1966).
Since prolactin and progesterone secretion increases during pseudopregnancy (Barkley et al., 1978; Choudary and Greenwald, 1969; Smith, Freeman and Neill, 1975) and prolactin (e.g., Myers et al., 1975; Ogle and Kitay 1979; Stern and Voogt, 1973/4; Vasquez and Kitay, 1978) and progesterone (Phillips and Poolsanguan, 1978; Rodier and Kitay, 1974) can alter pituitary-adrenal function, the effect of grouping may not necessarily be directly behavioural.

In Chapter 7 I have argued that if the pro-oestrus rise in plasma corticosterone was oestrogen mediated, most of the corticosterone would be bound to corticosteroid-binding-globulin (CBG), since oestrogen induces CBG (Gala and Westphal, 1966; Fortier et al., 1970). If the increase was stress-induced much of the corticosterone would be unbound (Knigge and Hoar, 1963). Since CBG bound and unbound corticosterone have different physiological actions (Ottenweller et al., 1979) a measure of total corticosterone would not necessarily reflect the degree of "stress", in terms of the General Adaptation Syndrome (Selye, 1950). Thus, the use of plasma corticosterone or adrenal weight as a measure of either "social stress" or "isolation stress" is confounded by the reproductive state of the animals, which can itself be altered by social conditions (Ryan and Schwartz, 1977) independently of stress.

The experiments reported here offer no support for either "isolation stress" (Sackler et al., 1969; Schwartz et al., 1974; Weltman et al., 1962, 1966) or "social stress" (Christian, 1971) in female groups. It remains unclear as to what social behaviours in females can cause the adrenal activation and reproductive failure found in freely growing
populations (Christian, 1956, 1961, 1963; Crowcroft and Rowe, 1957, 1958; Snyder, 1968). Apart from the type of adrenal measure used to assess hyperactivity, two factors may explain the differences between laboratory and "semi-natural" studies; the genetic strain of mouse and the presence of males. Most studies with freely growing populations have used wild mice, whereas laboratory work has used inbred or albino mice. Both TO outbred albino strain and C57 Bl/10 inbred mice were relatively unaffected by grouping in this study. However, Levine and Treiman, (1969) have shown that even C57 Bl mice have smaller stress response than wild mice.

Little is known of the influence of males on female adrenocortical function (Chapter 6). However, male mice can affect the oestrous cycle and pregnancy quite dramatically (eg. Parkes and Bruce, 1961; Whitten, 1966; Chapter 8).
CHAPTER 5

Effect of Social Environment on Plasma Corticosterone: Circadian Rhythm and Stress Responsiveness.

Introduction

Studies on the effect of housing conditions on pituitary-adrenal function in female mice have yielded inconsistent results (eg. Brain, 1975). In Chapter 4, looking at basal plasma corticosterone levels at the presumed circadian peak, no difference was found between individually and group housed female mice, except when pro-oestrus values were omitted from the analysis. With the development of a method for repeated blood sampling from mice it has been possible to carry out a longitudinal study of mice subjected to different housing conditions. In this Chapter the circadian rhythm and stress responsiveness of adrenocortical function have been investigated. Since knowledge of the oestrous cycle is important in such studies (Chapter 4) and daily handling or disturbance can alter adrenocortical function (Smolensky et al, 1978; Hennessey and Levine, 1977) the effect of daily vaginal smearing on plasma corticosterone levels was also examined.

The circadian rhythm of pituitary-adrenal activity is considered endogenous and is usually entrained to the light-dark regime, (Halberg et al, 1958). However, many factors can alter both the steroid response at different times of the day and the rhythm phase. Thus food and water deprivation can alter the phasing of the cycle of plasma corticosterone (Gray et al, 1978; Johnson and Levine, 1974; Krieger, 1974; Wilkinson et al, 1979) as can acute and chronic stressors (Paris and Ramaley, 1974; Brodish, 1974). Likewise,
reproductive condition can alter the adrenal rhythm. Male and female rats have different phasing of corticotrophin releasing factor (CRF), (Hiroshige, 1974) and ovariectomy advances the peak of CRF. Also, it has been shown that pregnant rats have a longer peak of plasma corticosterone (Grota and Ader, 1970), lactating rats have no rhythm (Endroczi, 1974) and infertile mice and rats may have no rhythm (or desynchronised free running rhythms), (Paris and Ramaley, 1974; Ramaley, 1975). Since reproductive condition can alter rhythmic pituitary-adrenal function and social factors, including housing conditions strongly influence ovarian function in female mice (eg. Whitten, 1966) it is of particular interest to examine the circadian rhythm of plasma corticosterone in individually and group housed female mice.

Apart from the circadian rhythm phase, pituitary-adrenal responsiveness to stressors can vary throughout the day. It has been shown with a variety of stressors that a higher corticosterone response occurs at the circadian trough than at the peak (Ader et al, 1967; Allen et al, 1975; Dunn et al, 1972; Engeland et al, 1977; Haus and Halberg, 1960; Ungar and Halberg, 1962, 1963; Ungar, 1964). In contrast, others have shown the same response at peak and trough (Retiene et al, 1967; Zimmerman and Critchlow, 1967). Brain and co-workers (Brain, 1975; Brain and Nowell, 1971; Goldsmith et al, 1976; Goldsmith et al, 1977) have argued that individually housed females may be more stress-responsive than group housed females. It is not known if this is apparent at all times of the day.
Methods

Sixty TO virgin female mice, housed in groups of four at weaning, were randomly assigned to twelve groups of five animals at the age of 86 days and rehoused individually. Twelve days later blood sampling commenced at 11 am, then at 4 hourly intervals for 48 hours. Ten animals were blood sampled under unstressed conditions (ie. within 3 minutes of disturbance) at each time point as described in Chapter 4. Each animal was sampled twice, the second sample being 12 or 36 hours after the first. A vaginal smear was taken after each blood sample, then daily for six days. The blood-sampling procedure was then repeated starting at 11 am with the same mice being sampled at the same time as previously sampled. A third blood sample (to assess stress response levels) was taken from each mouse 15 minutes after the second.

All the animals were then rehoused into six groups of ten animals in large cages, ensuring that blood samples could be taken from the groups of ten mice at the same time of day as before without disturbing their cage for 12 hours prior to blood sampling. Seventeen days after grouping all the mice were blood sampled at the same times as used previously. Smearing was the same and after six days resampling took place.

To summarise, a total of ten blood samples were taken from each mouse. Two blood samples from unstressed mice, 12 or 36 hours apart were taken from animals in each of four experimental conditions; individually housed (I), individually housed and smeared (Is); group housed (G) and group housed and smeared (Gs). In addition two "stress" samples were taken,
one at the end of individual housing and one at the end of group housing. Different groups of animals were sampled to give values at four hourly intervals throughout 48 hours.

In a second experiment 40 virgin TO females, aged 120 days, housed in groups of four from weaning, were randomly assigned to four experimental groups, two groups of 10 and two groups of 10 individually housed. All animals were undisturbed for 18 or 19 days then blood sampled at 11 am. One group of 10 and 10 individually housed mice were sampled for basal levels then resampled 15 minutes later, for a stress responsiveness level. The other animals were etherised only (until unconscious) then blood sampled 15 minutes later.

All blood samples were stored and assayed for plasma corticosterone as described in Chapter 3.
Results

Mean basal plasma corticosterone levels for the first experiment are shown in Figure 1. Friedman ANOVA revealed significant differences between the four conditions, I, Is, G, and Gs at 3 am, 7 am and 11 am (p<0.01; p<0.01; p<0.001) but not at 3 pm, 7 pm or 11 pm. Further analysis using the Wilcoxon test revealed significant differences between individually housed (I) and group housed (G) values at 3 am, 7 am, and 11 am (p<0.01; p<0.01; p<0.005) and between individually housed and smeared (Is) and group housed and smeared (Gs) at 3 am, 7 am, and 11 am (p<0.025; p<0.005; p<0.025).

Smearing had a statistically significant effect only at 7 am in group housed mice and at 11 am in individually housed mice (p<0.025; p<0.05). At both these times unsmeared animals had lower values than the equivalent smeared.

Stressed and unstressed mean plasma corticosterone values for individually housed and smeared and group housed and smeared are shown in Figure 2. Kruskal Wallis ANOVA of stress values throughout the day reveal no significant difference in either grouped (p>0.1) or individually housed (p>0.3) values implying an absence of circadian rhythm in stress levels. However, Wilcoxon signed rank test for differences between matched pairs revealed a highly significant difference between individually and group housed animals (p<0.00001). The latter had the higher value.

Mean plasma corticosterone values for experiment 2 are shown in Figure 3. There are no significant differences between individually and group housed unstressed or ether
stressed values. However in agreement with the first experiment group housed mice had a significantly higher stress level after blood sampling and ether than individually housed mice ($p < 0.05$).
Fig. 1 Plasma corticosterone levels throughout the day in female mice subjected to different housing conditions

- Individually housed
- Individually housed and smeared
- Group housed
- Group housed and smeared

<table>
<thead>
<tr>
<th>Time of day</th>
<th>3AM</th>
<th>7AM</th>
<th>11AM</th>
<th>3PM</th>
<th>7PM</th>
<th>11PM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Test</td>
<td>p&lt;0.01</td>
<td>p&lt;0.01</td>
<td>p&lt;0.001</td>
<td>p&gt;0.7</td>
<td>p&gt;0.5</td>
<td>p&gt;0.5</td>
</tr>
<tr>
<td>Friedmann ANOVA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Fig. 2  Basal and Stress Corticosterone levels throughout the day in female mice subjected to different housing conditions.

- Basal: Individually housed
  - Group housed

- Stress: Individually housed
  - Group housed

Plasma corticosterone (ng/ml) vs. Time of day:
- 3AM, 7AM, 11AM, 3PM, 7PM, 11PM
Fig. 3  Plasma corticosterone levels in differentially housed female mice

- Individually housed
- Group housed

Plasma corticosterone (ng/ml)

MWU test  N.S.  p<0.05  N.S.

Unstressed  Blood sampled  Ether stress
and ether stress
Discussion

The finding that grouped female mice have significantly higher basal plasma corticosterone levels than individually housed mice at the circadian peak contrasts with other results where no significant difference was found (e.g., Chapter 4; Champlin, 1969). However, it has been shown that basal plasma corticosterone levels of animals at pro-oestrus are elevated (Chapter 4 and 7) and that when these values are excluded from a comparison of grouped and individually housed animals, the former have higher values (Chapter 4). On average, only 16% of individually housed mice were at pro-oestrus when sampled compared to 37% in a previous experiment (Chapter 4). This could explain the significant difference found here but not elsewhere. However, the absence of any significant difference between individually and group housed animals at 3 pm, 7 pm, and 11 pm in this experiment persists even when pro-oestrus values are excluded from the analysis.

The significance of a difference in plasma corticosterone in differently housed female mice at only some times of the day is unclear. It is possible that the higher basal levels at and before the circadian peak are related to increased social interaction in a group, as postulated by Christian, (1971). The effect of an extremely active or otherwise aberrant individual on others in a group cannot be ignored as an explanation for higher basal levels found in groups. As discussed in Chapter 4, the difference may not necessarily be a direct behavioural effect on adrenocortical function but may be mediated by the reproductive system.

The argument that the difference in basal levels
found may reflect methodological factors (Brain, 1975; Goldsmith et al, 1976, 1977) eg. previous disturbance, blood sampling, social stability or differential stress responsiveness seems unlikely since elevation of values for grouped animals occurred at only three times of the day out of six sampling points. Further, it has been shown that plasma corticosterone levels are unaffected by blood sampling 12 hours (Chapter 3) or one week (Chapter 4) previously or social instability.

One week of daily vaginal smearing at the circadian peak of adrenal activity did elevate plasma corticosterone significantly but only at and around this time of day. This is in agreement with others (Hennessey and Levine, 1977; Smolensky et al, 1978) who found that the pituitary-adrenal system in mice is sensitized by daily "training" rather than habituated to it.

The presence of a circadian rhythm of basal plasma corticosterone in mice with highest values, just before lights off is confirmed here (Halberg et al., 1959; Spackman and Riley, 1974; Solem, 1966; Ungar and Halberg, 1962). However, neither housing nor smearing had any apparent effect on the rhythm phase. Paris and Ramaley, (1974) found a change in phase of plasma corticosterone rhythms in mice as a result of bi-daily stress (but not vaginal smearing) and noted altered fertility. Ramaley, (1975) found that a large percentage of rats that had no apparent corticosterone rhythm also had irregular vaginal smears. It is well known that grouping female mice leads to spontaneous pseudopregnancy (Ryan and Schwartz, 1977) and anoestrus (Whitten, 1966). The evidence presented here indicates that the altered reproductive state,
brought about by grouping, does not affect the phase of pituitary-adrenal activity.

The finding that there is no circadian rhythm of stress levels and therefore, that the corticosterone response to a stressor is greater at the trough than at the peak, is in agreement with others (in rats: Ader et al., 1967; Allen et al., 1975; Dunn et al., 1972; Engeland et al., 1977; Pfister and King, 1976; in mice: Haus and Halberg, 1960; Ungar and Halberg, 1962, 1963; Ungar, 1964). However, contrary to other reports (Brain, 1975; Brain and Nowell, 1971; Goldsmith et al., 1976, 1977) stress levels were higher in grouped animals than individually housed ones, using blood sampling and ether as stressors. This result was consistent throughout the day and was found in mice previously unused or disturbed. However, in animals subjected to ether stress there was no difference in plasma corticosterone levels, in agreement with Brain and Nowell, (1971).

It is known that pituitary-adrenal response to stressors can be dependent on the type of stressor and its intensity (Ader et al., 1967; Friedman et al., 1967; Dallman and Jones, 1973). Blood sampling and ether may constitute a more intense or different type of stress than ether alone. Activation of the pituitary-adrenal system can operate through different pathways (Dallman and Yates, 1968) and it has been shown that ether can stimulate the system at the median eminence, without higher brain centres (Matsuda et al., 1964). Since higher brain centres would be involved in social stress (Bajusz, 1964; Christian, 1971) the relevance of ether stress responsiveness to social behaviours is questionable.
The problem of which parameter of pituitary-adrenal activity to use as a measure of stress, remains unresolved (Chapter 1). Ader et al. (1967) discussed this in relation to the stress response; the duration of elevation of corticosterone levels. The measurement of basal levels of plasma corticosterone as a correlate of stress is equally problematical being open to interference from reproductive factors, for example.

In this and the previous chapter, basal levels, circadian rhythm phase and stress levels (at a fixed time point after stimulation) have been examined in relation to housing density of female mice. Whilst some differences have been found, higher basal levels of corticosterone before and at the circadian peak (excluding pro-oestrus values) and higher levels in response to blood sampling and ether in groups of ten mice, there is no evidence of the permanently elevated basal levels associated with the chronic stress of Selye's G.A.S. and implied in Christian's hypothesis of regulation of population size by social behaviour (Christian, 1971).
CHAPTER 6

Effects of Male Presence and Male Urine on Plasma Corticosterone Levels.

Introduction

In Chapter 4 it was concluded that since no evidence was found of social stress in all-female groups, that the presence of a male could be important in eliciting the adrenocortical activity and failure in reproduction found in freely growing confined populations of house mice (e.g. Crowcroft and Rowe, 1957, 1958; Christian, 1956, 1961, 1963). This factor has been ignored in most studies of differential housing of female mice. Christian, (1960, 1961) demonstrated that the adrenal response to grouping female mice, in terms of adrenal weight, was smaller in the absence of males than when males were present. It has been presumed that this is a by-product of inter-male aggression (e.g. Bronson, 1967) although the evidence for this is scanty (Calhoun, 1956). Little else is known of the influence of males on pituitary-adrenal function.

It is known that male presence can radically influence female ovarian function in the mouse (e.g. Whitten, 1966; Parkes and Bruce, 1961) and that these effects are mediated by pheromones found in male mouse urine (e.g. Marsden and Bronson, 1965; Dominic, 1966). It is also known that pheromones from grouped males can stimulate adrenocortical activity in other males (Ropartz, 1966; Archer, 1969) perhaps by some aggression modifying cues (Mugford and Nowell, 1970). It is not known if pheromones from male urine can activate female adrenocortical activity. If this were so it may offer an alternative explanation to the suggestion
Experiments were performed to assess the effects of male presence and male urine on basal and "stress" levels of plasma corticosterone. Since basal plasma corticosterone values of females at pro-oestrus can be nearly twice the values at other stages of the oestrous cycle (Chapters 4 and 7), pro-oestrus values are considered separately from others.

that accidental male attacks on females cause social stress (eg. Calhoun, 1956). Crowcroft and Rowe, (1957) reported that they could find no evidence of males attacking females.
Methods

Virgin TO female mice aged 14-16 weeks, were randomly assigned to four experimental treatments; individually housed with one male, kept in a separate wiremesh holder in each cage (Im), two groups of ten females with one male in a separate wiremesh holder in each cage (Gm), individually housed and given male urine daily (Imu) and a group of ten given male urine daily (Gmu). Mice were undisturbed for 14-16 days after rehousing, then blood sampled one hour before lights out as described in Chapter 4. A second blood sample was taken from each mouse 20 minutes after the first. A vaginal smear was taken after blood sampling, then daily for a week.

Sexually mature male TO mice were used for housing with females and for urine collection. Eight males were housed in a cage with a wiremesh floor and aluminium funnel to collect urine. A glass ball at the bottom of the funnel diverted faeces. No food was provided so the males were changed daily for a different eight. 2 ml of fresh male urine per female mouse was dispensed onto bedding daily i.e. group housed females had 20 ml daily in the cage.

A second experiment was performed to assess the short term effect of male urine on plasma corticosterone levels in females. Virgin females were randomly assigned to individual housing or group housing in tens and undisturbed for two weeks after rehousing. Thirty minutes after lights out, at a time when mice are usually active (eg. Halberg et al, 1959), 2 ml of male urine per female mouse was dispensed into half the cages and 2 ml of water into the remaining cages. Care was taken to do this quietly and rapidly. Blood
samples were taken from each treatment group 30 minutes, 4 hours or 24 hours after urine or water application.

Plasma samples were stored and assayed for corticosterone as described in Chapter 3.
Results

Mean basal and stress corticosterone levels of female mice differentially housed with males or given male urine daily are shown in Table 1. Individually housed mice given male urine daily had significantly higher basal levels than the group given urine or individually or group housed mice with a male, (Mann Whitney U test, p<0.01). The group given male urine had significantly lower stress levels than the other treatments (Mann Whitney U test, p<0.01). The stress levels were otherwise not significantly different.

A breakdown of data into mean plasma corticosterone values taken from mice at pro-oestrus and those at other stages of the oestrous cycle is shown in Table 2. Non pro-oestrus basal values for individually housed mice given male urine daily were significantly higher than the other categories (Mann Whitney U test, p<0.01). However pro-oestrus values were only significantly higher in the mice individually housed with males (Mann Whitney U test, p<0.05). There were too few pro-oestrus values in the group given male urine to be included in the statistical analysis. There were no significant differences in stress levels in any of the categories with either pro-oestrus or non pro-oestrus values.

Mean plasma corticosterone levels from the second experiment are shown in Table 3. Kruskal Wallis ANOVA revealed a significant difference between categories in values taken 30 minutes after application of male urine (H=13.07, p<0.01) but not 4 or 24 hours after. Females individually housed given urine and sampled 30 minutes later had significantly higher values than the controls given water only (Mann Whitney U test, p<0.001) but there was no difference
between grouped animals.

Further analysis of data from animals sampled at 30 minutes, separating pro-oestrus and non pro-oestrus values, is shown in Table 4. Individually and group housed females given male urine had significantly higher non pro-oestrus plasma corticosterone levels than the water controls (Mann Whitney U test, p < 0.025; p < 0.05). Individually housed females given urine also had higher pro-oestrus values than water controls (Mann Whitney U test, p < 0.05).
TABLE 1

Plasma Corticosterone Levels in Grouped and Individually housed Female mice either given Male Urine daily or housed with a Male.

<table>
<thead>
<tr>
<th></th>
<th>Basal</th>
<th>Stress</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individually housed with male (Im)</td>
<td>168 ± 13</td>
<td>567 ± 23</td>
</tr>
<tr>
<td>n=20</td>
<td>n=20</td>
<td></td>
</tr>
<tr>
<td>Group housed with male (Gm)</td>
<td>170 ± 16</td>
<td>552 ± 24</td>
</tr>
<tr>
<td>n=19</td>
<td>n=19</td>
<td></td>
</tr>
<tr>
<td>Individually housed and male urine (Imu)</td>
<td>264 ± 18</td>
<td>566 ± 30</td>
</tr>
<tr>
<td>n=14</td>
<td>n=14</td>
<td></td>
</tr>
<tr>
<td>Group housed and male urine (Gmu)</td>
<td>134 ± 11</td>
<td>435 ± 39</td>
</tr>
<tr>
<td>n=10</td>
<td>n=10</td>
<td></td>
</tr>
</tbody>
</table>
**TABLE 2**

Plasma Corticosterone Levels in Grouped and Individually Housed Female mice either given Male Urine daily or housed with a Male: Separation of Pro-oestrus and other values.

<table>
<thead>
<tr>
<th></th>
<th>Basal Pro-oestrus Values</th>
<th>Basal Other Values</th>
<th>Stress Pro-oestrus Values</th>
<th>Stress Other Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individually housed with male (Im)</td>
<td>223 (\pm) 16</td>
<td>150 (\pm) 13</td>
<td>609 (\pm) 67</td>
<td>552 (\pm) 22</td>
</tr>
<tr>
<td></td>
<td>n=5</td>
<td>n=15</td>
<td>n=5</td>
<td>n=15</td>
</tr>
<tr>
<td>Group housed with male (Gm)</td>
<td>235 (\pm) 40</td>
<td>159 (\pm) 16</td>
<td>552 (\pm) 71</td>
<td>553 (\pm) 26</td>
</tr>
<tr>
<td></td>
<td>n=3</td>
<td>n=16</td>
<td>n=4</td>
<td>n=15</td>
</tr>
<tr>
<td>Individually housed and male urine (Imu)</td>
<td>289 (\pm) 31</td>
<td>240 (\pm) 14</td>
<td>562 (\pm) 60</td>
<td>569 (\pm) 19</td>
</tr>
<tr>
<td></td>
<td>n=7</td>
<td>n=7</td>
<td>n=7</td>
<td>n=7</td>
</tr>
<tr>
<td>Group housed and male urine (Gmu)</td>
<td>175</td>
<td>124 (\pm) 12</td>
<td>352</td>
<td>456 (\pm) 46</td>
</tr>
<tr>
<td></td>
<td>n=2</td>
<td>n=8</td>
<td>n=2</td>
<td>n=8</td>
</tr>
<tr>
<td></td>
<td>After 30 minutes</td>
<td>After 4 hours</td>
<td>After 24 hours</td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>------------------</td>
<td>---------------</td>
<td>----------------</td>
<td></td>
</tr>
<tr>
<td><strong>Individually housed and water</strong></td>
<td>137 ± 12</td>
<td>91 ± 8</td>
<td>136 ± 13</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=10</td>
<td>n=10</td>
<td>n=10</td>
<td></td>
</tr>
<tr>
<td><strong>Individually housed and male urine</strong></td>
<td>339 ± 58</td>
<td>106 ± 14</td>
<td>148 ± 11</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=9</td>
<td>n=9</td>
<td>n=9</td>
<td></td>
</tr>
<tr>
<td><strong>Group housed and water</strong></td>
<td>192 ± 44</td>
<td>108 ± 9</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=10</td>
<td>n=10</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Group housed and urine</strong></td>
<td>210 ± 31</td>
<td>118 ± 18</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td>n=9</td>
<td>n=10</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 4
Plasma Corticosterone Levels in Grouped and Individually housed Female mice 30 minutes after application of Male Urine or Water to the cage: Separation of Pro-oestrus and Other values.

<table>
<thead>
<tr>
<th></th>
<th>Plasma Corticosterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pro-oestrus</td>
</tr>
<tr>
<td>Individually housed and water</td>
<td>165 ± 29</td>
</tr>
<tr>
<td></td>
<td>n=3</td>
</tr>
<tr>
<td>Individually housed and urine</td>
<td>532 ± 93</td>
</tr>
<tr>
<td></td>
<td>n=3</td>
</tr>
<tr>
<td>Group housed and water</td>
<td>560</td>
</tr>
<tr>
<td></td>
<td>n=1</td>
</tr>
<tr>
<td>Group housed and male urine</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Discussion

The continual presence of a male seems to have only a small effect on plasma corticosterone levels in individually and group housed female mice. Both basal and stress levels are similar to those found in females in the absence of males (Chapter 5). However, it is noted that the differences in both basal and stress levels of plasma corticosterone in individually and group housed females found previously (Chapters 4 and 5) did not occur in this experiment, presumably as a result of male presence.

The most striking feature of the results here is that male urine can activate the pituitary-adrenal system of the female. Daily application of male urine elevated plasma corticosterone only in individually housed females. It has been demonstrated that daily disturbance, novelty or handling, which are known stressors of mice (e.g., Levine and Treiman, 1964) can lead to a sensitization of the pituitary-adrenal system and increase plasma corticosterone levels at the time of daily disturbance (Hennessey and Levine, 1977; Smolensky et al., 1978). Thus it is possible that male urine acts as a stressor and when given daily increases plasma corticosterone levels. The failure of male urine given daily to stimulate grouped females, which in fact have generally lower values, is puzzling.

The acute adrenal response to urine is not so easily interpreted as a response to disturbance or novelty, as significant differences over similar doses of water were recorded (Table 3) although it might be argued that an odorous substance would have been a better control than water. The elevation of plasma corticosterone certainly
follows the same time course as the novelty response recorded by Pfister and King, (1976). Thus 30 minutes after application to the cage bedding of male urine, plasma corticosterone levels are elevated over water controls and by 4 hours this difference has disappeared. The adrenal stimulation by male urine after 30 minutes is apparent in both grouped and individually housed females when pro-oestrus values are excluded. The response to male urine of individually housed females at pro-oestrus is large and of a similar order to the stress response after blood sampling and ether exposure. This large response implies that females at pro-oestrus are either generally highly stress responsive or are responding to a specific cue in male urine.

Given the known pheromone-bearing properties of mouse urine, it is more likely that the animals are responding to sexual or other information. Urine from mature male mice is known to be an attractant to female mice (Caroom and Bronson, 1971; Scott and Pfaff, 1970; Hayashi and Kimura, 1973) so it is possible that male-induced adrenal activation is "arousal" primarily associated with sexual stimulation. It is not known if female urine has a similar effect on females. It has been demonstrated that exposure of young female mice to a male can lead to acute changes in serum LH and plasma oestradiol (Bronson and Desjardins, 1974). Since both LH and oestradiol can stimulate adrenocortical activity (Phillips and Poolsanguan, 1978; Kitay, 1963; Coyne and Kitay, 1969) it is possible that adrenocortical activation of adult females by males is mediated by these reproductive hormones.

An alternative explanation is that the adrenal is activated by an alarm pheromone. Since the males used to
collect urine were subjected to severe stress (food deprivation, handled, rehoused daily and grouped) and stressed mice can produce an alarm pheromone (Rottman and Snowdon, 1972; Muller-Velten, 1966; Carr et al, 1970) it is possible that a stress pheromone activates the adrenal. Even the presence of a "strange mouse" odour may be sufficient stimulus for adrenocortical activation, an explanation which would account for the absence of effect of continual male presence. Rowe and Redfern, (1969) found a low level of aggressiveness in family groups of mice due to the olfactory recognition of individuals and found increased aggressive encounters on the introduction of a strange mouse.

It is clear that the role of pheromones in social interactions in mice is still not fully understood. It is suggested that this may be a fruitful area of research.
CHAPTER 7

Plasma Corticosterone Fluctuations during the Oestrous Cycle.

Introduction

It has been shown that activity of the pituitary-adrenal axis varies with the oestrous cycle in the rat, (eg. Critchlow et al, 1963). Buckingham et al, (1978) and Raps et al, (1971) have demonstrated that plasma corticosterone levels are higher both in the morning and evening of the day of pro-oestrus than at other times in the cycle, although there is no interruption in the normal circadian pattern of variation. Champlin, (1969) found no difference in plasma corticosterone at different stages of the cycle in mice. However, in this study, pro-oestrus values were elevated at the circadian peak compared with others (Chapter 4).

The purpose of this experiment was to examine variations in plasma corticosterone in the mouse more thoroughly in respect to the time of the day sampled and the stage of the oestrous cycle. This was considered important because in any attempt to correlate stress-induced adrenocortical activity and inhibited reproductive activity the details of the interaction of the pituitary-adrenal system and the normal reproductive cycle need to be known.

Since Schwartz, (1969) has shown that five day cycling rats differ in their hormonal balance from four day cyclers, only mice on four day cycles were used. These were induced by the presence of males, as four day cycles are otherwise uncommon in mice (Bingel and Schwartz, 1972).
Methods

Two hundred virgin TO females obtained from the supplier were used. They were allowed three weeks to settle into the new animal quarters, then randomly assigned to six experimental groups for blood sampling at four hourly intervals. Half the mice were kept in reversed lighting (L:D, 14:10 lights on at 2200 hours) and half on normal lighting (L:D, 14:10 lights on at 0800 hours). Results are expressed in relation to normal lighting photoperiod. Mice were individually housed each with a male in an inner wire cage, undisturbed for two weeks then blood sampled once by retro-orbital puncture as before, (Chapter 4) at the different times of the day. One vaginal smear was taken 1-3 days before, and 5 consecutive daily smears starting immediately after blood sampling. Plasma samples were stored and assayed for plasma corticosterone as described previously, (Chapter 3). Only 4 day cycling mice were included in the data. Values for metoestrus and dioestrus were combined and means compared by the Mann Whitney U test.
Results

The circadian rhythm of plasma corticosterone concentration was maintained through all stages of the oestrous cycle, (Figure 1) with highest values always obtained at 2100 hours, one hour before lights out. Compared with values recorded at metoestrus and dioestrus elevated corticosterone levels were recorded throughout the day and night of pro-oestrus and only subsided late in the day of oestrus. Statistically significant differences are shown in Table 1.
Fig. 1  Plasma Corticosterone levels at different times of the day during the Oestrous cycle of the mouse
### TABLE 1
Concentration of Plasma Corticosterone at different times of the day during the Oestrous Cycle of the Mouse.

<table>
<thead>
<tr>
<th>Time</th>
<th>Pro-oestrus ng/ml</th>
<th>Oestrus ng/ml</th>
<th>Metoestrus/Dioestrus ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>0500</td>
<td>42.5 ± 12.6</td>
<td>93.0 ± 14.3</td>
<td>45.6 ± 4.6</td>
</tr>
<tr>
<td></td>
<td>n=6 NS</td>
<td>n=7 p&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>0900</td>
<td>96.2 ± 14.9</td>
<td>89.9 ± 19.1</td>
<td>51.6 ± 8.7</td>
</tr>
<tr>
<td></td>
<td>n=7 p&lt;0.01</td>
<td>n=8 p&lt;0.025</td>
<td></td>
</tr>
<tr>
<td>1300</td>
<td>91.6 ± 19.4</td>
<td>65.3 ± 22.4</td>
<td>30.5 ± 4.3</td>
</tr>
<tr>
<td></td>
<td>n=9 p&lt;0.001</td>
<td>n=4 NS</td>
<td></td>
</tr>
<tr>
<td>1700</td>
<td>68.2 ± 18.0</td>
<td>58.0 ± 17.9</td>
<td>34.5 ± 5.8</td>
</tr>
<tr>
<td></td>
<td>n=12 NS</td>
<td>n=8 NS</td>
<td></td>
</tr>
<tr>
<td>2100</td>
<td>214 ± 28.5</td>
<td>114 ± 24.5</td>
<td>120 ± 10.5</td>
</tr>
<tr>
<td></td>
<td>n=10 p&lt;0.01</td>
<td>n=7 NS</td>
<td></td>
</tr>
<tr>
<td>0100</td>
<td>165 ± 11.3</td>
<td>94.6 ± 10.6</td>
<td>107 ± 11.9</td>
</tr>
<tr>
<td></td>
<td>n=9 p&lt;0.05</td>
<td>n=7 NS</td>
<td></td>
</tr>
</tbody>
</table>

Mean Values ± SE. Significance calculated with reference to metoestrus/dioestrus values.

n = number of animals per group.
Discussion

Whilst most endocrine studies of the oestrous cycle have been carried out on rats, a few studies indicate that the mouse is similar (Michael, 1976; Murr et al, 1973). The data presented here are in general agreement with those from the rat (Buckingham et al, 1978; Raps et al, 1971; Phillips and Poolsanguan, 1978; Critchlow et al, 1963) showing corticosterone levels higher in the morning and evening of pro-oestrus than at other stages of the cycle, but the data here show the elevation persisting well into the day of oestrus in mice.

The reason why some previous work with the mouse failed to show corticosterone elevation at pro-oestrus (Champlin, 1969) may be the high variability of the oestrous cycle in mice (Bingel and Schwartz, 1969). This is particularly apparent in all-female groups (Whitten, 1966) with the absence of males and with daily vaginal smearing (Bingel and Schwartz, 1972) where only a few mice show regular 4-day cycles.

Oestrogen may be the key to the increase in corticosterone at pro-oestrus. ACTH (Coyne and Kitay, 1969) and corticosterone (Kitay, 1963) are depressed by ovariectomy and restored by oestradiol-17-B in rats. Oestrogen induces increased thyroid stimulating hormone (TSH) and thyroxine secretion, which in turn raise circulating levels of corticosterone binding globulin (CBG), (Gala and Westphal, 1966). Since 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 pro-
oestrus in the 4-day cycling rat (eg. 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). It is not known whether there is a rise in CBG at pro-oestrus, but it has been shown that CBG levels can fluctuate daily in the male rat (and mouse) and are higher in the female (Ottenweller et al, 1979).

Phillips and Poolsanguan, (1978) have argued that corticosterone elevation at pro-oestrus in the rat is biphasic, a morning rise due to high oestrogen and low progesterone; the afternoon elevation due to LH (Butcher et al, 1974) which has a stimulatory effect on the adrenal both in vitro and in vivo (Vinson et al, 1976). However Buckingham et al, (1978) have shown a peak of ACTH in the afternoon of pro-oestrus, higher than at other stages of the cycle, implying that the afternoon increase in corticosterone is mediated by ACTH.

The extension of the pro-oestrus elevation of corticosterone reported here may reflect a more prolonged secretion of oestrogen which Bingel and Schwartz, (1969) have suggested occurs in the mouse.

The functional significance of increased corticosterone levels at pro-oestrus is as yet unclear. It is of importance to know whether the increase is of unbound or bound corticosterone since their physiological activities differ. As discussed in the previous chapter, the function of corticosterone elevation may be "arousal" associated with sexual behaviour.

Whilst it has been shown that non-physiological doses of corticosterone can trigger ovulation in hens (eg. Sharp and Beuving, 1978) most of the data does not support
the suggestion that corticosterone has a role in the initiation or facilitation of ovulation (although ACTH secretion may be important), (Wilson et al, 1978).

In Chapter 2 the notion that adrenal progesterone could inhibit ovulation was discussed. There is growing evidence however, that pre-ovulatory adrenal progesterone can facilitate the LH surge triggering ovulation, in conjunction with oestradiol (Wilson et al, 1978; Feder et al, 1971; Cambell et al, 1977; Mann et al, 1976; Lawton, 1970, 1972; Nequin and Schwartz, 1971; Rodgers et al, 1974). Clearly the timing of the adrenal progesterone peak (or surge) is important (eg. Lisk, 1969).

What emerges from this study is that the stage of the oestrous cycle can seriously interfere with the use of plasma corticosterone as a measure of stress (unless the pro-oestrus rise is considered "stress"). In view of all the endocrine changes during pro-oestrus (eg. Butcher et al, 1974) it is useful to treat pro-oestrus and non-pro-oestrus female mice separately in any investigation of stress.
CHAPTER 8
Effects of Social Environment on the Oestrous Cycle

Introduction

It is well known that the oestrous cycle of mice is strongly influenced by social cues (eg. Whitten, 1966). Pheromonal activity has been identified as an important factor in many of the effects on reproductive activity, such as in spontaneous pseudopregnancy (Lee-Boot Effect), synchronous oestrus (Whitten Effect), blocked implantation of embryos (Bruce Effect) and accelerated sexual maturation (Vandenbergh Effect). However it is not known whether social stress may be an important factor in initiating these effects nor is it clear what part these effects may play in regulating population size.

It has been shown that mice kept in all-female groups can show spontaneous pseudopregnancy (Lee and Boot, 1955, 1956; Dewar, 1959; Mody, 1963; Caschera, 1960), lengthened oestrous cycles (mostly prolonged dioestrus), (Lamond, 1958, 1959; Champlin, 1971; Kimura, 1971; Andervont, 1944) and anoestrus (Whitten, 1957, 1959). The precise nature of these lengthened cycles is not understood. It has been suggested that the type of oestrus suppression is dependent on the size of the group (Whitten, 1966).

The social environment of juvenile female mice can determine their age of sexual maturation (eg. Vandenergh, Drickamer and Colby, 1972). Thus increased density of grouped juvenile females (Castro, 1967; Drickamer, 1974) and exposure of juveniles to grouped females urine (Colby and Vandenergh, 1974) can delay vaginal opening and first
oestrus by up to twenty days compared to singly housed females. Male presence (Vandenberg, 1967) and male urine (Colby and Vandenberg, 1974) are effective at accelerating female sexual maturation. However Christian, (1956, 1963) produced some evidence of prolonged inhibition of sexual maturation in females, in a dense population of mixed sex. Notably young females had delayed involution of the x-zone of the adrenal cortex, implying a delay in first pregnancy and depressed ovarian and uterine weights in females up to the age of 110 days compared to control females.

On the basis of this data and other (Crowcroft and Rowe, 1957), Christian, (1971) has argued that inhibition of sexual maturation is an important factor in limiting reproductive success at high density and hence regulating population size. Since in dense populations it is only the socially dominant females that are reproductively successful (eg. Retzlaff, 1938) and that the juveniles are mostly of subordinate social rank it is plausible that inhibition of sexual maturation is a result of social position.

Individually housing female mice leads to short oestrous cycles; mostly four, five or six day cycles (Bingel and Schwartz, 1969; Champlin, 1971; Marsden and Bronson, 1965). Grouped females showing lengthened cycles can be induced by the presence of a male, to return quickly to oestrus (Whitten, 1956, 1957, 1958, 1959; McKinney, 1972; Marsden and Bronson, 1965; Chipman and Fox, 1966). Male presence can also induce short cycles (Whitten, 1957) of predominantly four days (Bingel and Schwartz, 1972). Male urine is effective at inducing oestrus (Marsden and Bronson, 1964) but it is not known if it is as effective at inducing four day
cycles as male presence. The object of the experiments described here were (a) to ascertain whether the size of groups of female mice determined the type of oestrous cycle suppression i.e. anoestrus, pseudopregnancy, (b) to ascertain whether prolonged inhibition of sexual maturation occurred when juvenile females were housed amongst groups of adult females, (c) to investigate the effect of male presence and male urine on the oestrous cycle of grouped and individually housed female mice.
Materials and Methods

Experiment 1

Fifty TO virgin female mice aged 81 days were randomly assigned to three experimental categories: 10 individually housed, 10 in a group and 30 in a group. A vaginal smear was taken daily for 17-19 days, starting one week after rehousing.

Experiment 2

Eighty TO virgin female mice were weaned at 21 days and randomly assigned to four experimental categories: 20 individually housed, 20 housed in two groups of 10, 20 housed in groups of 10 with 5 adult females per cage and 5 weanlings, and 20 housed in groups of 30 with 20 adult females and 10 weanlings per cage. Adult females were approximately 160 days old at the start. Fifty days later the younger mice were smeared daily for ten days.

Experiment 3

Ninety two TO virgin female mice aged 14-16 weeks old were randomly assigned to six experimental categories: individually housed in a "male smell free" room (n=28), group of 10 in a "male smell free" room (n=10), individually housed with 1 male kept in a separate wiremesh holder in each cage (n=20), two groups of 10 with one male per cage, kept in a wiremesh holder (n=20), individually housed given male urine daily (n=14) and group of 10 given male urine daily (n=10). Details of male urine collection and application given in Chapter 6. Mice were undisturbed for 14-16
days then smeared daily for 8 days.

Experiment 4

Sixty TO virgin female mice aged approximately 32 weeks old were randomly assigned to six groups of ten, housed with one male in a wiremesh holder per cage. They were smeared daily for 9-10 days, then rehoused in a "male smell free" room in groups of ten (5 experimental mice and 5 other females of similar age, previously housed together). Daily smearing continued for a further 10 or 11 days. The females were rehoused when at dioestrus, pro-oestrus or oestrus between 2 and 5 hours after lights out.

Vaginal Smearing: This was performed and the smears staged as previously described in Chapter 3. Pseudopregnant cycles were defined as having 5 or more consecutive days of dioestrus or metoestrus. Abnormal cycles were defined as 4 or more days of oestrus.

Statistics

Student t tests were performed for comparisons of pairs of means. Chi-squared tests for independent samples were used for frequency data.
Results

Experiment 1

The effect of numbers of female mice in a group, on frequency of ovulation and type of oestrous cycle are shown in Tables 1 and 2. Individually housed females show significantly higher mean frequency of pro-oestrus/oestrus smears in 16 days and a higher proportion of 4, 5 and 6 day cyclers than longer cyclers both in groups of 10 and group of 30, (p<0.001). There was no statistically significant difference between groups of 10 and group of 30 with respect to these measures, although it was noted that in the group of 30, 4 mice showed abnormal cycles (prolonged oestrus).

Experiment 2

The effect of housing immature female mice in groups and with adult females on the frequency of ovulation and the type of oestrous cycle are shown in Tables 3 and 4. Individually housed females showed statistically significant higher mean frequency of pro-oestrus/oestrus, (p<0.002) and higher proportion of 4, 5 and 6 day cyclers to long cyclers (p<0.01) than groups of females. There were no significant differences in these measures in groups of 10, groups of 10 housed (5 juveniles plus 5 adults) or groups of 30 housed (20 adults plus 10 immature).

Experiment 3

Table 5 shows the percentage of animals in each category with 4, 5, 6 day or longer oestrous cycles. It can be seen that male presence with individually housed females induces a majority of 4 day cyclers. Male urine given daily to individually housed females induces 4 and 5 day cyclers.
in equal numbers. The cycles found in grouped females are predominantly longer than those in individually housed females, and show signs of pseudopregnant cycles eg. mucification of the smear. Male presence and male urine increase the proportion of shorter cycles in a group and prevented the high percentage of pseudopregnant cycles found in a group without males present. Table 6 summarizes the results of the effects of social environment on the oestrous cycle. It is apparent that individual housing is effective in maintaining short cycles regardless of male presence.

Experiment 4

Table 7 shows the effects of grouping female mice at different stages of the oestrous cycle on the number of days to the next oestrus and the percentage of females showing long cycles immediately. Females grouped at dioestrus and pro-oestrus did not show immediate long cycles and ovulation usually occurred within three days whereas 69% of females grouped at oestrus showed immediate long cycles.
**TABLE 1**

Effect of Numbers of Female Mice in a group on frequency of Pro-oestrus/Oestrus Smears.

<table>
<thead>
<tr>
<th></th>
<th>Mean Frequency of Pro-oestrus/Oestrus in 16 days + SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individually</td>
<td>n</td>
</tr>
<tr>
<td>housed</td>
<td>10</td>
</tr>
<tr>
<td>Group 10</td>
<td>10</td>
</tr>
<tr>
<td>Group 30</td>
<td>30</td>
</tr>
</tbody>
</table>

Student t test

* p < 0.001

N.S. Not significant
**TABLE 2**

Effect of Numbers of Female Mice in a group on the type of Oestrous Cycle.

<table>
<thead>
<tr>
<th></th>
<th>Number of 4, 5 and 6 day cyclers</th>
<th>Number of Longer cyclers</th>
<th>Chi-squared test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individually housed</td>
<td>9/10 (90%)</td>
<td>1/10 (10%)</td>
<td>p&lt;0.001</td>
</tr>
<tr>
<td>Group 10</td>
<td>0/10 (0%)</td>
<td>10/10 (100%)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Group 30 (a)</td>
<td>2/26 (8%)</td>
<td>24/26 (92%)</td>
<td></td>
</tr>
</tbody>
</table>

(a) 4 mice had abnormal cycles
<table>
<thead>
<tr>
<th></th>
<th>Mean Frequency of Pro-oestrus/Oestrus smears in 10 days</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
</tr>
<tr>
<td>Individually housed</td>
<td>20</td>
</tr>
<tr>
<td>Groups 10</td>
<td>20</td>
</tr>
<tr>
<td>(5 adults + 5 immature)</td>
<td>20</td>
</tr>
<tr>
<td>Groups 30</td>
<td>20</td>
</tr>
<tr>
<td>(20 adults + 10 immature)</td>
<td></td>
</tr>
</tbody>
</table>

Students t test
* p<0.002
N.S. Not significant
TABLE 4

Effect of Housing Immature Female Mice in groups and/or with adult females on the type of Oestrous Cycle.

<table>
<thead>
<tr>
<th>Group</th>
<th>Numbers of 4, 5 and 6 day cyclers</th>
<th>Number of Longer cyclers</th>
<th>Chi-squared test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individually housed</td>
<td>19/20 (95%)</td>
<td>1/20 (5%)</td>
<td>p&lt;0.01</td>
</tr>
<tr>
<td>Group 10</td>
<td>10/20 (50%)</td>
<td>10/20 (50%)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Group 10 (5 adults + 5 immature)</td>
<td>7/20 (35%)</td>
<td>13/20 (65%)</td>
<td>N.S.</td>
</tr>
<tr>
<td>Group 30 (20 adults + 10 5/20 (25%)</td>
<td>15/20 (75%)</td>
<td>N.S.</td>
<td></td>
</tr>
</tbody>
</table>

N.S. Not significant
TABLE 5
Effect of Male Presence and Male Urine on the type of Oestrous Cycle.

<table>
<thead>
<tr>
<th></th>
<th>Number of 4 day cyclers</th>
<th>Number of 5 day cyclers</th>
<th>Number of 6 day cyclers</th>
<th>Number of Pseudo-pregnant or Abnormal cyclers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individually housed with male</td>
<td>18/20 (90%)</td>
<td>1/20 (5%)</td>
<td>-</td>
<td>1/20 (5%)</td>
</tr>
<tr>
<td>Group housed with male</td>
<td>6/20 (30%)</td>
<td>7/20 (35%)</td>
<td>4/20 (20%)</td>
<td>3/20 (15%)</td>
</tr>
<tr>
<td>Individually housed with male urine</td>
<td>7/14 (50%)</td>
<td>7/14 (50%)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Group housed with male urine</td>
<td>3/10 (30%)</td>
<td>3/10 (30%)</td>
<td>2/10 (20%)</td>
<td>2/10 (20%)</td>
</tr>
<tr>
<td>Individually housed</td>
<td>14/28 (50%)</td>
<td>9/28 (32%)</td>
<td>3/28 (11%)</td>
<td>2/28 (7%)</td>
</tr>
<tr>
<td>Group housed</td>
<td>1/10 (10%)</td>
<td>2/10 (20%)</td>
<td>-</td>
<td>7/10 (70%)</td>
</tr>
</tbody>
</table>
TABLE 6

Summary of Results: Percentage of Females in different Social Environments with Short or Long Cycles.

<table>
<thead>
<tr>
<th></th>
<th>4,5 or 6 day cyclers</th>
<th>Abnormal and Long cyclers</th>
<th>n</th>
<th>Number of days smeared</th>
</tr>
</thead>
<tbody>
<tr>
<td>Individually housed plus male</td>
<td>95%</td>
<td>5%</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>Individually housed plus male urine daily</td>
<td>100%</td>
<td>-</td>
<td>14</td>
<td>8</td>
</tr>
<tr>
<td>Individually housed</td>
<td>93%</td>
<td>7%</td>
<td>38</td>
<td>8-10</td>
</tr>
<tr>
<td>Individually housed from weaning</td>
<td>95%</td>
<td>5%</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Group of 10 plus male</td>
<td>85%</td>
<td>15%</td>
<td>20</td>
<td>8</td>
</tr>
<tr>
<td>Group of 10 plus male urine daily</td>
<td>80%</td>
<td>20%</td>
<td>10</td>
<td>8</td>
</tr>
<tr>
<td>Group of 10 from weaning</td>
<td>50%</td>
<td>50%</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Group of 10</td>
<td>34%</td>
<td>66%</td>
<td>60</td>
<td>8-10</td>
</tr>
<tr>
<td>Group of 10 (5 from weaning and 5 adults)</td>
<td>35%</td>
<td>65%</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Group of 30</td>
<td>33%</td>
<td>67%</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>Group of 30 (10 from weaning and 20 adults)</td>
<td>25%</td>
<td>75%</td>
<td>20</td>
<td>10</td>
</tr>
</tbody>
</table>
TABLE 7
Average Number of Days until Oestrus Phase after Grouping Females away from Males at different Stages of the Oestrous Cycle.

<table>
<thead>
<tr>
<th>Stage</th>
<th>No. of days to Oestrus</th>
<th>% Showing Long cycles immediately</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dioestrus (n=18)</td>
<td>2.94 ± 1.25</td>
<td>6%</td>
</tr>
<tr>
<td>Pro-oestrus (n=22)</td>
<td>1.09 ± 0.29</td>
<td>0%</td>
</tr>
<tr>
<td>Oestrus (n=16)</td>
<td>8.75 ± 3.24</td>
<td>69%</td>
</tr>
</tbody>
</table>
Discussion

The results presented here are in agreement with those of a recent study, (Ryan and Schwartz, 1977) that grouping female mice leads to pseudopregnancy and not anoestrus. Thus all the mice individually housed or in groups of ten or thirty showed at least one period of pro-oestrus/oestrus in sixteen days of smearing (Experiment 1). In contrast Whitten, (1959) found true anoestrus in groups of thirty females with cessation of oestrus for periods of up to forty days, lighter ovaries and fewer corpora lutea compared to individually housed mice. It was proposed that the failure to induce anoestrus in other studies (eg. Lee and Boot, 1956) was due to small group size or to the presence of male odour, (Whitten and Champlin, 1973).

These discrepancies in results may represent a genuine genetic strain difference. An alternative is that in Whitten's study oestrus smears were missed perhaps due to heavy vaginal mucification. It was noted in this study that the pro-oestrus and oestrus smear stages after a pseudopregnant cycle were sometimes rapid, both stages passing in twenty four hours and the cell types at oestrus, whilst recognisably cornified, were unusual (Chapter 3).

The results from the second experiment indicated that neither duration of grouping nor housing of juveniles with adult females induced anoestrus. Thus whilst grouping young female mice can delay the first oestrus by up to twenty days (eg. Drickamer, 1973), the all-female social environment does not seem to inhibit sexual maturation in the same way or for the same duration as a mixed-sex population, (Christian, 1956). The failure to observe anoestrus
lends support to the notion that social stress is not found in all-female groups, regardless of the housing density.

The cue for group-induced pseudopregnancy is believed to be pheromonal, since individually housed females show pseudopregnancy when exposed to soiled bedding material from a group of females, (Champlin, 1971). There is some evidence that the pheromone may operate through taste rather than smell, (Kimura, 1971). An unresolved problem is the type of social behaviour in females which stimulates the production of a pseudopregnancy-inducing pheromone. It is not known if all the females in a group produce the pheromone. It has been observed here and elsewhere, (Whitten, 1959) that usually in a group of females, a few mice will continue to have short oestrous cycles. Similarly, in mixed groups it has been found that only a few females will be reproductively active, (Retzlaff, 1938) and that these are socially dominant, (Lloyd and Christian, 1969). It is possible though unproven, that it is the socially dominant females which continue to have short cycles and produce the pheromone inducing pseudopregnancy. How an individual female mouse achieves dominance is unclear, (Chapter 2).

A second unresolved problem is that of the function of group-induced pseudopregnancy in a natural habitat, if indeed it is more than a laboratory artefact. Whilst it is a form of reproductive inhibition the fact that mating is successful with the introduction of a male, (Whitten, 1966) implies that it is not an important mechanism of population regulation. Clearly, the reproductive inhibition of females found in some studies of semi-natural populations, (Crowcroft and Rowe, 1957) is quite different from the pseudopregnancy
found in all-female groups.

The results of experiment 4 here and those of Ryan and Schwartz, (1977) indicate that the stage of the oestrous cycle when females are grouped determines the onset of pseudopregnancy, with grouping at oestrus being most effective. This is a time when fresh corpora lutea are in the ovary. It has been proposed that grouping acts as a luteotrophic stimulus since it did not inhibit gonadotrophin secretion, (Ryan and Schwartz, 1977). Smith et al, (1975, 1976) demonstrated the existence of a bi-circadian rhythm of prolactin in rats after sterile mating and no change in gonadotrophin secretion in support of this notion. A bi-circadian rhythm of prolactin was found in pregnant mice, (Barkley et al, 1978) but it is not known if similar elevations of prolactin occur during pseudopregnancy in the mouse. It is noted that the luteotrophic secretion in the mouse is believed to be prolactin and FSH, (Choudary and Greenwald, 1969). Also some differences have been found between sterile mating and group-induced pseudopregnancy in mice, (Ryan and Schwartz, 1977). No evidence has been found supporting the view that social stress and adrenocortical activity initiate group-induced pseudopregnancy.

The ability of male presence or male urine to shorten cycles, (Marsden and Bronson, 1965; Bingel and Schwartz, 1972) is confirmed here. Male urine was less effective at inducing four day cycles than male presence. However this may simply be because inadequate urine was supplied (approximately one third to one half of a male's daily urine production was given per day) or urine was supplied only once a day.
It has been proposed that a male pheromone is able to block luteotrophic secretion thus preventing pseudopregnancy, (Ryan and Schwartz, 1977; Milligan, 1978). Pseudopregnancy induced by sterile mating has been blocked effectively by male pheromone, (Dominic, 1966) and pregnancy block, caused by the odour of strange males, has been shown to be due to a failure of prolactin secretion, (Parkes and Bruce, 1961; Dominic, 1966). The mechanism involved in the maintenance of four day cycles is not clear but it would seem unlikely that it is further blocking of the luteotrophic secretion. It is noted that male presence has a minor protective effect on cycle length with mice kept in continuous light, (Cambell et al, 1976).

Overall, there is a continuum of social environment affecting the length of oestrous cycles in female mice (Table 6). Individual housing is effective at preventing long, pseudopregnant cycles that occur in grouped mice. Similarly, male presence and male urine are largely able to prevent pseudopregnancy in groups of ten females. Male presence seems to have an additional effect in inducing four day cycles in individually housed females. These various social factors may be quantitative in their effects such that, for example, an increase in size of a group of females may increase the number of females entering pseudopregnancy and the introduction of a male into the group decreases the number showing pseudopregnant cycles.
CHAPTER 9

General Conclusions

The specific aim of this study has been to test the hypothesis that at increased housing density, female mice are stressed, as judged by adrenocortical activity, and become anoestrus. No evidence has been found to support this view. No fighting behaviour was observed, even at high density. There was no mortality of female mice. Subjectively animals appeared in good health even at very high density (30 mice per cage). Assessment of adrenocortical activity revealed some differences in functioning, none of which could be positively identified as stress in terms of the General Adaptation Syndrome. Lengthening of oestrous cycles was believed to be pseudopregnancy rather than persistent anoestrus at high density. It seems clear that with the strains of mice used in this study there is no social stress in groups of females. There is similarly little evidence of isolation stress. It would seem that social stress in females, if it in fact exists, would be a result of male presence. Some preliminary evidence has been presented indicating that a pheromone in male urine can activate adrenocortical activity in females. Other possible mechanisms of stimulating social stress in females have been discussed.

Attention has been paid to the relationship between stress and adrenocortical activity. As discussed earlier (Chapter 1) it is not useful to consider adrenocortical activation and stress as synonymous. However, adrenal activity can perhaps be a useful indicator of stress, since it is a reliable respondent of stress. One problem however, centres around how to measure adrenal activation.
Conventional measures are adrenal weight and plasma glucocorticoid levels. Hypertrophy of the adrenal gland does not always correlate with cortical hyperfunction (eg. Sung et al, 1976).

If it were accepted that the prime function of glucocorticoid secretion was to facilitate adaptation to the environmental demands (through metabolic and psychological means) then one might expect plasma levels of glucocorticoid would be a good indicator of stress. However, these assumptions are not necessarily valid.

Most assays of rat or mouse plasma glucocorticosteroids claim to measure total corticosterone, (although in some fluorometric methods only 30% of the total estimate can be attributed to corticosterone; Diez et al, 1976). However, over 90% of plasma corticosterone is usually bound to a protein corticosteroid binding globulin (CBG), (eg. Fortier et al, 1970) When an animal is acutely stressed by, for example, fighting, anaesthetic injection or given ACTH, the increased glucocorticoid is primarily unbound (Knigge and Hoar, 1963; Bronson and Eleftheriou, 1965; de Moor et al, 1962). Consequently the measurement of total corticosterone would tend to underestimate the change from unstressed to stress levels of free (unbound) steroid.

Negative feedback of corticosterone on ACTH secretion operates only through the unbound corticosterone fraction (eg. Fortier et al, 1970). Since in unstressed situations CBG levels effectively regulate the proportions of bound and unbound steroid, CBG levels can regulate unstressed total corticosterone levels by the negative feedback loop. If it is assumed that it is the physiological actions of unbound corticosterone which are the important
respondents of stress it follows that measurement of total plasma corticosterone is not necessarily an accurate estimate of stress since situations which raise CBG levels will reveal increased adrenocortical activity but no increase in unbound steroid levels. There are several situations in which CBG levels are raised. Barlow et al., (1975) showed that female mice on day 16 of pregnancy had total corticosterone levels of 1400 ng/ml yet the unbound fraction was only 1.5% of this or about 20 ng/ml. Others have shown an increase of CBG activity during pregnancy (eg. Daughaday, 1959) and it has been suggested that this is due to elevation of oestrogen since oestrogen administration also increased CBG levels (Westphal, 1974; Sandberg and Slaunwhite, 1959). There is a decline in CBG levels during lactation (Koch, 1969; van Baelen et al., 1977). Gala and Westphal, (1966) showed that TSH from the pituitary was necessary for the increase in CBG after oestrogen treatment. During pregnancy there is an increase in oestrogen, and this can stimulate thyroid activity (eg. Fortier et al., 1970). It remains a possibility that CBG levels can be in part determined by thyroid activity. This would agree with the finding that cold increases total glucocorticoid but not unbound steroid (Knigge and Hoar, 1963) since cold also stimulates thyroid activity (eg. Yamada, 1974). It is noted that testosterone and corticosterone can depress CBG levels (Gala and Westphal, 1966) but whether these may be tied to thyroid activity is not known.

The possible importance of the distinction between bound and unbound corticosteroid has been demonstrated. In a study of a dasyurid marsupial it was found
that after breeding, males but not females, suffered heavy mortality. Whilst total corticosteroid levels were not particularly elevated, unbound corticosteroid levels were high in males but not females, due to a sudden decline in CBG levels, and this was identified as the cause of death (Bradley et al., 1976). In this study it has been postulated that the rise in corticosterone at pro-oestrus may be due to an increase in CBG, induced by oestrogen, and consequently of little importance to any notions of stress. There is clearly a need for studies to identify the functions of bound and unbound corticosterone during stress and to further clarify alterations in their ratio attributable to non-stress factors such as lactation or the oestrous cycle.

As noted, a variety of hormones affect CBG levels, and hence presumably, corticosterone levels. However, it has been shown that some hormones can affect the adrenal directly, effectively increasing corticosterone levels. For example LH can stimulate the adrenal cortex directly (Phillips and Poolsanguan, 1978; Vinson et al., 1976) and oestrogens minimise the conversion of corticosterone to by-products in the adrenal (eg. Kitay et al., 1971). Prolactin inhibits corticosterone release but only in the absence of the ovaries (or presumably oestrogen) since prolactin and oestrogen act synergistically to elevate corticosterone output (Ogle and Kitay, 1979; Vasquez and Kitay, 1978). Progesterone can depress corticosterone output (Rodier and Kitay, 1974; Phillips and Poolsanguan, 1978). In view of these hormone interactions how sure can we be when estimating plasma corticosterone levels (even unbound steroid) that we are measuring the degree of stress and not some specific result
of a hormone interaction.

Another difficulty in the use of plasma corticosterone levels as an indicator of stress centres around the notion of stress responsiveness. (It is difficult in itself to disentangle the terminology). For example, Brain and Nowell, (1971) found a difference in basal corticosterone levels in grouped and isolated mice, the latter having lower levels. However, both had similar levels after a "standard stress" and it has been concluded that the isolated mice were more stress responsive. A similar situation is seen concerning the peak and trough of the circadian rhythm, where stress responsiveness is supposedly greater at the trough than the peak (eg. Allen et al, 1975) because there is no circadian rhythm in "stress" levels.

However, it has not been unequivocally demonstrated that a quicker or more prolonged or higher stress response has any benefit over a shorter or slower or lower response. It is not inconsistent with most data to say that the benefit (in adaptation terms) of a stress response of adrenal activity is an all-or-none action. Certainly the response can vary with different intensity of stimulus (eg. Friedman and Ader, 1967) but this does not necessarily imply that the stress response receptors need any more than above a threshold level or rate. Variations in stress response levels may then be due to conditioning factors for example, Barnett, (1960) demonstrated higher adrenal stress responses after oestrogen treatment. The stress response levels of corticosterone measured may also reflect the degree of negative feedback on ACTH (Yates and Maran, 1974). Dallman and Jones, (1973) demonstrated two types of stress response:
a feedback sensitive and a feedback insensitive.

When using a "standard stress" eg. ether exposure, the levels of corticosterone may accurately reflect the degree of stress but may also be of little relevance to the problem at hand, since activation of the pituitary-adrenal system can operate through different pathways (eg. Dallman and Yates, 1968; Matsuda et al, 1964).

What emerges from this analysis of the measurement of stress by adrenocortical activity is that we still cannot accurately identify the function of corticosteroid secretion during stress (Yates and Maran, 1974). It is known that corticosteroids affect glucose metabolism (eg. Schulster et al, 1976), blood pressure (Yates and Maran, 1974), inflammatory responses and disease resistance (eg. Selye, 1950; Joassoo and McKenzie, 1976) and various behaviour (eg. Brain, 1978). The relative importance of these actions in coping with environmental demands is not so well understood. Additionally corticosterone has been demonstrated to have functions which seem to have little connection with stress in terms of adaptability. For example, corticosterone plays an indispensable role in mammary development and initiation of lactation (Talwaker et al, 1961; Meites et al, 1963). In birds it has been shown that temporal synergism between prolactin and corticosterone leads to gonadal development (Meier et al, 1971). This would seem to indicate that the rhythm of corticosteroid can have functions apart from stress effects.

The general objective of this thesis has been to investigate some physiological aspects of social stress, particularly overcrowding. No evidence has been found of
stress in female mice housed at different densities. The dangers of extrapolating conclusions from mice to men has been recognised (Brain, 1975; Brain and Benton, 1979; Daly, 1973). It is hard enough to draw the same conclusions from different strains of house mice. What can be stated is that high density does not necessarily cause stress and does not necessarily have deleterious effects. Any general rules on the effects of "crowding" must recognise species-specific characteristics. It seems that it is not density per se which is the critical factor in determining crowding stress but the nature and quantity of social interactions.
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