"Social Stress in Female Mice: Effects of Differential

Housing on Adrenocortical Activity and the Oestrous Cycle."

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Doctor of Philosophy

1980

UNIVERSITY OF KEELE

Thesis for degree of Ph.D.

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

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    (iii) That no part of the work incorporated in the above
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thesis submitted by me for a Higher Degree at any
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Acknowledgements

I would like to ackowledge the practical help
given to me at various times throughout my stay at the
Universtiy of Keele by many of the members of the
Department of Biology. In particular, I would like to
thank Dave Boswell for the meticulous care he has taken
in looking after the laboratory animals. I thank Dr.
Tony Polwart for helping to write a computer program for
the assay.

My thanks also go to Sue Carr both for her
accurate typing of an illegible scrawl and for her gentle
encouragement.

Finally, I am indebted to Dr. Pete Chevins, my
supervisor, for his continuous help and encouragement and
for the time he has always generously given in discussing
ideas and problems.

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 over-crowding.

The term crowding is usually used rather loosely to mean a large numbe   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 vertbrates 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 (<u>Mus musculus L</u>) 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 Christan (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 re-
cognised 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 defor-
mation in a body, as distinct from strain which is deforma-
tion 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 po-
tentially 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_2)

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 per-
turbation 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 dis-
order 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 im-
plies 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 seperate theoretical view-points 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 be-haviourists, because it is inappropriate to a relatively inocuous 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 en-vironmentally 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 fac-tors 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).

Glucocorticoids also influence the metabolism of cate-
cholamines (Coupland, 1968) suppress the inflammatory res-
ponse (eg. Schulster et al, 1976) suppress the immune system
(Joassoo & McKenzie, 1976) and alter behaviour (eg. Brain,
1978).

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 environ-
mental 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 encoun-
tered in stress. It is because the adrenal cortex is consis-
tently 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 res-
ponse 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_2) 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 re-
lation 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 hypothalmic factors re-
gulating adrenal activity.

In addition to quantitative output the qualita-
tive 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 ex-
ceptions, 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 mal-
adaptive 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 meta-
bolic function, mobilising energy reserves for a fight or
flight reaction. This assumption has been challenged (Yates
& Maran, 1974_1).

Peripheral metabolic effects of high doses of glu-
cocorticoid 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 convin-
cingly proven that changes in glucocorticoid during stress
responses have beneficial or significant effects on periphe-
ral 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, 1974_2), (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, (eg. Dallman &
Yates, 1968; Dallman & Jones, 1973; Yates & Maran, 19741;
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 hypothalmic-hypophyseal axis. Stimulation
from higher brain centres causes release of a neuro-trans-
mitter, as yet uncharacterised, Corticotrophin Releasing
Factor (CRF) from the hypothalmus. This passes to the an-
terior pituitary via vascular portal vessels causing the
release of adrenocorticotrophic 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, hypothalmus 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, (eg. 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 deoxycortico-
sterone, a relatively minor adrenal product, the overall
conclusions do not differ substantially. Some of the patho-
logical 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 acti-
vity. Thus, apart from the effects of other pathological dis-
orders, 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 gonado-
trophin production, in order to mobilise energy for survival.
The evidence in favour of the simplistic notion is over-
whelmed 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 maladap-
tive functioning brought about by prolonged activation (stress).
Smith, (1973) wrote "as a respondent to emotional arousal,
glucocorticoid levels are reliable, non-specific and insen-
sitive". The hypothesis that emotional arousal is a respon-
dent 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 critisised the importance

placed upon the adrenal in adaptation. Mason, (1968$_2$) pro-
posed that rather than a single endocrine response to stress
there is an overall response of all the different endocrine
glands ie. thyroid, pituitary, gonads, adrenals. This con-
clusion was based on extensive data from experiments sub-
jecting monkeys to avoidance learning for several days and
measuring a wide variety of hormones throughout the pro-
cedure. Some hormone levels increased during the period of
learning ie. adrenaline, noradrenaline, glucocorticoids,
growth hormone and thyroxine, all of which have a catabolic
function in metabolism. The other hormones, insulin, oes-
trogen 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 hor-
monal response. This notion is supported by many modern en-
docrinological 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 res-
ponse, 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 corres-
pond to any one physiological system. Stress refers to the
failure of normal physiological and psychological homeostatic
systems to cope with environmental demands. The initial res-
ponse to these environmental demands is adaptive; prolonged

exposure leads to maladaption 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).

CHAPTER 2

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, 1955_1, 1955_2, 1956, 1959, 1960, 1961, 1963_1, 1963_2, 1968, 1970, 1971_1, 1971_2, 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 (eg. 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_1;
Valzelli, 1973; de Feudis, 1975; Brain, 1975_1; 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 ad-
renal measure in the apparent absence of conventional limi-
ting 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 (eg. 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 adap-
table, flourishing in a wide diversity of habitats, inclu-
ding cold stores (Laurie, 1946), open fields (Linduska, 1950)
heathland (Berry, 1968), grassland (Lidicker, 1965) salt
marsh (Breakey, 1963), urban areas including warehouses,

TABLE 1

Correlations between Population Density and
Reproductive Efficiency in Natural Populations.

SPECIES	REPRODUCTIVE MEASURE	REFERENCE
Rats	Number of pregnancies	Davis, 1953
Rattus norvegicus		
Rabbits	Number of young	Myers, 1966
Oryctolagus euniculusk	surviving	
Voles		
Clethrionomys rufocanus	(1) Inhibited maturation	Kalela, 1957
	(2) Litter size	
Microtus montanus	(1) Ovulatory rate	Hoffmann, 1958
" californicus	(2) Number of pregnancies	Hamilton, 1937
" pennsylvanicus	(3) Birth rate	Christian, 1971$_2$
	(4) Litter size	
" agrestis	(1) Inhibited maturation	Chitty, 1952
	(2) Inhibited reproduction	
" ochrogaster	Litter size	Fitch, 1957
Lemmings	Inhibited reproduction	
Lemmus trimucronatus		Rausch, 1950
Dicrostony groenlandicus		Barralow, 1952
Mice		
Peromyscus maniculatus bairdii	(1) Inhibited reproduction	Christian, 1971$_2$
Peromyscus leucopus	(2) Inhibited maturation	
Mus musculus	(1) Number of pregnancies	Southwick, 1958
	(2) Number of fetal resorptions	Evans, 1959

. . . .

TABLE 2

Correlations between Population Density and

Adrenal Measure in Natural Populations.

SPECIES	ADRENAL MEASURE	REFERENCE
Rats	Adrenal weight	Christian, 1959
Rattus norvegicus		
Rabbits	" "	Wodzicki and
Oryctolagus cuniculus L.		Roberts, 1960
Voles		
Microtus montanus	" "	Adams et al, 1959
Microtus pennsylvanicus	" "	Louch, 1956
Microtus agrestis ♀	" "	Chitty, 1960
Lemmings		
Lemmus trimicronatus	(1)Adrenal weight	Andrews, 1968
	(2)Adrenal secretory function	
Mice		
Peromyscus maniculatus bairdii ♂	Adrenal weight	Christian, 1971_2
Peromyscus leucopus ♀	" "	" "

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_1, 1955_2; Calhoun, 1956). In confined populations, low temperature (Southwick, 1955_1) 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$_1$). It must be concluded that it is the social interactions among individuals that limit growth (Petrusewicz, 1957). Laboratory studies grouping males and females together 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 territorial behaviour amongst males (Crowcroft, 1955; Crowcroft and Rowe, 1963; Anderson and Hill, 1965; Mackintosh, 1970). Male mice, given sufficient area, will form distinct defended 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 between 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_2). Southwick, (1955_2) 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

<u>Summary of Endocrine and Reproductive changes found</u>

<u>within confined, freely growing populations of</u>

<u>Mus musculus: Effects of increasing density.</u>

<u>REPRODUCTION</u>	<u>REFERENCE</u>
(a)Reduced female fertility	Southwick, 1955[1]
(i)inhibition of gonadal function	Christian, 1956, 1959, 1961 Crowcroft & Rowe, 1957, 1958 Lloyd & Christian, 1969
(ii)inhibition of sexual maturation	Christian, 1956, 1961 Lloyd & Christian, 1969
(iii)increased time between ovulation	Lloyd & Christian, 1969
(b)Reduced number of pregnancies	
(i)male sexual behaviour abnormal	Southwick, 1955[1]
(c)Reduced litter size	
(i)abortion or resorption of embryos	Christian, 1959
(ii)reduced number of ova	
(d)Increased infant mortality	
(i)nest disturbance	Christian, 1956 Brown, 1953 Southwick, 1955[1]
(ii)suppressed lactation	Christian, 1959

<u>ENDOCRINE</u>	
(a)Increased adrenal weight	Christian, 1956, 1961, 1963[1]
(b)Increased zone widths of adrenal cortex	
(c)Decreased ovary weight	
(d)Decreased testis weight	
(e)Decreased seminal vesicle weight	
(f)Decreased thymus weight	

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$_1$). 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 Lemunyan, (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_1, 1961, 1963_1) 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_3). 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_1, 1965_2, 1965_3). 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 prenantal effects on growth, white blood cell count and "Evasiveness" (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). Con-
tinuous 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 be-
come 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 luteini-
sing hormone (LH), such that anoestrus occurs when the feed-
back 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
'zeitgeiber' (Hoffmann, 1978) or prolonged secretion of
oestradiol. However, this induction of anoestrus is not be-
lieved 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 neverthe less 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, 1964_1, 1964_2). 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 (1971_2) 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$_2$).

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 in-
fant 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 domi-
nance behaviour. It is not known how or if social stress
occurs in females. The mechanisms of reproductive ineffi-
ciency 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 $18^{o}C - 23^{o}C$. 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 arbitary 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_1, 1965_2; Snell et al, 1940) it is not known whether this is so during pseudopregnant or abnormal cycles. Oestrous 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_1). Even then there are studies which fail to
correlate adrenal weight with plasma corticosterone (eg.
Brain and Nowell, 1971_2; 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 chroma-
tographic 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, vali-
dated in the mouse by Grad and Khalid, (1969), with some

modifications. A RIA, using rabbit anti-corticosterone-21-thyroglobulin serum, based on the method of Gross et al, (1972) with modifications.

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 $4^{o}C$.

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 $4^{o}C$.

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

Labelled corticosterone (1,2 ^{3}H corticosterone, specific activity approximately 50Ci/mmol, Radiochemical Centre) was diluted with ethanol on arrival to 4µCi/ml and stored in 5ml aliquots at $-20^{o}C$. 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 $45^{o}C$ 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 ultra-
sonically. 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 stan-
dards 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 $2\mu Ci$ ^3H-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 ^3H-corticosterone made up to the same radio-
active concentration as binding solution. Tubes were held at
$37^{\circ}C$ for 30 minutes and shaken twice. They were then placed
in an ice bath ($3.5^{\circ}C$) 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 an-
tibody and $1\mu Ci$ ^3H-corticosterone made up to 70 ml with
borate buffer) was dispensed to all tubes except charcoal
blanks, which had 0.7 ml ^3H-corticosterone and borate buffer.
After 25 minutes at room temperature tubes were vortexed.
Tubes were placed in a water bath at $37^{\circ}C$ for 1 hour and vor-
texed. 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 $4^{\circ}C$, 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 $4^{o}C$. 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

Murpny, (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 proges-
terone, (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_2; 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

<u>Specificity of Corticosteroid Binding Protein in</u>

<u>Dog Plasma.</u>

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

* 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.

	Est. Conc. Cortico- sterone with solvent wash (ng/ml)	Est. Conc. Cortico- sterone without solvent wash (ng/ml)
Blood samples n=12	49 ± 17	56 ± 19
Pool n=8	49.3 ± 1	52.5 ± 1.7
Pool + 100ng/ml progesterone n=8	52.3 ± 1	80.2 ± 2.3

TABLE 3

Specificity of rabbit anti-corticosterone-21-
thyroglobulin (data from Miles/Yeda).

Steroids Tested	% Cross reaction calculated B/Bo = 50% *
Corticosterone	100
Progesterone	20.6
Deoxy corticosterone	10.9
20α hydroxy progesterone	7.6
Cortisol	5.8
20B hydroxy progesterone	5.7
Testosterone	3.8
17 hydroxy progesterone	3.5
Androstenedione	2.4
Aldosterone	1.0
11 deoxy cortisol	11.5
5α dihydrotestosterone	0.5
Androsterone	0.4
Cortisone	0.3
Dihydroxyepiandrosterone	0.3
Estrone	0.1
Estradiol 17B	0.1
Estriol	0.1

* 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 $\pm$ SD   Slope = -1.0865 $\pm$ 0.054

      Intercept = -0.637 $\pm$ 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 $\pm$ 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

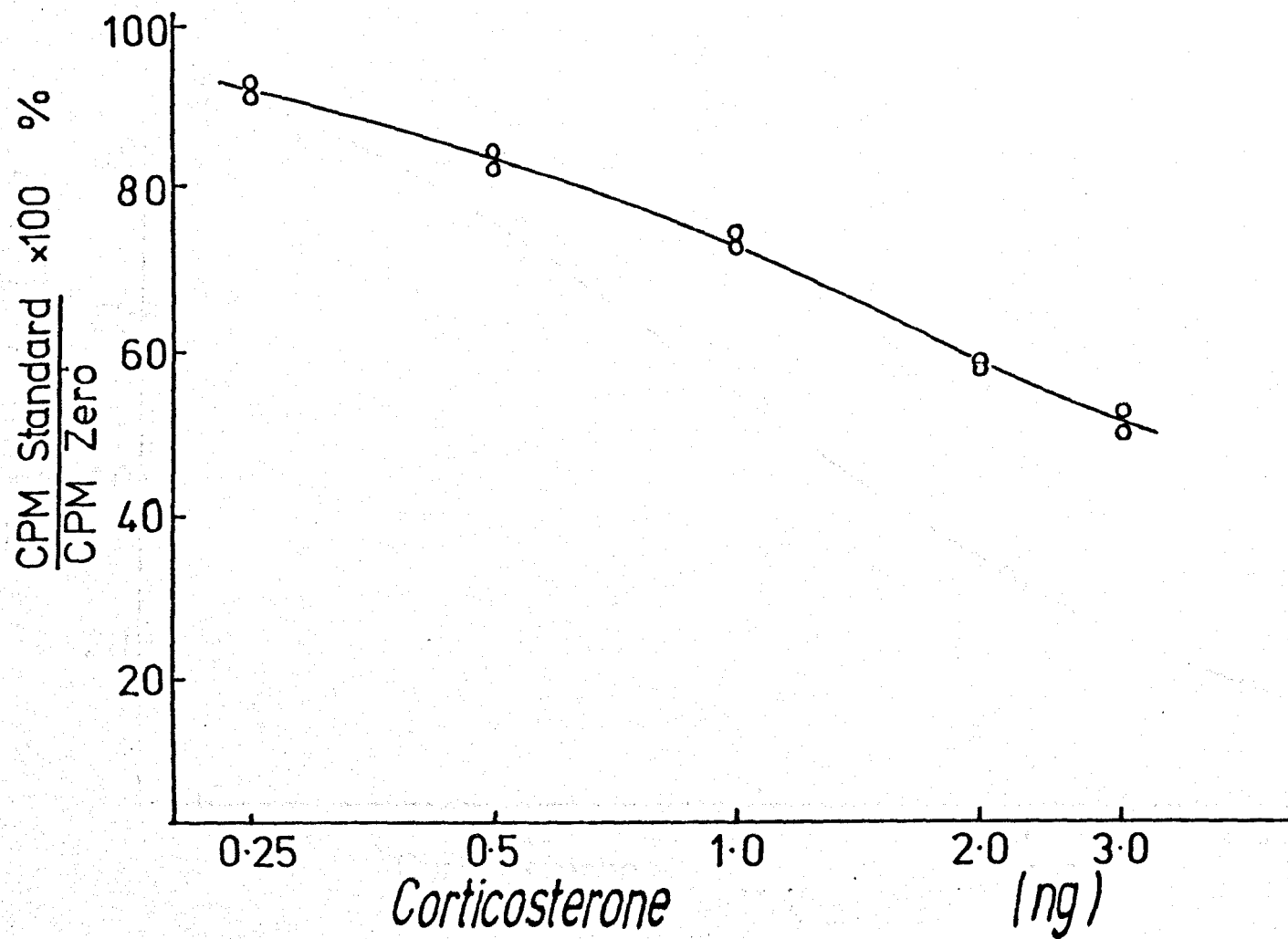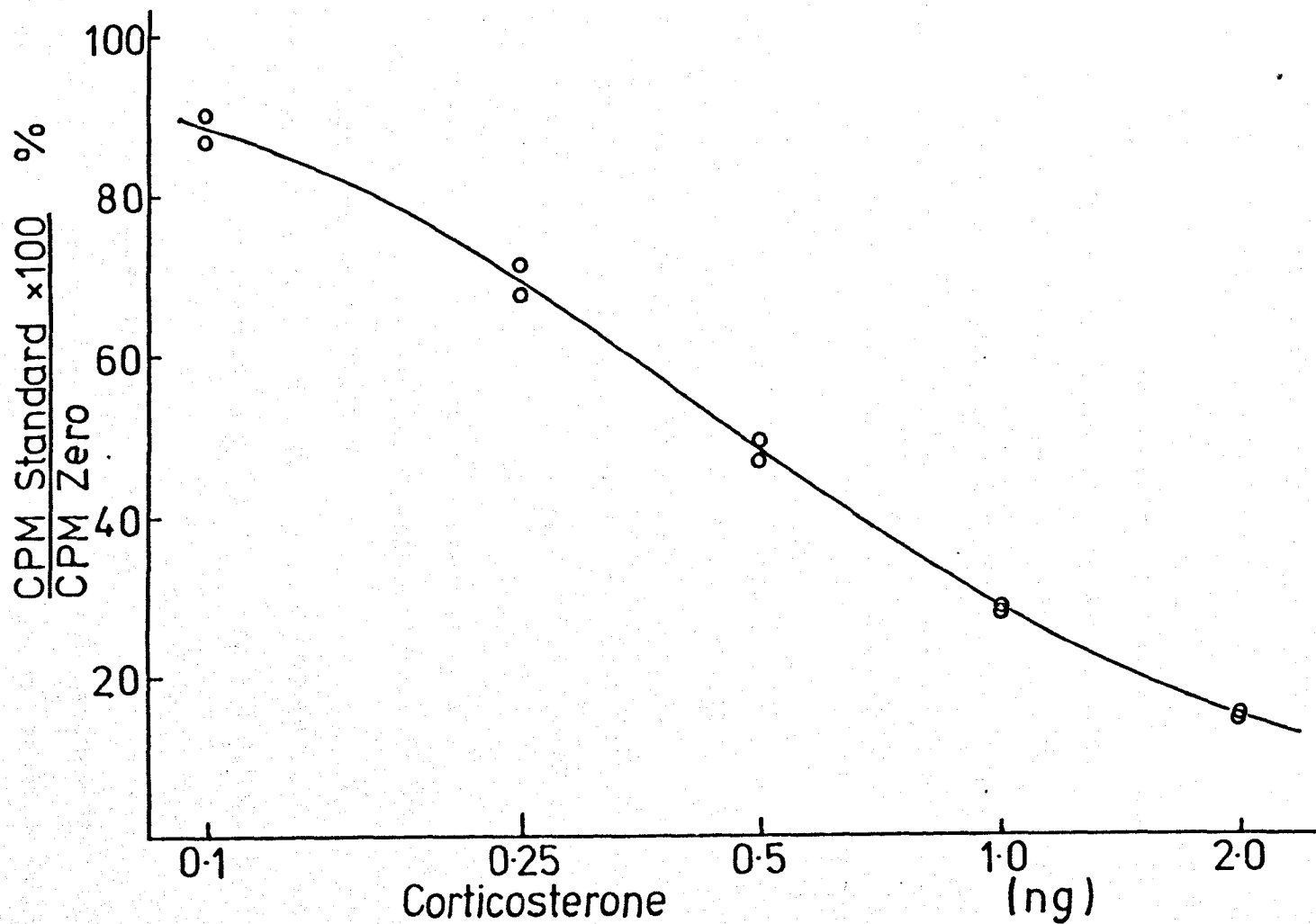Fig. 1   Typical Standard Curve : CPB Method

Fig. 2   Typical Standard Curve : RIA Method

significantly different from sensitivity.

Sensitivity = 40 $\pm$ 34 pg for nine assays.

Plasma treated with charcoal to remove all steroids gave a concentration of 2.0 ng/ml $\pm$ 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 $\qquad$ 100.9 $\pm$ 3.4%, n=19 (3 experiments)

With solvent wash $\qquad$ 93.8 $\pm$ 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

Amount of Corticosterone added	Amount Determined		% Recovery
(ng/ml)	(ng/ml)	n	
0	22.1	3	
50	61.8	3	79
100	115	3	106
150	160	4	90
200	215	4	110
300	318	4	103

TABLE 5   Estimation of Recovery of Corticosterone: RIA

Amount of Corticosterone added	Amount Determined		% Recovery
(ng/ml)	(ng/ml)	n	
0	86.6	5	
50	135	2	96.8
150	232	4	97
250	322	5	90

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

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

TABLE 7   Between Assay Variation: CPB

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

Precision: RIA

Within and between assay variation was assessed in six buffer pools with known concentrations of cortico-sterone added. Two or three samples were included in over twenty assays spanning a year, (Table 8).

TABLE 8   Within and Between Assay Variation: RIA

Conc. of Cortico-sterone in Pool (ng/ml)	Number of Samples	Conc. of Cortico-sterone (ng/ml)	Standard Deviation	Coefficient of Variation (%)	Recovery (%)
C_1 12.5	8	10.7	± 1.3	± 12.1	85.6
C_2 25	13	22.0	± 2.5	± 11.4	88
C_3 50	16	45.8	± 2.7	± 5.9	91.6
C_4 100	19	88.5	± 7.7	± 8.7	88.5
C_5 200	13	183	± 17	± 9.3	91.5
C_6 400	14	355	± 25.5	± 7.2	88.8

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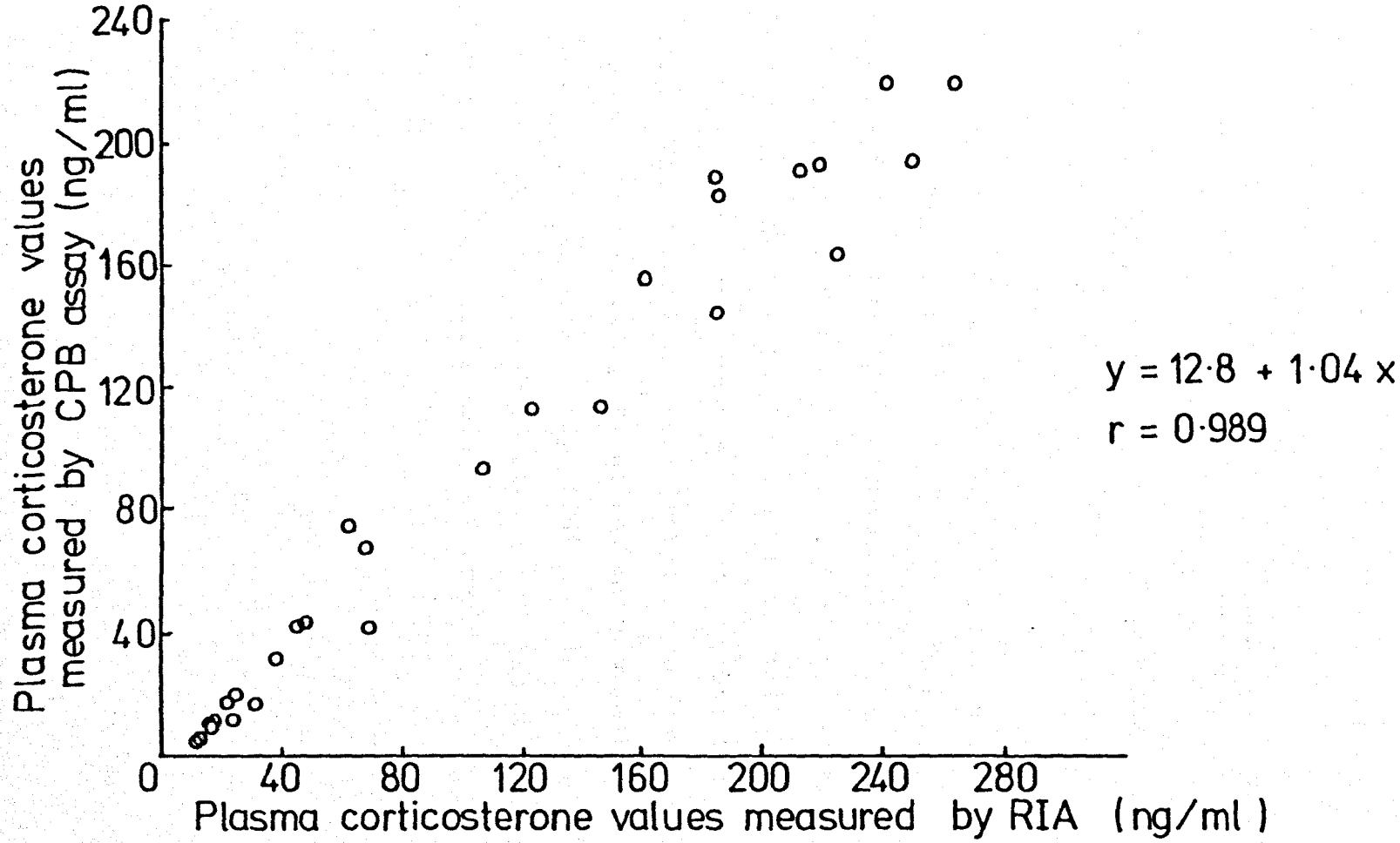
Mean Coefficient of Variation = ± 9.1%

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

$y = 12.8 + 1.04\,x$

$r = 0.989$

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

Plasma corticosterone values measured by RIA (ng/ml)

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.

		n	Mean $\pm$ SEM Plasma Corticosterone (ng/ml)
Group A	Eye—sampled at peak	8	148 $\pm$ 36
	Decapitated at trough	8	29.4 $\pm$ 5.7
Group B	Eye—sampled at trough	8	41.5 $\pm$ 11.4
	Decapitated at peak	8	120 $\pm$ 22

Using Mann Whitney U test there are no significant differences between peak or trough values. These values are "basal" levels ie. 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, (1971$_2$); 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 inter-ference 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 con-trolling 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 $\pm$ 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 sta-tistical 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 indi-
cated 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 func-
tion (eg. Brain, 1975_1). Most work using adrenal weight as
a measure of adrenocortical activity has shown that indivi-
dually housed female mice have larger, heavier adrenals
than group housed mice (Brain and Nowell, 1971_2; 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 adreno-
cortical 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,
1971_2; 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_2; 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 $\pm$ standard error. Friedman ANOVA and Wilcoxon matched pairs signed ranks test were used for statistical analysis of correlated samples ie. 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 (GR10), 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.

		Mean Plasma Corticosterone Level (ng/ml)		
		1st Blood sample	2nd Blood sample	Wilcoxon Test[*]
Group of 10 G10	n=10	163 ± 25	140 ± 12.7	N.S.
Individually housed I	n=10	139 ± 14	172 ± 17.5	N.S.
Groups of 4 Unfamiliar GR4 UNFAM	n=16	141 ± 12.3	152 ± 10.5	N.S.
Groups of 4 Familiar GR4 FAM	n=15	171 ± 18	169 ± 13	N.S.
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 ie. 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 2

The Effect of changing housing conditions on Basal

Plasma Corticosterone Levels in Female mice.

Mean Plasma Corticosterone Levels
(ng/ml)

	1st Blood sample	2nd Blood sample	3rd Blood sample	Friedman ANOVA*
GIG***n=9	148 ± 22	102 ± 7	144 ± 23	N.S.
IGI***	169 ± 13.7	124 ± 12	175 ± 20	N.S.
Mann Whitney U test**	N.S.	N.S.	N.S.	

*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 ± 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
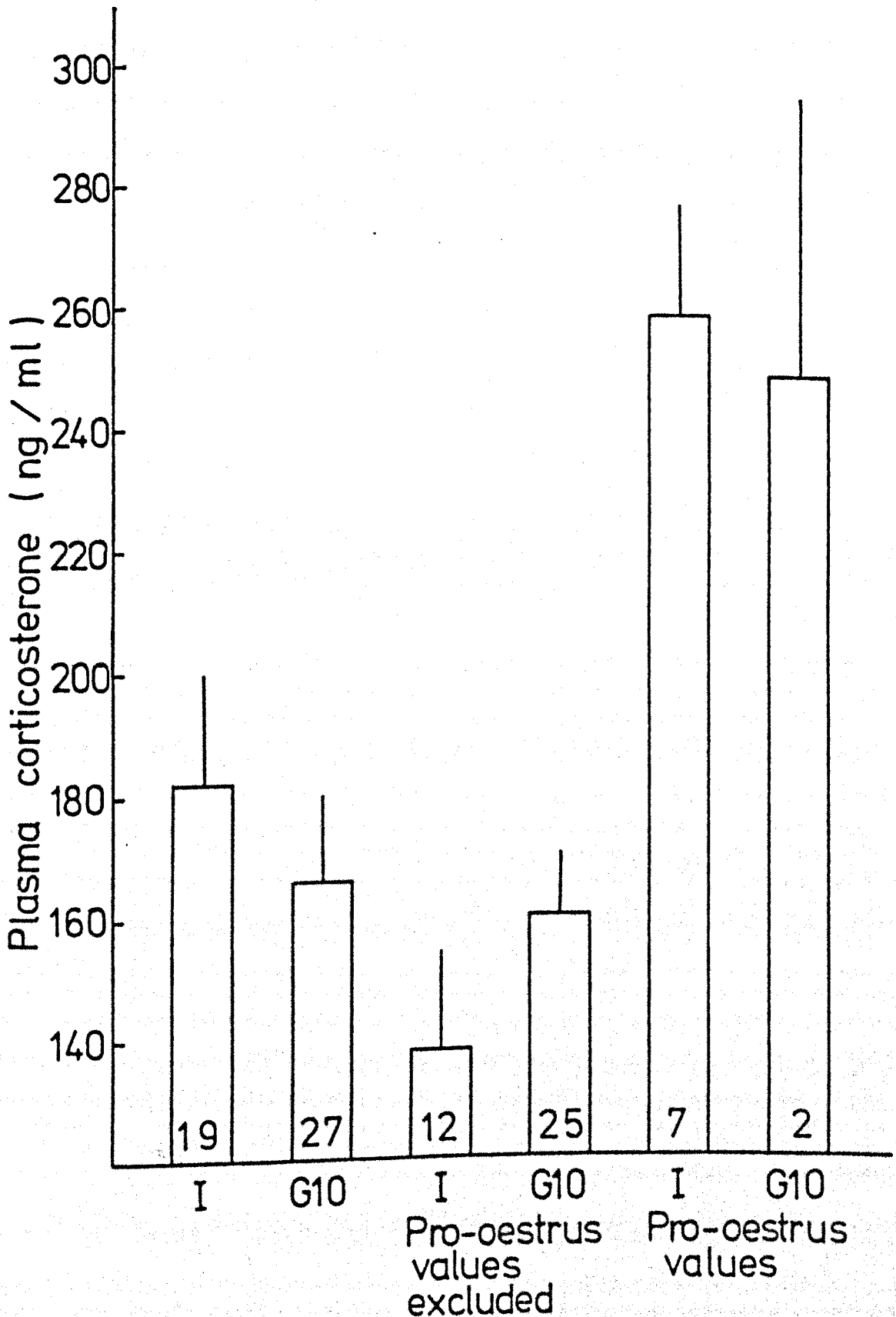
Experiment 5

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
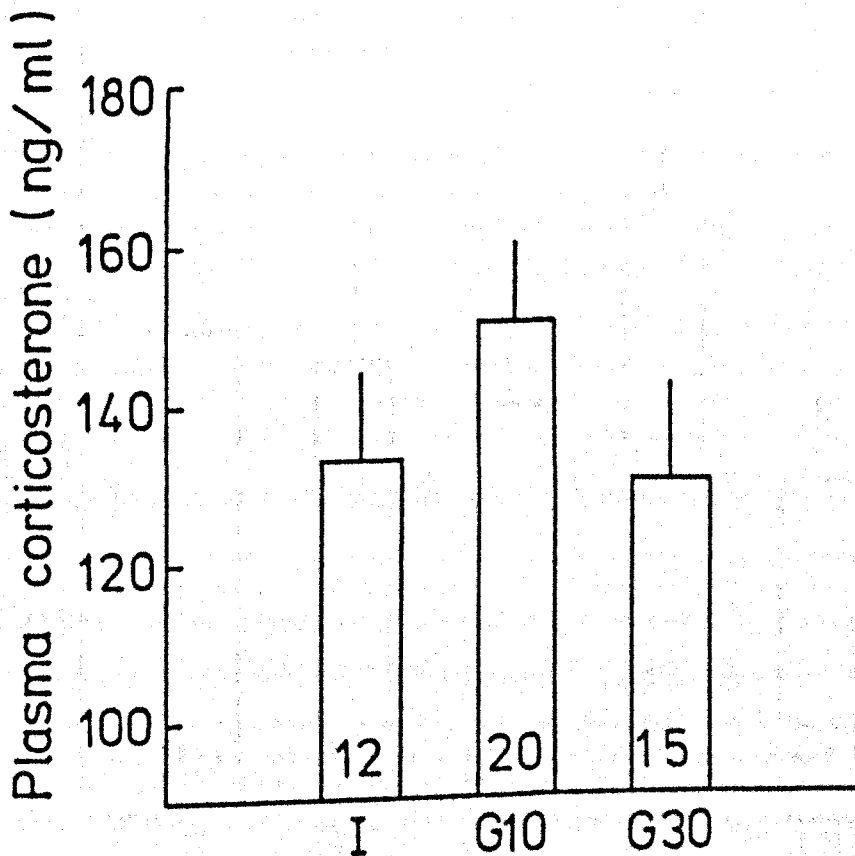
Fig. 3   Summary of results of differential
          housing in female TO mice
           I - Individually housed
          G4 - Groups of 4
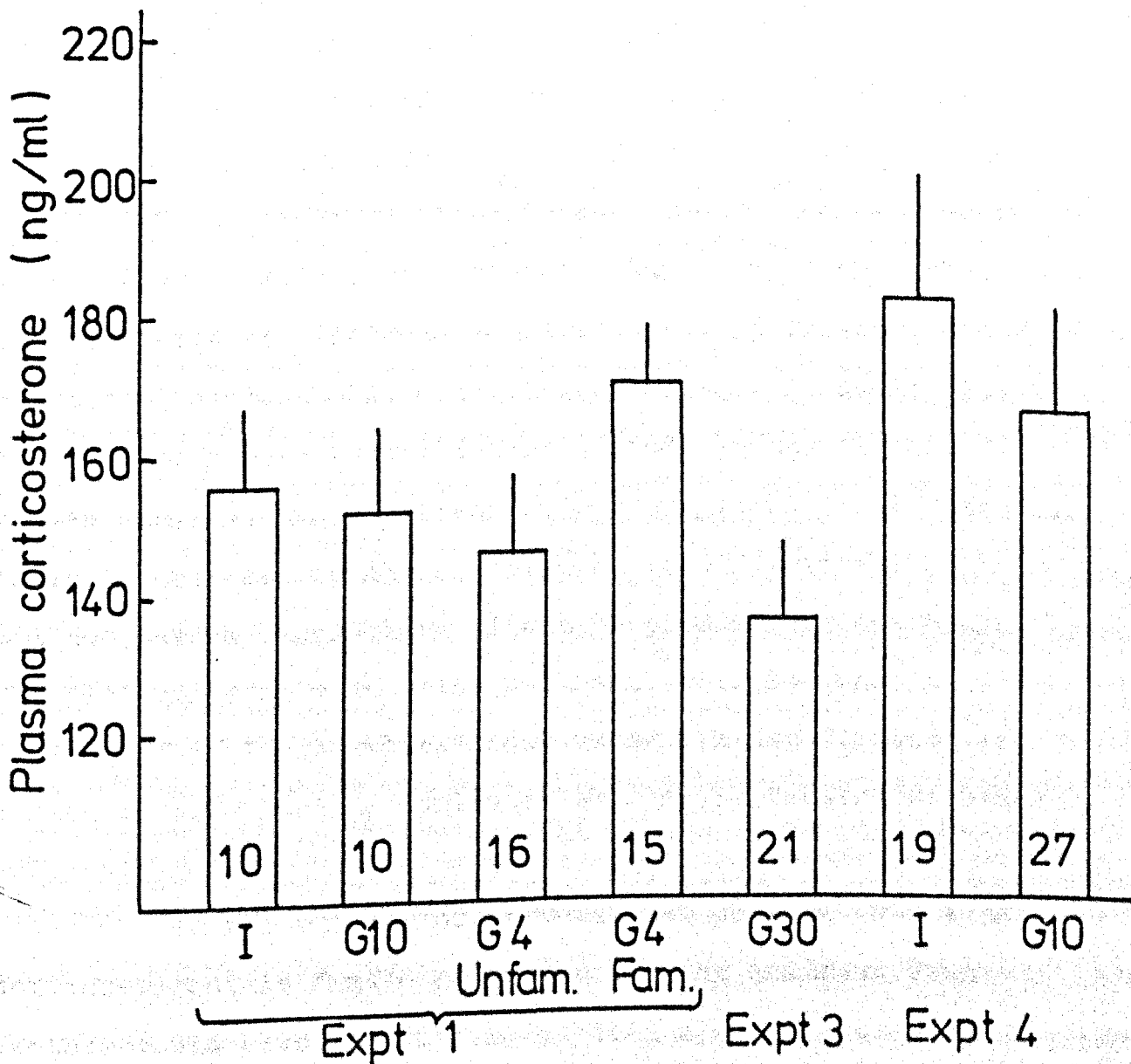   G10 - Groups of 10
          G30 - Group of 30
          Number of mice sampled is shown
          in columns
          Data from Expt 1 are means of blood
          sample 1 and 2
          KWANOVA   p > 0·1

Discussion

A characteristic feature of the results presented here and elsewhere (eg. Brain and Nowell, 1971[2]; 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_2; 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 (eg. Whitten, 1966). Brain and Nowell, (1971_2) 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) ex-cluding 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 (eg. 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 (eg. 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 un-bound (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_2) 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

<u>Effect of Social Environment on Plasma</u>
<u>Corticosterone: Circadian Rhythm and Stress Responsiveness</u>.

<u>Introduction</u>

Studies on the effect of housing conditions on pituitary-adrenal function in female mice have yielded inconsistent results (eg. Brain, 1975_1). 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 <u>et al</u>, 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 <u>et al</u>, 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 <u>et al</u>, 1978; Johnson and Levine, 1974; Krieger, 1974; Wilkinson <u>et al</u>, 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 preg-
nant 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 parti-
cular 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 con-
trast, others have shown the same response at peak and trough
(Retiene et al, 1967; Zimmerman and Critchlow, 1967). Brain
and co-workers (Brain, 1975[1]; Brain and Nowell, 1971;
Goldsmith et al, 1976; Goldsmith et al, 1977) have argued
that individually housed females may be more stress-respon-
sive 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 ran-
domly 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 un-smeared 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
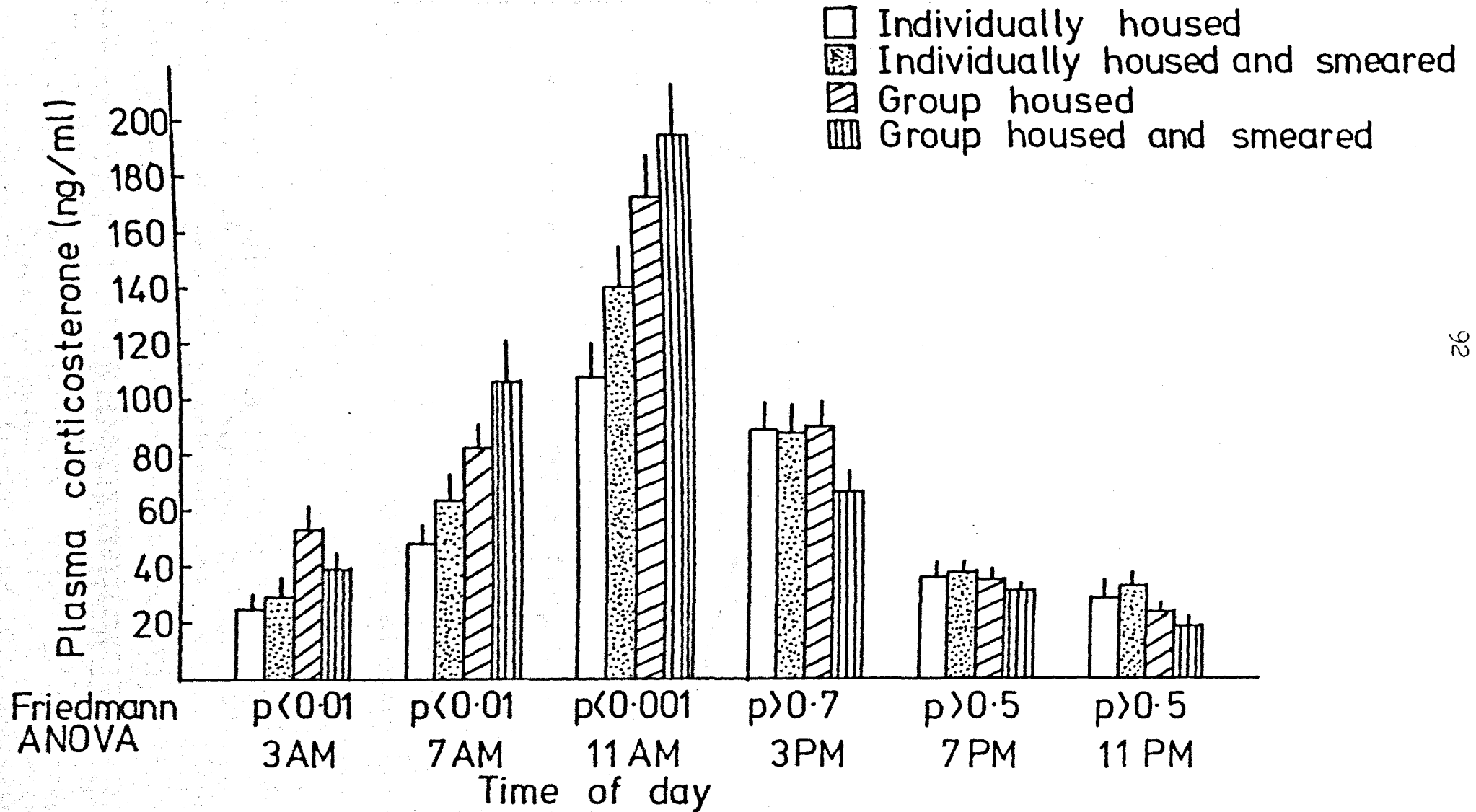
Legend:
☐ Individually housed
▨ Individually housed and smeared
▧ Group housed
▥ Group housed and smeared

Y-axis: Plasma corticosterone (ng/ml)

Friedmann ANOVA:
$p < 0.01$ (3 AM)
$p < 0.01$ (7 AM)
$p < 0.001$ (11 AM)
$p > 0.7$ (3 PM)
$p > 0.5$ (7 PM)
$p > 0.5$ (11 PM)

Time of day

Fig. 2 Basal and Stress Corticosterone levels throughout the day in female mice subjected to different housing conditions

Fig. 3 Plasma corticosterone levels in differentially housed female mice
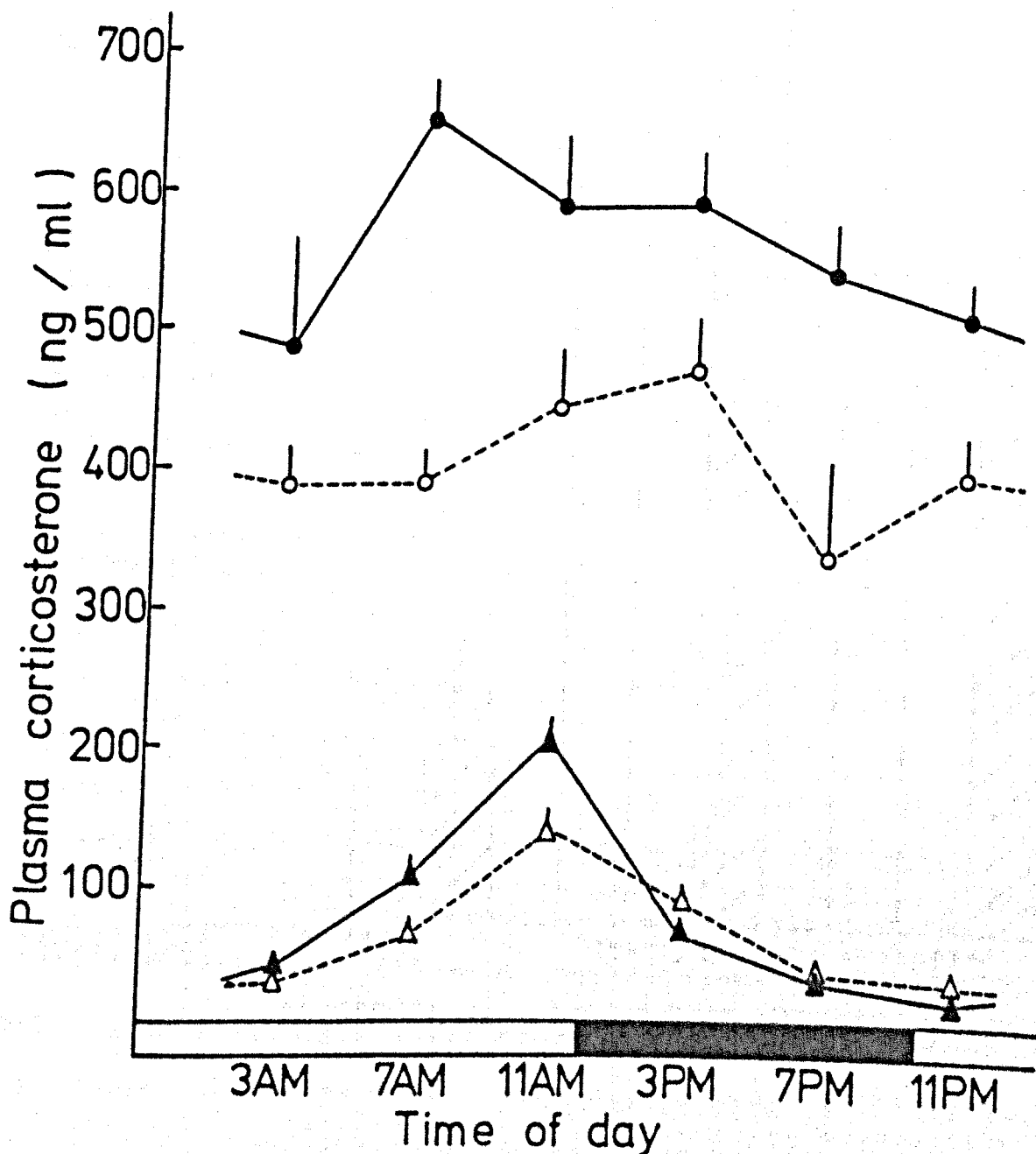
□ Individually housed
▨ Group housed
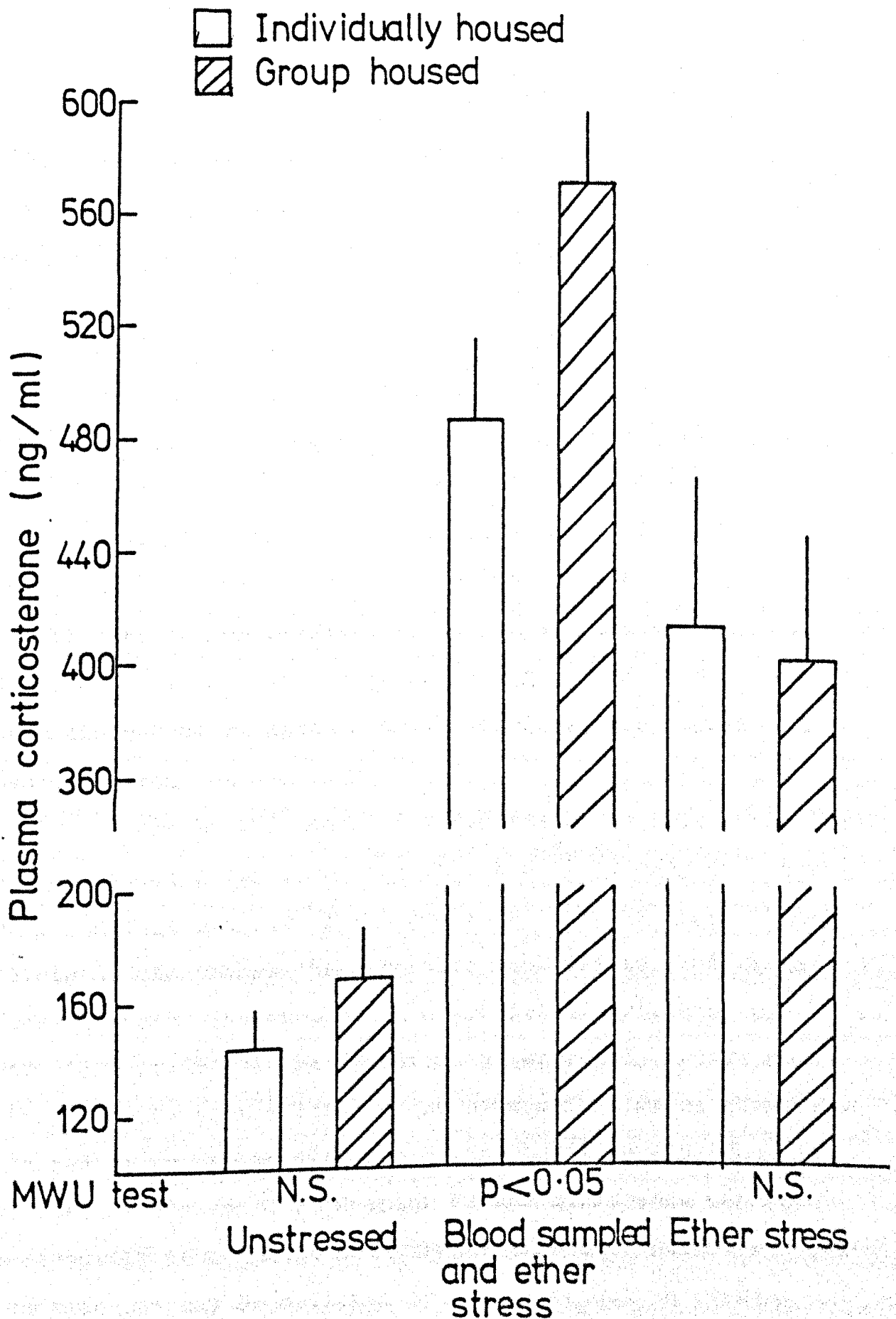
Discussion

The finding that grouped female mice have signi-
ficantly higher basal plasma corticosterone levels than
individually housed mice at the circadian peak contrasts
with other results where no significant difference was found
(eg. 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 indi-
vidually 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 sig-
nificant difference found here but not elsewhere. However,
the absence of any significant difference between indivi-
dually 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 cortico-
sterone 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 in-
creased social interaction in a group, as postulated by
Christian, (1971_2). 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[1];
Goldsmith et al, 1976, 1977) eg. previous disturbance, blood
sampling, social stability or differential stress respon-
siveness 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 circa-
dian peak of adrenal activity did elevate plasma cortico-
sterone 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 percen-
tage 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[1]; Brain and Nowell, 1971[2]; 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[2]).

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[2]) 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_2).

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 (eg. Crowcroft and Rowe, 1957, 1958; Christian, 1956, 1961, 1963_1). 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 (eg. 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 (eg. Whitten, 1966; Parkes and Bruce, 1961) and that these effects are mediated by pheromones found in male mouse urine (eg. 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

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.

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.

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 ie. 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.

	Plasma Corticosterone (ng/ml)	
	Basal	Stress
Individually housed with male (Im)	168 ± 13 n=20	567 ± 23 n=20
Group housed with male (Gm)	170 ± 16 n=19	552 ± 24 n=19
Individually housed and male urine (Imu)	264 ± 18 n=14	566 ± 30 n=14
Group housed and male urine (Gmu)	134 ± 11 n=10	435 ± 39 n=10

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.

	Plasma Corticosterone (ng/ml)			
	Basal		Stress	
	Pro-oestrus values	Other values	Pro-oestrus values	Other values
Individually housed with male (Im)	223 ± 16 n=5	150 ± 13 n=15	609 ± 67 n=5	552 ± 22 n=15
Group housed with male (Gm)	235 ± 40 n=3	159 ± 16 n=16	552 ± 71 n=4	553 ± 26 n=15
Individually housed and male urine (Imu)	289 ± 31 n=7	240 ± 14 n=7	562 ± 60 n=7	569 ± 19 n=7
Group housed and male urine (Gmu)	175 n=2	124 ± 12 n=8	352 n=2	456 ± 46 n=8

TABLE 3

Plasma Corticosterone Levels in Grouped and Individually
housed Female mice 30 minutes, 4 and 24 hours after
application of Male Urine or Water to the cage.

	Plasma Corticosterone (ng/ml)		
	After 30 minutes	After 4 hours	After 24 hours
Individually housed and water	137 ± 12 n=10	91 ± 8 n=10	136 ± 13 n=10
Individually housed and male urine	339 ± 58 n=9	106 ± 14 n=9	148 ± 11 n=9
Group housed and water	192 ± 44 n=10	108 ± 9 n=10	–
Group housed and urine	210 ± 31 n=9	118 ± 18 n=10	–

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.

	Plasma Corticosterone (ng/ml)	
	Pro-oestrus	Others
Individually housed and water	165 ± 29 n=3	125 ± 11 n=7
Individually housed and urine	532 ± 93 n=3	242 ± 27 n=6
Group housed and water	560 n=1	151 ± 17 n=9
Group housed and male urine	–	210 ± 31 n=9

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 (eg. 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
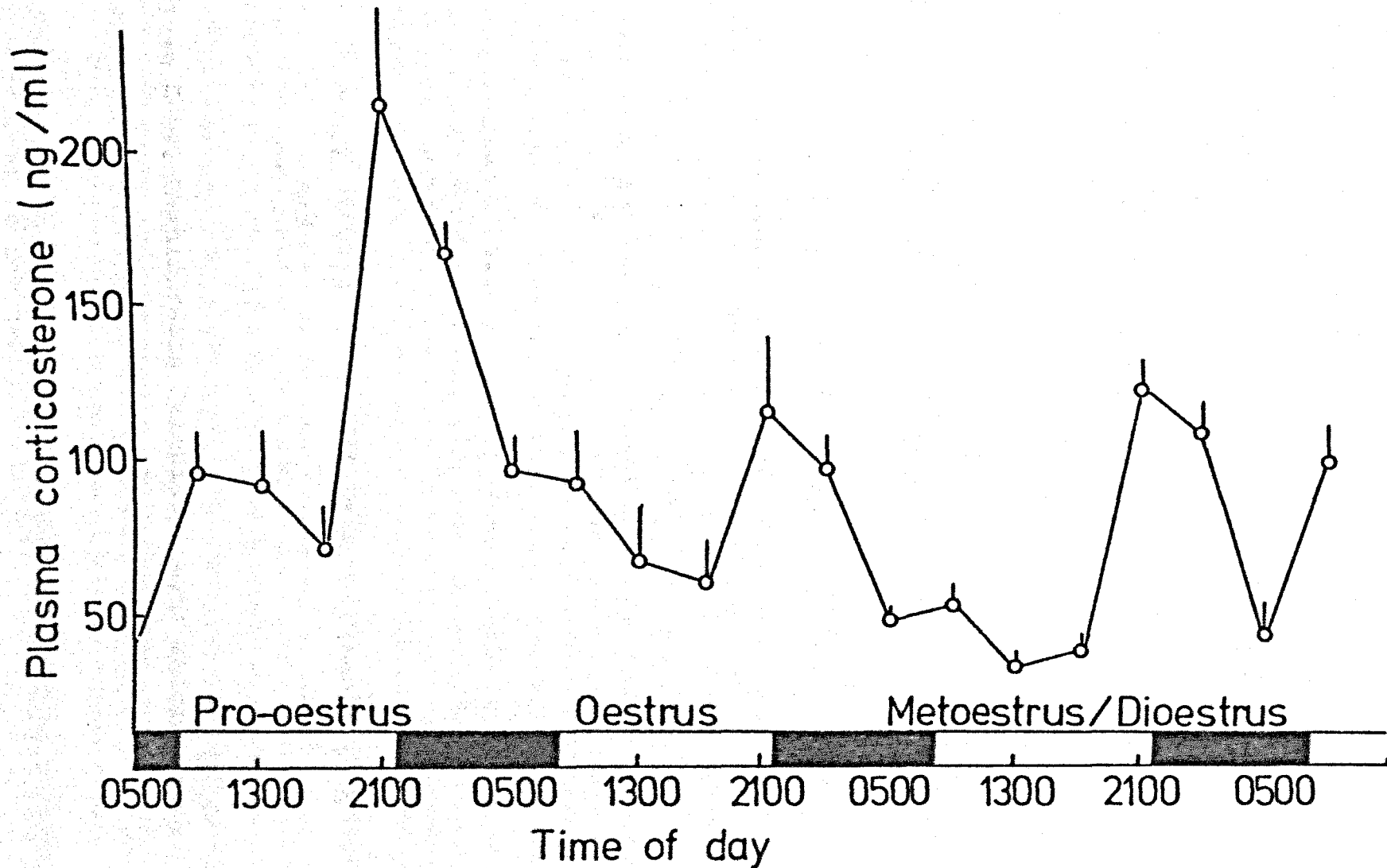
TABLE 1

Concentration of Plasma Corticosterone at different times
of the day during the Oestrous Cycle of the Mouse.

Time	Pro-oestrus ng/ml	Oestrus ng/ml	Metoestrus/ Dioestrus ng/ml
0500	42.5 ± 12.6 n=6 NS	93.0 ± 14.3 n=7 $p<0.01$	45.6 ± 4.6 n=17
0900	96.2 ± 14.9 n=7 $p<0.01$	89.9 ± 19.1 n=8 $p<0.025$	51.6 ± 8.7 n=14
1300	91.6 ± 19.4 n=9 $p<0.001$	65.3 ± 22.4 n=4 NS	30.5 ± 4.3 n=15
1700	68.2 ± 18.0 n=12 NS	58.0 ± 17.9 n=8 NS	34.5 ± 5.8 n=7
2100	214 ± 28.5 n=10 $p<0.01$	114 ± 24.5 n=7 NS	120 ± 10.5 n=16
0100	165 ± 11.3 n=9 $p<0.05$	94.6 ± 10.6 n=7 NS	107 ± 11.9 n=13

Mean Values $\pm$ 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. Vandenbergh, 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 Vandenbergh, 1974) can delay vaginal opening and first

oestrus by up to twenty days compared to singly housed females. Male presence (Vandenbergh, 1967) and male urine (Colby and Vandenbergh, 1974) are effective at accelerating female sexual maturation. However Christian, (1956, 1963_2) 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_2) 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 ie. 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 pseudoprenant 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 di-oestrus 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.

	n	Mean Frequency of Pro-oestrus/ Oestrus in 16 days + SEM	
Individually housed	10	3.9 ± 0.28	
			*
Group 10	10	1.9 ± 0.23	
			N.S.
Group 30	30	1.8 ± 0.14	

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.

	Number of 4,5 and 6 day cyclers	Number of Longer cyclers	Chi-squared test
Individually housed	9/10 (90%)	1/10 (10%)	
			$p < 0.001$
Group 10	0/10 (0%)	10/10 (100%)	
			N.S.
Group 30 (a)	2/26 (8%)	24/26 (92%)	

(a) 4 mice had abnormal cycles

TABLE 3

Effect of Housing Immature Female Mice in groups and/or
with adult females, on the frequency of Pro-oestrus/
Oestrus Smears.

	n	Mean Frequency of Pro-oestrus/ Oestrus smears in 10 days ± SEM	
Individually housed	20	2.2 ± 0.12	
Groups 10	20	1.75 ± 0.14	*
Groups 10 (5 adults + 5 immature)	20	1.65 ± 0.17	N.S.
Groups 30 (20 adults + 10 immature)	20	1.60 ± 0.17	N.S.

N.S. spanning groups

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.

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

N.S. Not significant

TABLE 5

Effect of Male Presence and Male Urine on the type of

Oestrous Cycle.

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

TABLE 6

Summary of Results: Percentage of Females in different

Social Environments with Short or Long Cycles.

	4,5 or 6 day cyclers	Abnormal and Long cyclers	n	Number of days smeared
Individually housed plus male	95%	5%	20	8
Individually housed plus male urine daily	100%	–	14	8
Individually housed	93%	7%	38	8-10
Individually housed from weaning	95%	5%	20	10
Group of 10 plus male	85%	15%	20	8
Group of 10 plus male urine daily	80%	20%	10	8
Group of 10 from weaning	50%	50%	20	10
Group of 10	34%	66%	60	8-10
Group of 10 (5 from weaning and 5 adults)	35%	65%	20	10
Group of 30	33%	67%	30	10
Group of 30 (10 from weaning and 20 adults)	25%	75%	20	10

TABLE 7

Average Number of Days until Oestrus Phase after Grouping Females away from Males at different Stages of the Oestrous Cycle.

	No. of days to Oestrus	% Showing Long cycles immediately
Dioestrus (n=18)	2.94 ± 1.25	6%
Pro-oestrus (n=22)	1.09 ± 0.29	0%
Oestrus (n=16)	8.75 ± 3.24	69%

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 luteotrophin 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_1) 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_2). The mechanism involved in the maintainance 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 gluco-
corticoid 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 psycho-
logical means) then one might expect plasma levels of glu-
cocorticoid would be a good indicator of stress. However,
these assumptions are not necessarily valid.

Most assays of rat or mouse plasma glucocorti-
costeroids 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 un-
stressed total corticosterone levels by the negative feed-
back 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 particulary 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 out-put (Ogle and Kitay, 1979; Vasquez and Kitay, 1978). Pro-gesterone can depress corticosterone output (Rodier and Kitay, 1974; Phillips and Poolsanguan, 1978). In view of these hor-mone 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 cortico-sterone 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 bene-fit (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 thre-shold 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 nega-tive feedback on ACTH (Yates and Maran, 1974[1]). 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$_1$). It is known that corticosteroids affect glucose metabolism (eg. Schulster et al, 1976), blood pressure (Yates and Maran, 1974$_1$), 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_1; 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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