INTERACTIONS BETWEEN PLASMA TESTOSTERONE AND SEXUAL BEHAVIOUR IN THE MALE HOUSE MOUSE (*MUS MUSCULUS*)

by

JENNIFER BATTY

Thesis presented for the Degree of Doctor of Philosophy of the University of Edinburgh, Faculty of Science.

NOVEMBER 1976

SUMMARY

The experiments described in this thesis investigate whether differences in measures of sexual behaviour between strains of the male house mouse (Mus musculus) are related to differences in the levels of plasma testosterone.

There was found to be a negative correlation between measures of sexual behaviour and levels of plasma testosterone across the eight lines of mice examined. This result was not mediated by an influence of copulation on plasma testosterone levels. This inverse relationship was confirmed for some of the measures of sexual behaviour in an F_2 analysis.

Testosterone levels were found to show rapid fluctuations when males were exposed to receptive females. In some strains, testosterone levels were increased after 15 minutes exposure to an inaccessible receptive 'teaser' female. Immediately after a sexual behaviour test, those males which showed sexual responses had increased plasma testosterone levels compared to males which showed no sexual responses. Testosterone levels were found to be at their highest at the initiation of mounting responses, and to gradually decline to low levels 30 minutes after ejaculation. It was suggested

that rises in testosterone levels, or the release of LH-RF and LH might be important in the mechanisms which initiate sexual responses.

The dose-response relationships of (1) measures of sexual behaviour and (2) seminal vesicle weights, with doses of testosterone propionate (TP) injected into castrate males of three strains were examined. It was found that the doses of TP necessary to maintain measures of sexual behaviour were greater than those necessary to maintain the weights of the seminal vesicles. The strains showed differences in the doses of TP necessary to maintain the weights of the seminal vesicles, but no differences were found in the doses of TP necessary to maintain behavioural measures. It was suggested that the 'basal' levels of plasma testosterone may be less than those necessary for the display of 'normal' sexual behaviour, and that these results support the hypothesis that 'acute' increases in plasma testosterone levels may be an important factor in determining the probability of sexual responses.

The effects of neonatal testosterone injections on adult sexual behaviour were investigated. It was suggested that differences in the 'neonatal' testosterone pulse might be related to the differences in sexual behaviour between the strains, and that 'standardizing' this pulse

by injections of TP neonatally might reduce the between strain variability in sexual behaviour, and perhaps in plasma testosterone levels. Measures of sexual behaviour were increased in some strains, and there was a decreased between strain variance. Testosterone levels were increased in one strain, and there was an overall decrease in between strain variability. It was suggested that differences in the neonatal pulse of testosterone may contribute to the between strain differences in sexual behaviour, but not necessarily to the between strain differences in testosterone levels.

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CHAPTER ONE

HORMONES AND SEXUAL BEHAVIOUR

1.1. INTRODUCTION

It has been known since ancient times that the castration of man and domestic animals suppresses the development of secondary sexual characters, including aggressive and sexual behaviour. However, Arnold Adolph Berthold in 1849 was the first to demonstrate scientifically that it was some chemical substance produced by the testes that was responsible for these effects. As described by Corner (1947), Berthold's experiments consisted of the castration of six immature cockerels. From two of these birds, he removed both testes, resulting in "typical capons - fat, docile, without combs, wattles and spurs", and also lacking in the "customary attention to hens". In two other birds, he removed the testes as before, but replaced one of them "among the intestines". Both these cockerels became typical cocks - with combs and wattles and the typical aggressive and sexual behaviour. On later removing the implanted testis, which had "acquired a good blood supply", the cock now fully castrated "reverted back to the status of a capon". From the other two birds, he removed one testis, and exchanged the remaining testes, giving each bird the other's sex gland, which he then implanted amongst the intestines. These birds also developed into normal cocks. The

experiment demonstrated that the testis exerts its effects on morphology and behaviour by some substance released into the blood stream.

Since these early days, a whole new field of biological research has developed - behavioural endocrinology. It is however, only in the last 10-15 years that research has been intensive (Beach, 1974). We now know that there are many hormones which have effects on a variety of behaviours, and not only in birds and mammals. Hormones are reported to have behavioural effects in reptiles, amphibians, fish and insects; and have been extensively reviewed (birds - Lofts and Murton, 1973; mammals - Davidson and Levine, 1972, Hart, 1974; reptiles - Crews, 1975; fish - Liley, 1969; insects - Riddiford and Truman, 1974).

The mechanisms by which hormones influence behaviour are not fully understood, and several complementary effects might be involved. For example, hormones are known to have 'central' and 'peripheral' effects. Central effects are the direct effects of hormones on specific brain regions, in particular areas of the hypothalamus. In the castrate rat, implantation of crystalline testosterone into some parts of the hypothalamus will restore sexual behaviour,

whereas implants into other brain areas are ineffective (Davidson, 1966b, Johnston and Davidson, 1972). Such implants have only very small effects on peripheral structures, and are considered to be directly influencing the neural substrates concerned with the behaviour. Peripheral effects of hormones are where there are effects on the sensory input to the central nervous system. For example, in the rat, the ejaculatory response is partially dependent on stimuli from the penis during intromission. Beach and Levinson (1950) have shown that after castration, there is a regression of the sensory penile spines, and the increased ejaculation latency may be a result of this decreased sensory feedback. After testosterone treatment the spines are restored, and ejaculation latency returns to precastrational levels.

We also know that there are effects of hormones during early development, which have permanent effects manifested in the adult. For example, if neonatal female mice are injected with testosterone, they will show an increased level of male sexual behaviour, such as mounting and intromission responses (Manning and McGill, 1974). These effects may also be considered as 'central' and/or 'peripheral'. The neonatal testosterone may have produced changes in the neuronal connections in the brain, or may have produced peripheral changes in hormone production and metabolism; and morphological

changes such as clitoral hypertrophy. All these influences may combine to result in the changed behaviour of the adult (Davidson and Levine, 1972).

It is often very difficult to assess whether a hormonal effect on behaviour is the result of central or peripheral changes. Sometimes it is possible to separate out particular peripheral effects, by for example selective denervation, or by examining different behavioural measures. For example, changes in mount latency are unlikely to be mediated by changes in the peripheral feedback from penile spines, although other peripheral effects, such as changes in the responsiveness of the olfactory mucosa cannot be excluded. Perhaps it is unwise to attempt to separate out particular changes as being the 'major' effects: hormones have effects on so many tissues that the changes in behaviour are most likely to be the product of a whole concert of small changes which summate together, resulting in the behaviour being displayed under appropriate social and environmental conditions.

The relation between hormones and sexual behaviour has been the most investigated area of behavioural endocrinology (Davidson and Levine, 1972). One of the main questions has been to determine how variations in sexual behaviour, between individuals and during seasonal

and sexual cycles, can be related to variations in hormone levels, and the extent to which such hormonal changes are instrumental in mediating behavioural change. The techniques for investigating this directly, by measuring plasma hormones, have however only been developed in the last few years, and much of the knowledge we have of the relationships between hormones and sexual behaviour comes from work involving removal of the hormone by castration, and replacement therapy with exogenously administered hormone. Experiments of this type are discussed, with reference to male sexual behaviour in mammals, in Sections 1.2.1 and 1.2.2. There are problems however in such an approach - castration and replacement therapy with hormones, such as androgens, produce so many changes in physiology, that there are difficulties in assessing which changes were in fact responsible for the changes in behaviour. For example, after castration, the pre-existing balance between hormones is upset, and there are increased plasma levels of luteinizing hormone (LH) from the pituitary, and a regression of peripheral structures such as the seminal vesicles. The resultant decline in behaviour may be a consequence of several neural and hormonal factors, not necessarily the specific loss of testicular hormones. We can overcome these problems to a certain extent by examining the 'natural' changes in hormone levels which occur during

development, or at different times of the year in seasonally breeding animals. If there is found to be a correlation between increasing levels of sexual responses, and increasing titres of the hormone, then this is good supporting evidence that the results of the hormone manipulation experiments were duplicating a 'natural' event. It is important that both these approaches be applied in investigating the role of particular hormones in controlling sexual behaviour. As has often been pointed out - correlations between hormones and behaviour are not by themselves evidence of causality. Some of the work relating changing levels of testosterone and sexual behaviour, during development and in seasonal breeders, is discussed with reference to male mammals in Section 1.2.3.

Experiments examining the behavioural effects of castration and replacement therapy with testosterone, and the correlations between testosterone levels and sexual behaviour in the developing animal and in seasonal breeders, suggests that testosterone is the hormone important in the control of sexual behaviour. We now know however that steroid hormones, such as testosterone, can readily be metabolized into other active substances, and one of these other metabolites might itself be influencing the behaviour. This is examined in Section 1.3, and the evidence that other hormones might be involved is discussed with reference

to male sexual behaviour in mammals. Although many metabolites can maintain or restore sexual behaviour in castrate males, and in some animals there is evidence that oestrogens in particular may be important, testosterone is a hormone which is found in the plasma of all male mammals so far examined, and also is a universal restorer of sexual behaviour in the castrate male mammal.

The evidence is very convincing that androgens from the testis, and in particular testosterone, are involved in the control of sexual responses in male mammals. We might therefore ask: can we explain differences in sexual behaviour between individuals in terms of differences in plasma testosterone levels? It is this aspect of behavioural endocrinology with which this thesis is concerned, and the discussion in Section 1.4 considers this question further.

1.2. ANDROGENS AND SEXUAL BEHAVIOUR IN THE MALE

1.2.1. Castration experiments

The most direct way to demonstrate the relationship between testicular hormones and sexual behaviour is by castration. In the rat, castration results in a progressive decline in the behaviour, with the ejaculatory responses being the first to disappear, followed by intromission and then mounting (Beach, 1944). In a more recent study, Davidson (1966a) observed that although some rats lose the ejaculatory pattern within a few weeks, others can continue to show the ejaculatory response for up to 5 months after castration. He also observed that if a rat was capable of showing intromissions, if he was left for long enough with a female, he would also show ejaculation. Mounts were however observed after the disappearance of intromissions and ejaculation. Differences in the dependence of the behaviour on circulating androgens are clearly shown by McGill and Manning (1976), in their study of the effects of castration in different strains of mice, where it was found that some strains lose the ejaculatory response within a matter of days, whereas other strains will continue to ejaculate for up to a year. Similarly, of the tropical goats studied by Hart and Jones (1975), only one of eight animals lost the ejaculatory reflex after 18 weeks, and in rhesus monkeys Phoenix, Slob and Goy (1973) report that 50% of males show ejaculation responses a year after castration.

There are differences in the degree of dependence of sexual behaviour on androgens between species, and between individuals of the same species. The mechanisms behind these differences are not known, but they are not the result of compensatory adrenal androgen production, since adrenalectomy has no effect on the maintenance of post-castration sexual behaviour in rats (Bloch and Davidson, 1968) and mice (Thompson, McGill, McIntosh and Manning, 1976).

1.2.2. Replacement therapy experiments

Testosterone is the major hormone produced by the testis of the adult rat (Resko, Feder and Goy, 1968), and esters of this hormone (which have longer lasting effects) such as testosterone propionate (TP) have been largely used in replacement therapy experiments.

After castrate animals have stopped showing sexual responses, TP treatment will restore all aspects of the behaviour, although there may be a 'lag period' of several weeks before complete behaviour is shown. Beach (1944) reported that TP treatment of castrate rats resulted in a reactivation of the mating pattern in 'reverse order' - thus mounts and intromissions were restored first, and ejaculation later. Sometimes elements of sexual behaviour can be shown to respond in a dose-dependent manner, and Beach and Holz-Tucker (1949) found that with increasing doses of TP there was a reduction in mount latency, and an increase in the proportion of animals showing intromissions.

Whalen, Beach and Kuehn (1961), examined the doses of TP necessary to restore copulatory behaviour after it had ceased following castration, and found that the doses were much greater than those found necessary by Beach and Holz-Tucker (1949) to maintain the behaviour. This has been further investigated by Davidson (1971), who found that eight times the dose of TP is needed to restore mating in rats castrated for two months, than is needed to maintain mating, when hormone treatment is started at the time of castration. Apparently, continued hormonal stimulation is necessary if the structures which mediate sexual behaviour are to remain responsive to the hormone.

1.2.3. Correlations between testosterone levels and sexual behaviour in:

a) seasonal breeders

Correlations between increasing levels of androgens and seasonal changes in behaviour were first established indirectly from examinations of testis weights, and androgen-responsive tissues such as the seminal vesicles. But now, with the availability of assays which measure plasma testosterone, we can examine directly the changes in hormone levels which are associated with changes in sexual behaviour. In the last few years there have been many papers reporting seasonal changes in testosterone levels. In the white-tailed deer, (Odocoileus virginianus borealis), McMillan, Seal,

Keenlyne, Erickson and Jones (1974), report that during the breeding season, plasma testosterone levels are increased sevenfold over the levels recorded out of the breeding season, 2.1 ng/ml as compared with 0.3 ng/ml. Similarly, plasma testosterone levels are reported to be elevated during the breeding season for the horse (Berndston, Pickett and Nett, 1974), and the rock hyrax (Neaves, 1973).

However, such correlations do not reflect an 'absolute' control of sexual behaviour by testosterone alone, since administration of exogenous androgens will not necessarily result in the behaviour being shown outside the normal season. For example, in the red deer stag (Cervus elaphus), although sexual behaviour is testosterone dependent, and only shown during the 'rut', administration of testosterone at other times of the year has no effect on sexual behaviour, and will not induce it out of season (Lincoln, Guinness and Short, 1976). Thus, increased plasma androgen levels during the breeding season are necessary, but not a sufficient stimulus for the normal display of sexual responses, and other as yet unknown events are also involved.

b) in sexual development

In immature males, plasma testosterone levels are low, but gradually increase until sexual maturity is reached. During this period elements of sexual behaviour start to appear. In rats, plasma levels of

testosterone start to increase beyond about day 30 after birth, and reach maximum levels at about day 90 (Resko, Feder and Goy, 1968), and during this period, between days 40-55, the adult mating pattern develops (Stone, 1924).

Injections of TP given to immature rats can hasten the onset of sexual responses (Beach 1942a, Baum, 1972, 1973), such that animals will intromit and ejaculate at an earlier age than controls. This suggests that in young animals, sexual behaviour is absent partially because of low levels of circulating androgens.

To summarise, the evidence that testosterone is involved in the control of sexual behaviour comes from three main lines of research. Firstly, castration results in a decline of sexual behaviour, which, secondly can be prevented by injections of TP; and thirdly there are correlations between increased testosterone levels and the occurrence of sexual behaviour in seasonally breeding animals, and in the developing individual.

1.3. TESTOSTERONE METABOLISM AND SEXUAL BEHAVIOUR

Although testosterone is the major plasma androgen, there is now an increasing amount of evidence suggesting that it may be converted to other active steroids at the target organ. For example, the prostate, seminal vesicle and brain of the rat can reduce testosterone to 5 α - dihydrotestosterone (DHT) (reviewed by Minguell and Sierralta, 1975) and also many tissues can aromatize testosterone to oestrogens - for example, brain tissues of monkeys, rabbits, rats and mice (reviewed by Naftolin and Ryan, 1975).

To determine whether such metabolic conversions reflect the normal mechanisms of action of testosterone, many studies have examined the effects of DHT and oestrogens on the maintenance and restoration of sexual behaviour and accessory sex-structures. In the castrate rat, administration of DHT will not restore sexual behaviour (McDonald et al, 1970; Feder, 1971), but will restore peripheral androgen dependent structures such as the seminal vesicles. The administration of oestrogens will stimulate sexual behaviour in the adult castrate rat (Davidson, 1969; Södersten, 1973). However, the most effective restorer of sexual behaviour has been reported to be a combination of DHT plus low doses of oestrogens, and Baum and Vreeburg (1973) report that

200 µg DHTP plus 2 µg oestradiol benzoate injected every day into castrate rats will prevent the decline of sexual behaviour. From these, and other similar results arose the 'Aromatization hypothesis', which proposed that the metabolic conversion of androgens to oestrogens is a necessary step in the mechanism of hormone dependent sexual behaviour. Further work, involving the administration of various androgens which, according to their structure, could or could not be converted to either DHT or oestrogens, were tested for their efficacy in maintaining and restoring sexual behaviour. Androgens which can be converted to oestrogens ('aromatizable androgens'), such as testosterone, androstenedione, androstenediol are capable of restoring the full pattern of sexual activity in castrate rats, (Beyer, Larsson, Perez-Palacios and Morali, 1973), whereas non-aromatizable androgens such as DHT, androstanedione, 3 α-androstostanediol and androsterone (Beyer et al, 1973) will not restore sexual behaviour, although will restore sexual accessory structures such as seminal vesicle weights and penile spines. These results show that in rats, the mechanism whereby androgen acts upon the neural tissues mediating sexual behaviour may differ from the mechanisms whereby androgen stimulates the growth of the accessory sexual structures, and that 'aromatization' may be important in the brain, whereas 5-α reduction may be important in peripheral structures.

However, there are some problems with this hypothesis, and it may not be applicable to other species. For example, DHT has been reported to restore sexual behaviour in the castrate mouse (Luttge, Hall and Wallis, 1974), and rhesus monkey (Phoenix, 1974). It may be that these effects are mediated by an increased peripheral feedback, since DHT will restore sensitivity through its action on the penile spines (Parrott, 1975), an effect which might be important in these sexually experienced animals. However, DHT will <u>initiate</u> behaviour, in sexually naive castrate guinea pigs (Alsum and Goy, 1974), rabbits (Beyer and Rivaud, 1973) and golden hamsters (Payne and Bennett, 1976), suggesting that in these species aromatization is not a necessary step in the mechanism of androgen action on sexual behaviour.

This discussion is not however critical to the questions under examination in this thesis and is further reviewed by Perez-Palacios et al (1975). Although the mechanism of androgen action at the molecular level may not be clear, the primary hormone is not disputed as being testosterone, and in all mammalian and avian species so far investigated, testosterone can maintain or restore sexual behaviour after castration.

1.4. CAN DIFFERENCES IN SEXUAL BEHAVIOUR BETWEEN INDIVIDUALS BE EXPLAINED BY DIFFERENCES IN THE LEVELS OF CIRCULATING ANDROGENS?

The observation that sexual behaviour declines after castration, and can be restored in a dose-dependent manner as described by Beach and Holz-Tucker (1949) raises the possibility that individual differences in sexual behaviour may be related to differences in the levels of circulating androgens. Attempts to stimulate behaviour in 'poor' copulators by injections of TP have been reported to increase sexual responses in rats (Stone, 1938; Beach 1942b), suggesting that sexually unresponsive rats may suffer from an androgen deficiency.

However, these results are not in agreement with those of Grunt and Young (1952, 1953), who found that three lines of guinea-pigs which differed in sexual behaviour, retained these differences when castrated and given equal doses of TP. Similarly, Beach and Fowler (1959) found that there was a correlation between pre- and post-castrational sexual behaviour in rats receiving equal doses of TP, and they concluded that individual differences in sexual behaviour could not be explained in terms of differences in androgen levels. These results were also confirmed by Larsson (1966) who examined the behaviour of two groups of male rats; one showing a low, and the other a high level of sexual

responses. After castration both groups were given high doses of TP (≏ 2 mg/rat/day) but the differences in behaviour, observed before castration, persisted.

These experiments suggest that differences in plasma levels of testosterone do not contribute to behavioural differences in these species and that as suggested by Young (1961) there are differences in tissue sensitivity to androgens. However, this result must be interpreted with caution - the sensitivity to a hormone, and the plasma levels of that hormone may be closely linked. For example, we might speculate that it is the sensitivity to a hormone that in part determines the levels of that hormone in the blood. In this case, testosterone release from the testis is controlled by trophic hormones from the pituitary, in a negative feedback loop - and rising levels of testosterone inhibit, either directly or indirectly via the hypothalamus, the release of trophic hormones. Differences in the sensitivity of the receptors involved in this loop may therefore have a direct effect on the levels of testosterone in the blood. We might therefore still expect there to be differences in the levels of plasma testosterone between animals showing differences in sexual behaviour.

It is now possible to re-examine this problem by directly measuring plasma levels of testosterone, in

animals which differ in sexual behaviour. The experiments described in this thesis investigate whether differences in measures of sexual behaviour in the male house mouse, such as those described by McGill (1962), are related to differences in plasma levels of testosterone. In Chapter Three, Experiment 1 examines these two measures in 8 lines of mice, and discusses the relations between them.

CHAPTER TWO

GENERAL MATERIALS AND METHODS

2.1 EXPERIMENTAL SUBJECTS

2.1.1. The House Mouse (Mus musculus)

The house mouse has a number of characteristics which make it a useful animal for laboratory study. They are easy to maintain and breeding is continuous throughout the year - gestation lasts three weeks, and the young reach adult size in about ten weeks. Further, many inbred lines are available which should enable comparisons to be made between results, from different workers, on many aspects of mouse physiology and behaviour.

2.1.2. Housing and Maintenance

Subjects were weaned at four weeks and housed, in single sex groups, in large cages (27 x 44 x 12 cm). At eight weeks, they were placed in experimental groups - either singly in small cages (33 x 16 x 12 cm), or in groups of eight in the large cages. (The 'large' and 'small' cages described were as used in all subsequent experiments.) Lighting was on a reverse 12/12 cycle (on 7.30 pm, off 7.30 am), and room temperature was maintained at 22°C. Standard laboratory chow and water were available ad libitum.

The strains and hybrids used, are listed in each experiment. The derivation of the F_1 hybrids is as listed overleaf.

	Parent Lines	
F_1 Hybrid	Female	Male
BDF_1	C57Bl/6Fa	DBA/2J
DBF_1	DBA/2J	C57Bl/6Fa
CBF_1	Balb/c	C57Bl/6Fa

Unless stated otherwise, all animals used were from stocks bred in the Zoology Department, University of Edinburgh.

2.2. BEHAVIOURAL OBSERVATION

2.2.1. General Description

Copulation in the male house mouse is characterised by a series of mounts and mounts with intromission which result in ejaculation (McGill, 1962). When a female is placed with a male, he approaches and will show genital investigation. After a few minutes he mounts, and if the female is in oestrus, she will adopt a lordosis position, with the back arched, and head and hind-quarters raised. If not in oestrus the female rears on her hind legs and may kick the male away. When the male mounts, he palpates the female's sides with his forelimbs, and shows rapid pelvic thrusting movements. As soon as intromission is achieved the rate of thrusting changes to a slow steady rate of about one per second. After about 10-30 seconds, the male dismounts, and both male and female will usually show genital grooming. After a series of intromission bouts, varying from one to over 30 depending on the strain of mouse, ejaculation occurs. This is characterised by an accelerated rate of thrusting, and a quiver of muscle contraction over the whole body. The female is firmly clutched by all four limbs, and the pair may fall over on to their sides, and remain motionless for up to 20 seconds. Male and female then separate and show

further genital grooming. No further sexual interest is shown by the male for a variable period of time, which may range from hours to several days, and is known as the refractory period.

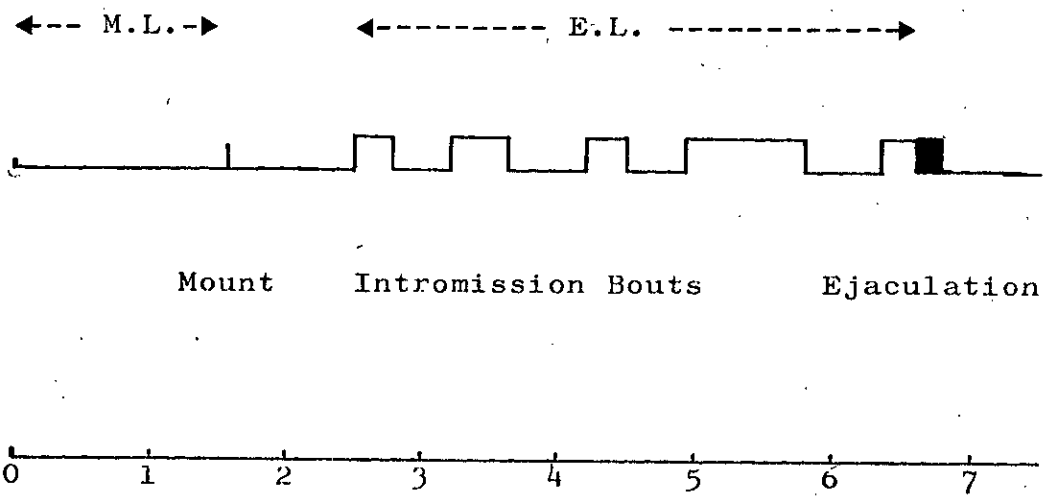
2.2.2. Testing Procedure

The stimulus females used in all behavioural tests were from the BDF_1 hybrid. Behavioural oestrus was induced by intraperitoneal injections of 100 µg oestradiol benzoate on the day prior to testing (0.02 ml from a solution of 5 mg/ml in Arachis oil: Benzyl Alcohol, 90:10 v.v., Intervet Laboratories, Bar Hill, Cambs.). Females from this hybrid were used in all tests in order to reduce variability in male behaviour due to differences in the behaviour of the females. Before being used in a behavioural test, all females were observed for a few minutes with 'stud' males, in order to check that all females were in fact receptive, that is showed little squeaking, some movement during mounting, but remaining still during intromission. As females became more experienced, they reliably showed high receptivity and thus pretesting was discontinued.

Behavioural tests were conducted during the dark phase of the lighting cycle, under dim red illumination. The test animals were observed in clear plastic cages (33 x 16 x 12 cm) with flat lids, and the floor covered

Figure 2.1

A typical copulation of the house mouse as shown on an event recorder tape.



by wood shavings. In a test, the male and a receptive female were placed in the cage and observed for 45 minutes. If no mounts occurred after 10 minutes, then the female was exchanged for another. The male was allowed a total of three females, the third remaining with the male until the end of the test. A male intromitting was left undisturbed until the end of the test, when animals were returned to their home cage.

The behavioural events were measured with an Edgecome - Peebles Event Recorder, and the behaviours recorded were: mounts, intromission bouts and ejaculation. Eight animals could quite comfortably be watched at once, and from the records the following measures were taken:

Mount Latency - the number of minutes from the start of the test to the first mount. (M.L.)

Intromission Latency - the number of minutes from the start of the test to the first intromission. (I.L.)

Ejaculation Latency - the number of minutes from the first intromission to ejaculation. (E.L.)

Total Intromission Time - the sum of the intromission bout lengths (only calculated if the animal ejaculated) (T.I.)

Number of Intromissions - the total number of intromissions before ejaculation. (No.I.)

A diagrammatic representation of a typical copulatory series is illustrated in Figure 2.1.

2.3 ASSAY OF TESTOSTERONE

2.3.1. Principle of the Assay

The measurement of hormone levels by radio-immunoassay (RIA) was first developed by Yalow and Berson in 1959, to determine insulin levels in human plasma. Since then, RIA has become a standard technique in many laboratories. In RIA, labelled and unlabelled hormone compete for binding sites on an antibody - the reaction obeys the law of mass action and, at equilibrium, the binding of the labelled hormone is inversely proportional to the concentration of unlabelled hormone. By counting the radioactivity associated with the antibody, the amount of unlabelled hormone can be inferred by reference to a "standard curve" (Figure 2.2.), which shows the effects of different amounts of unlabelled hormone on the binding of a constant amount of labelled hormone to the antibody. This assay principle is shown in Figure 2.3.

An RIA for testosterone was established by Dr. Charles Corker, at the MRC Reproductive Biology Unit, Edinburgh; where facilities for doing the assays were very kindly made available to myself. The method was based on that of Furuyama, Mayes and Nugent (1970), and is summarised schematically in Figure 2.4. For completeness a full assay description will be given

Figure 2.2

A typical standard standard curve - showing the decrease of labelled testosterone which can bind to the antibody as increasing amounts of unlabelled testosterone are added .

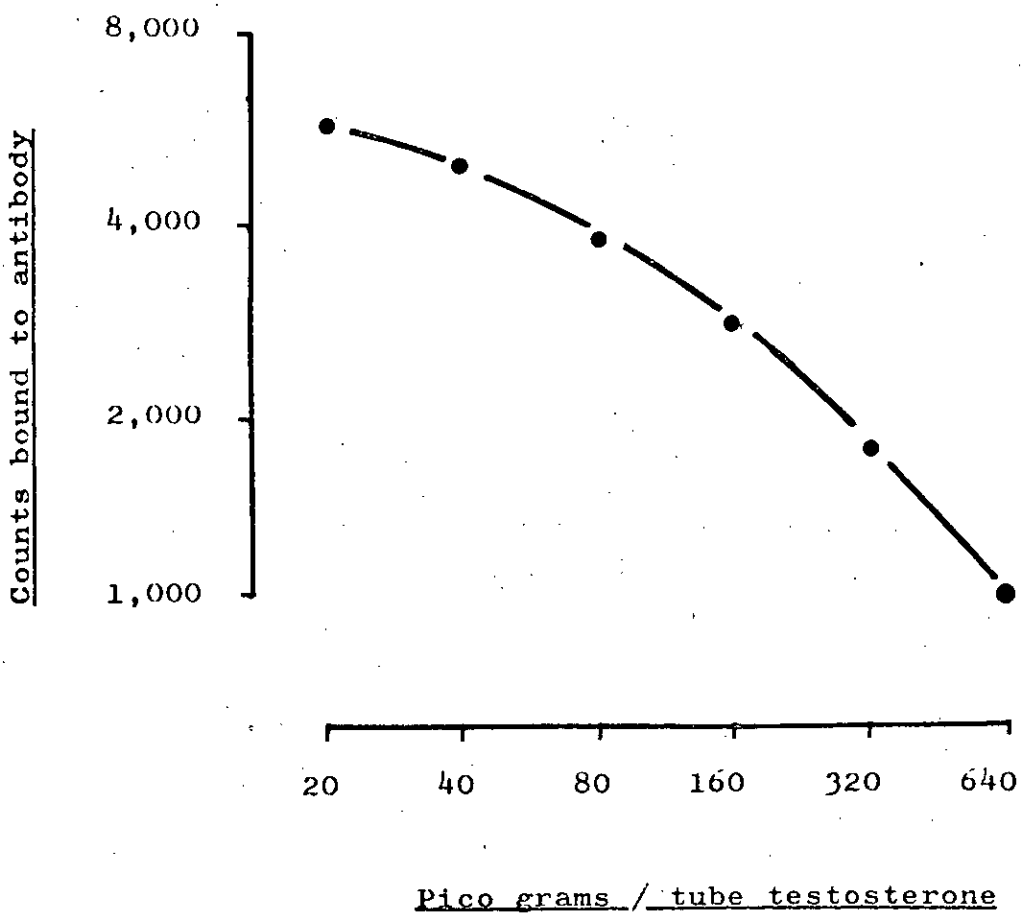
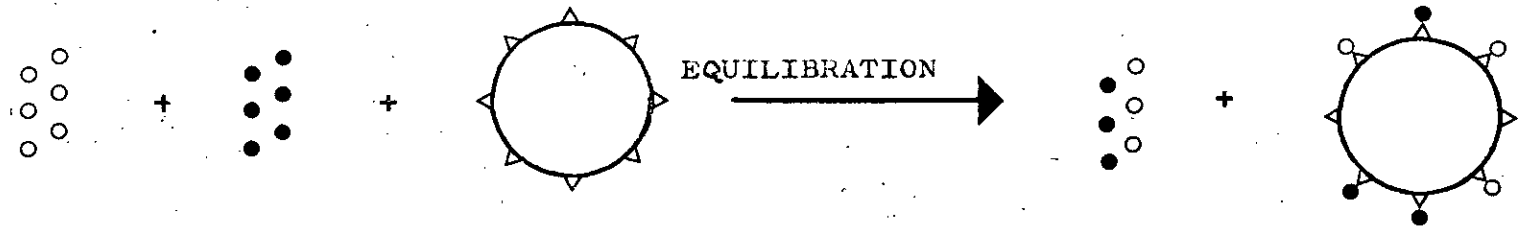


Figure 2.3

The Principle of Radioimmunoassay



Hormone

Radioactive Hormone

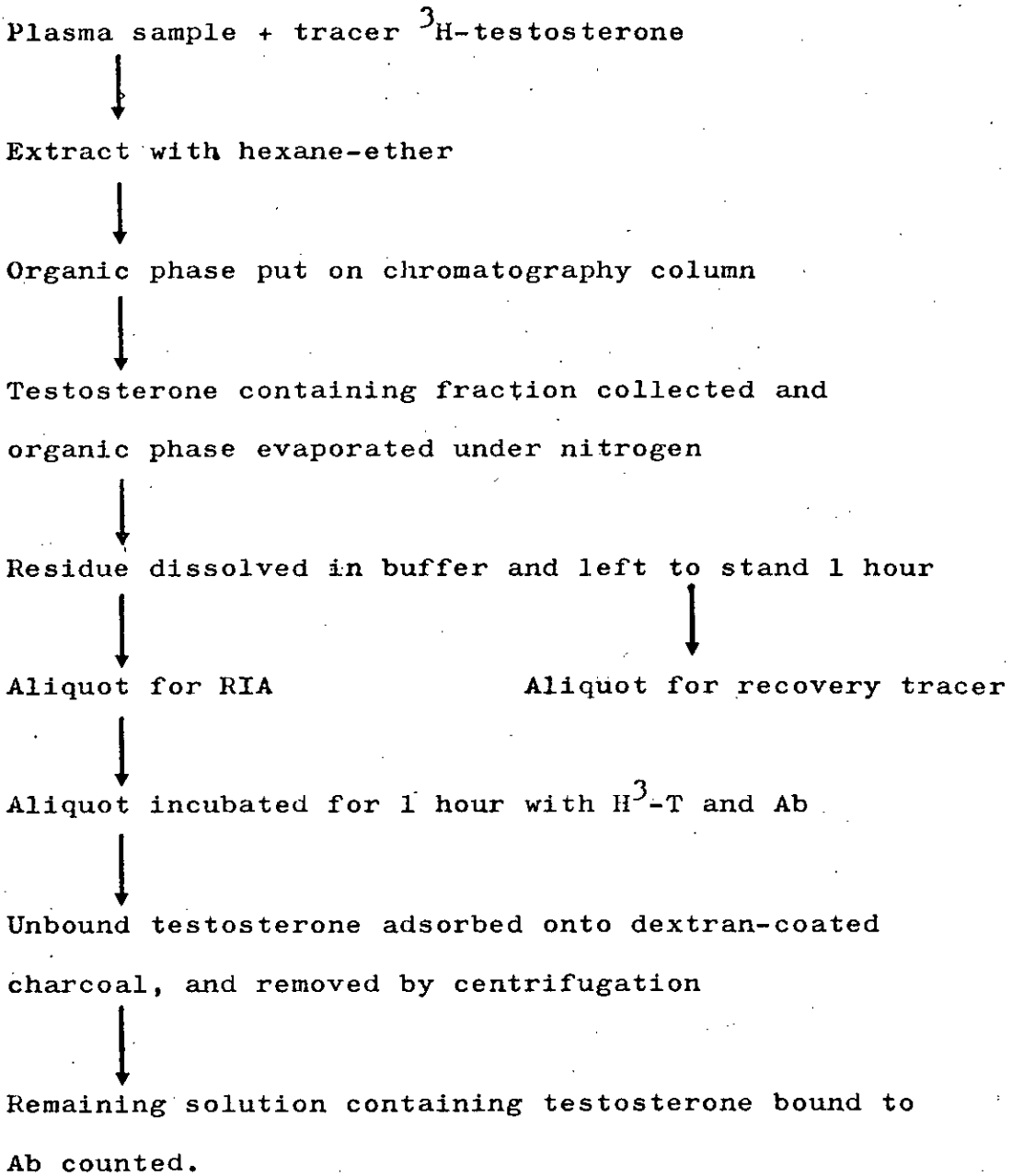
Antibody - offering limited binding sites

Hormone- "free"

Hormone- "bound"

Figure 2.4

Method outline of the testosterone assay.



here but the method has since been published by Dr. Corker (in press).

2.3.2. Materials

Solvents and reagents

The assay reagents, and their suppliers, were as follows:

Alumina (for chromatography) - Savory and Moore Ltd., London

Diethyl ether (analar) - British Drug Houses, U.K.

Hexane - May and Baker Ltd., Dagenham, U.K.

Dextran T70 - Pharmacia, London.

Norit A charcoal, and gelatin - Sigma Ltd., London

Toluene - A.J. Beverage Ltd., Edinburgh

Triton X100 - Koch-Light, U.K.

PPO and dimethyl-POPOP - Nuclear Enterprises Ltd., Scotland.

1,2-^{3}H-testosterone (SA 46.7 Ci/mM)-Radiochemical Centre, Amersham, U.K.

Antiserum

The antiserum (E01) was raised in goat against testosterone -3-carboxymethyl oxime-BSA, and donated by Dr. S. Tillson (1973). When used at a final dilution of 1:18,000, three hundred microlitres bound 60% of the tracer (46pg).

Buffer

The buffer (pH 7.2) was prepared by dissolving 0.01 g merthiolate, 9g sodium chloride, 8.6g disodium hydrogen phosphate, 6.0g sodium dihydrogen phosphate and 1g gelatin in distilled water, and then diluting to 1000 ml.

Extraction Solvent

Extraction solvent was prepared freshly for each assay from Hexane:Ether, 1:4 by volume.

Chromatography

Alumina columns

Glass columns, 11 cm long and 0.5 cm in internal diameter, were prepared from disposable glass pipettes by shortening the constricted part. Each tube was packed with 3 cm of alumina (Al_2O_3) after plugging the base with glass wool. Before use columns were washed twice with ethanol, 4 times with methanol, 3 times with methanol:dichloromethane (1:1) and three times with dichloromethane. Each wash volume was 1.6 ml.

Dextran - coated charcoal

Charcoal (0.25 g) and Dextran T70 (0.025 g) were added to 100 ml buffer and shaken thoroughly. The mixture was stored at $4^{\circ}C$.

Standards

Testosterone (10 mg) was dissolved in 100 µl etnanol. One ml of this solution was diluted to 100 ml with ethanol, 640 µl were removed, evaporated to dryness, and the residue dissolved in 100 ml of buffer giving a concentration of 640 pg/100 µl. This was then diluted to give concentrations of 320, 160, 80, 40 and 20 pg/0.01 ml.

Radioactivity measurement

Aqueous samples were dissolved in 10 ml scintillation fluid and counted in a Packard 3375 Tricarb counter (efficiency 56%). The scintillation fluid was prepared by dissolving 10 g PPO and 0.75 g POPOP in 2500 ml toluene and then adding 1250 ml Triton - x100.

Blood Samples

Animals were exsanguinated under ether anaesthesia, during the first half of the dark phase of the cycle (between 10-12 p.m.). Blood was collected in tubes containing EDTA as anticoagulant (Sarstedt Laboratories, Leicestershire) and centrifuged, within 30 minutes at 1000 rpm for five minutes. Plasma was then removed and stored at -5°C until assayed.

2.3.3. Method

2.3.3.1. Extraction of samples. a) With chromatography. To each 50 µl of plasma was added 20 µl of tritiated testosterone (ca 10,000 cpm) as a tracer, and tubes were run on a whirlimixer for a few seconds. Extraction solvent (1 ml) was then added and tubes whirlimixed for thirty seconds, twice. The tubes were then placed in dry ice/ethanol mixture, until the aqueous layer was frozen, and the organic layer could be decanted onto washed alumina columns. Columns were then washed with 1.6 ml extraction fluid, followed four times with 1.6 ml

0.4% ethanol in hexane, and the testosterone fraction eluted with 3.2 ml of 14.% ethanol in hexane. This fraction was then evaporated to dryness in a stream of nitrogen. Buffer (200 µl) was then added, and tubes left to stand for one hour, to allow the testosterone to dissolve. A recovery aliquot of 50 µl, and an aliquot of 100 µl for assay were then removed.

b) without chromatography

Samples were treated as above, but evaporated to dryness immediately after extraction, with no chromatography step.

2.3.3.2. Radioimmunoassay.

A standard curve, containing 0, 20, 40, 80, 160, 320 and 640 pico grams per tube was set up in duplicate. Antiserum, 100 µl of a $^{1}/_{6000}$ dilution, was then added to both standards and unknowns, followed by approximately 10,000 cpm of tritiated testosterone in 100 µl buffer. Two tubes were included which received 100 µl buffer in place of the antiserum, and which gave a measure of the non-specific binding. Tubes were then whirlimixed, and left to equilibrate for 1 hour at room temperature, or overnight at 4°C. The unbound testosterone was then removed by the addition of 1 ml dextran-coated charcoal. After whirlimixing for a few seconds, tubes were left to stand for 15 minutes, on a bed of crushed ice. Tubes were then centrifuged at 2,500 rpm for 5 minutes, and the supernatant decanted into vials for counting.

Scintillation fluid was added, and after equilibration in the counter for at least 1 hour, the vials were counted for 4 minutes or 10,000 counts each.

2.3.3.3. Calculations.

The results were calculated on an Olivetti Programma 101, using a programme which linearises the standard curve by a reciprocal transformation of the number of counts per tube. The programme (Appendix I) calculates the regression between labelled testosterone counts and unlabelled standard testosterone amounts. The programme then calculates the values of the unknown samples, using the constants of the equation, which are expressed as picograms of testosterone per tube.

Testosterone values were then corrected for recovery and size of aliquots taken, and expressed as ng/ml plasma.

2.3.4. Characteristics of the assay

2.3.4.1. Specificity

Specificity refers to the amount of cross-reactivity of the antibody with other compounds. Of 16 different steroids examined, only dihydrotestosterone was found to interfere with the assay (cross-reactivity = 25%). (Data from Dr. C. Corker.)

The specificity of the assay method, as applied to mouse plasma, was checked by comparing results of samples analysed with and without chromatography. No significant

differences were found between samples, with and without chromatography, of four strains of mice (Table 2.1). This suggests that mouse plasma contains no steroids which interfere with RIA of testosterone, as also found by Lucas and Abraham (1972). The chromatography step was therefore omitted from the assay.

2.3.4.2. Sensitivity

The sensitivity of an assay method is defined as the smallest amount of compound the method will detect. The sensitivity was 8 pg (determined by Dr. C. Corker), which is equivalent to 0.4 ng/ml plasma.

2.3.4.3. Precision

The precision of an assay method represents the reproducability of estimation. The between assay variation was calculated from the results of a quality control run in each assay. This gave a value of 7.7 ng/ml, SE $\pm$ 0.3, n = 27. After several assays had been performed, the recoveries were found to show little variation within, and between, assays (Table 2.2), therefore a constant recovery factor of 85% was used in subsequent calculations.

2.3.4.3. Accuracy

The accuracy of a method is defined as the possible deviation between the estimated amount and the true amount present in the sample, and therefore indicates the systematic errors of a method. The percentage

Table 2.1

Plasma testosterone levels measured after hexane-ether extraction only compared to extraction followed by chromatography.

Strain	With chromatography ng / ml	Without chromatography ng / ml
Balb/c	13.8	15.0
Balb/c	14.4	13.6
Balb/c	20.5	19.4
BDF_1	17.6	15.4
BDF_1	4.6	3.8
BDF_1	1.2	1.0
CBA/H	8.0	7.5
CBA/H	10.3	9.5
CBA/H	11.1	11.4
Mean (Ln $\pm$ SE)	2.24 $\pm$ 0.27	2.17 $\pm$ 0.28
ng / ml	9.4	8.8

Table 2.2

Recovery of tracer testosterone - results from four assays.

a) Within assays

Recovery (%)		Assay A	B	C	D
Mean	$\bar{x}$	82	91	86	84
Standard Error	SE	0.9	1.1	1.7	0.9
Standard Deviation	SD	4.5	5.3	3.8	4.3
Number	n	21	22	5	22

b) Between assays
(using data from above)

Mean recovery	SE	SD	n
85.4	1.9	3.9	4

recovery of known amounts (2-6 ng/ml) of testosterone added to plasma was 105.6 ± 9, after correction for recovery. (Data from Dr. C. Corker.)

2.4. STATISTICAL ANALYSIS

2.4.1. Data Distribution

Experimental results were examined by parametric statistical methods, wherever possible. These may only be used, however, on data which is normally distributed, and therefore preliminary results, from observations of Balb/c and C57Bl/6Fa, were examined to determine the distribution of the data.

The method used was as described in Sokal and Rholf (1969), Program A 3.1. The programme computes the mean and median of the data; G1 and G2 which are measures of skewness and kurtosis respectively; and 95% confidence limits. If the mean and median are not significantly different, and the 95% C.L. for G1 and G2 are symmetrical about zero, then the data is normally distributed. If G1 is positive, then the distribution is skewed to the right; and if negative skewed to the left. This skew will also be shown in the 95% confidence limits which will not be symmetrical about zero. Similarly, a positive G2 indicates a "peaked" curve; and a negative, a "flattened" curve. If the data does not satisfy the conditions necessary for a normal distribution, then a transform can be applied. For example, data showing a skew to the right can often be normalised by the reciprocal or logarithmic transform.

2.4.1.1. Testosterone levels.

Testosterone levels for the two strains showed a positively skewed distribution, which was normalised by logarithmic transformation, as shown in Table 2.3 and 2.4. All testosterone data was subsequently transformed to logarithms for analysis, but for clarity, group mean values are also expressed in the usual way of ng/ml.

2.4.1.2. Behaviour.

The number and total time of intromissions preceeding ejaculation, and the ejaculation latency, were all normally distributed. Mount latency was however skewed to the right and was normalised by the logarithmic transform.

Behavioural data was further examined, to determine the relation between the different measures of sexual behaviour. If two measures are found to be highly correlated, then it is likely that they are indices of the same phenomenon, and continued measurement of both may be unnecessary. In the Balb/c and C57Bl/6Fa strains, the ejaculation latencies were significantly correlated with the number of intromission bouts (Balb/c $r = 0.8$, $df = 22$, $p < 0.001$; C57Bl/6Fa $r = 0.9$, $df = 25$, $p < 0.001$), and the total intromission time (Balb/c $r = 0.8$, $df = 22$, $p < 0.001$; C57Bl/6 $r = 0.9$, $df = 27$, $p < 0.001$). Because of the high correlations between these three measures, it was decided to measure only the latency to ejaculation in the behavioural tests.

Table 2.3

Frequency distribution statistics for testosterone data.

Balb/c Strain Number of animals = 73.

1) using no transform

	Statistic	Stand.Error	Confidence Limits (95 per cent)	
Mean	8.90	0.85	7.21	10.61
Median	6.70	1.07	4.60	8.83
G1	1.09	0.28	0.54	1.64
G2	0.77	0.55	-0.32	1.85

Note that the mean > median; G1 is positive, and its confidence limits are not symmetrical about zero. This data is therefore NOT normally distributed, but shows a marked skewness to the right.

2) using a logarithmic transform

	Statistic	Stand.Error	Confidence Limits (95 per cent)	
Mean	0.78	0.05	0.68	0.87
Median	0.82	0.06	0.70	0.95
G1	-0.42	0.28	-0.97	0.12
G2	-0.72	0.55	-1.81	0.36

Note that mean is not significantly different from the median; G1 indicates a skewness to the left, but confidence limits are symmetrical about zero. This data can therefore be assumed to approximate to a normal distribution.

Table 2.4

Frequency distribution statistics for testosterone data.

C57Bl/6 Strain Number of animals = 52

1) using no transform

	Statistic	Stand.Error	Confidence Limits (95 per cent)	
Mean	5.46	0.65	4.15	6.76
Median	3.30	0.82	1.66	4.93
G1	0.91	0.33	0.26	1.55
G2	-0.41	0.65	-1.68	0.86

Note that mean > median ; G1 is positive , and its confidence limits are not symmetrical about zero. This data is therefore not normally distributed, but shows skewness to the right.

2) using a logarithmic transform

	Statistic	Stand.Error	Confidence Limits (95 per cent)	
Mean	0.55	0.05	0.44	0.67
Median	0.52	0.07	0.37	0.66
G1	-0.05	0.33	-0.69	0.60
G2	-1.26	0.65	-2.53	0.02

Note that the mean is not significantly different from the median; G1 indicates a slight skewness to the left but confidence limits are symmetrical about zero. This data is therefore normally distributed.

2.4.2. Statistical Tests

Where possible parametric tests were used throughout, using appropriate transformations to normalise the distribution of the data where necessary. Homogeneity of variance was determined by the F_{max} test - where the ratio of the two variances is calculated and the value read from "F" tables with degrees of freedom of one minus the sample size for each group. Ratio values greater than "F" at $p < 0.05$ show there to be significant differences in variance. Subsequent statistical tests on such data incorporated correction factors for heterogeneity of variance, as described in Sokal and Rohlf (1969) and Snedecor and Cochran (1967). Differences between groups were analysed by Analysis of Variance (Anovar), and Student Newman-Keuls a posteriori tests. Correlations between sets of data were determined by the Pearson Correlation coefficient, using transformations as necessary to ensure linearity.

Where non-parametric statistical tests were necessary, these were as described in Siegel (1956).

CHAPTER THREE

PLASMA LEVELS OF TESTOSTERONE AND SEXUAL BEHAVIOUR IN STRAINS OF THE HOUSE MOUSE (*MUS MUSCULUS*)

3.1. GENERAL INTRODUCTION

If we are to examine the role of testosterone in the control of male sexual behaviour, it is first necessary to discuss the various components of the behaviour. Sexual behaviour is built up from many units, and each unit may be related in different ways to plasma testosterone. There is no single measure which describes sexual behaviour, but a series of measures which relate to different aspects. Behaviour has components of "start", "continue" and "stop", which in this context may be equivalent to mount, intromission and ejaculation, and each of these may be controlled by very different factors. Evidence that these components may be differentially controlled comes from three lines of experimentation: the effects of castration and testosterone replacement therapy; the mode of inheritance of the components; and the effects of spinal transection.

Beach (1944) examined the effects of castration on the sexual behaviour of the male rat, and found that the different components of mounts, intromissions and ejaculation declined differentially. The ejaculatory response was lost by all rats after 25-30 days, then intromissions declined, until only a few of the rats even mounted. Replacement therapy with testosterone

propionate restored the behaviours in the 'reverse' order - that is mounts, then intromissions and finally ejaculation. Similarly, castration in the mouse results in a differential decline of the three components, with the ejaculatory response being the first to disappear. When androgen therapy was given mount and intromission responses were restored before animals exhibited ejaculatory responses (Champlin, Blight and McGill, 1963). These observations suggest that the three behavioural components have different dependencies on androgens, and might therefore be controlled by different mechanisms.

A further line of evidence comes from observation on the mode of inheritance of mount, intromission and ejaculation responses. Work on the sexual behaviour of hybrids between inbred lines of mice (McGill and Blight, 1963a; McGill and Ransom, 1968) suggests that the latencies to the occurrence of the behaviours can be inherited relatively independently. For example, the mount latency characteristic of one strain, and the ejaculation latency characteristic of a second strain, may be found in association in the hybrid between the strains. Similarly, between inbred lines of mice, the latencies to mounting are not necessarily related to the latency to ejaculation. For example, in a study by Vale and Ray (1972) on the inheritance

of male sexual behaviour in the house mouse, the latencies to mounting and ejaculation of the inbred lines and hybrids were not correlated - for example one of the inbred lines, DBA/2J, showed the longest ejaculation latency and yet also showed the second shortest mount latency of the nine inbred lines and hybrids examined. Thus, the mechanism which 'starts' the behaviour, mounting, may not be the same as that which 'terminates' the behaviour, ejaculation. Although this must seem inherently obvious, the two measures are sometimes taken as being 'general' indicators of sexual motivation, or sexual performance, as discussed by Dewsbury (1975). Evidence that the ejaculatory response may be independently controlled comes from the work of Hart (1968) on the sexual responses of the spinally transected rat. Such animals can exhibit erection and ventral flexion after suitable genital stimulation, which closely resemble the ejaculatory response. Thus, ejaculation can be considered to be a spinal reflex, and not necessarily under 'control' from the brain structures mediating sexual behaviour.

All these experimental results suggest that the different components of sexual behaviour may be relatively independent, and should be considered separately. Beach (1956) proposed that there were in

fact two mechanisms involved - one concerned with arousal of sexual behaviour (the arousal mechanism AM) and another with the execution of the copulatory pattern (the copulatory mechanism CM). Thus, the AM mediates the initiation and maintenance of the behaviour, and as the level of arousal increases, the copulatory threshold is reached, at which point the male mounts the female. Achievement of intromission results in activation of the CM and with successive intromissions the level of excitement within the CM increases until the ejaculatory threshold is reached. Although this model has since fallen into disuse, the terminology remains of value, and the 'AM' and 'CM' will be used in this thesis, but only as a means of describing the factors involved in the initiation of a sexual response, and those involved in the maintenance and completion of that response. Thus I shall consider that the AM is represented by the latency to, and the occurrence of mounting responses, and the CM by the latency to, and the occurrence of ejaculatory responses. The experiments to be described in this chapter, on the relations between plasma testosterone levels and sexual behaviour, will attempt to evaluate the role of the hormone in both of these aspects.

3.2. Experiment 1

The relation between plasma levels of testosterone and sexual behaviour in several strains of the house mouse

3.2.1. Introduction.

To investigate whether differences in sexual behaviour can be related to differences in plasma testosterone levels, these two measures were examined in as many strains as were readily available. The three strains (Balb/c, C57Bl and DBA/2J) described by McGill (1962) were included in the experiment as these were already known to show differences in sexual behaviour.

3.2.2. Method.

Male mice were housed in groups of 6-8, in large cages, under the conditions previously described. The strains examined were CBA/H and Balb/c (from stock bred in the Zoology Department, Edinburgh); C57Bl/6Fa (from the Genetics Department, University of Edinburgh); DBA/2J(F) (from Fisons Ltd., Loughborough) and DBA/2J (from the M.R.C. Animal Breeding Centre, Carshalton). Three F_1 hybrids were also used - from stock bred in the Zoology Department, Edinburgh, using parental strains as described - BDF_1, CBF_1 and DBF_1. When the mice were 9-10 weeks old, testing of male sexual behaviour was begun, using the method previously described. Tests were given twice a week, and the animals were killed three days after the fifth test, and blood was removed for analysis of plasma testosterone.

3.2.3. Results.

Plasma testosterone levels

Within each strain there was a large range in plasma testosterone levels, with considerable overlap between the strains. The lower values recorded, of some animals within each of the strains, were at the threshold of the assay - 0.4 ng/ml, and higher values of over 8 ng/ml were found. In two of the strains, CBA and Balb/c, individual values of over 20 ng/ml were recorded. Despite the high overlap, there were significant differences in mean testosterone levels across the strains - Analysis of Variance $F_{(17,103)} = 2.15$ $p < 0.05$. Table 3.1 shows the testosterone levels for the 8 strains - in both the transformed logarithmic state, on which statistical analysis was conducted, and also the data expressed as ng/ml.

Sexual Behaviour

The number of tests in which mount, intromission and ejaculation responses occurred was calculated for each mouse, and the average response for each strain determined. This is expressed as the average percentage of tests with the behaviour. The mount and ejaculation latencies were also calculated for each mouse over the tests, and the strain average determined. However the latencies from the first behavioural tests were found to be greater than subsequent tests, and therefore the strain average latency was actually calculated from

Table 3.1

Mean plasma testosterone levels of several strains of the house mouse.

Strain	Number of Animals	Testosterone Ln.	± SE	Testosterone ng / ml
Balb/c	15	1.75	0.32	5.8
CBA/H	15	1.67	0.36	5.3
DBA/2J (F)	16	1.71	0.22	5.5
CBF_1	13	1.29	0.29	3.6
C57Bl/6	15	1.16	0.28	3.2
DBA/2J (M)	14	0.89	0.37	2.4
BDF_1	16	0.91	0.25	2.5
DBF_1	8	0.52	0.33	1.7

Analysis of Variance :

"F" = 2.14 , $p < 0.05$, df = 7,104

data of tests 2-5 only. As described in Chapter 2, the mount latency data was logarithmically transformed before any analysis was conducted.

The measures of sexual behaviour, and Analysis of Variance, are shown in Table 3.2. There were highly significant differences on all the behavioural measures of the 8 groups examined. The strains exhibiting the most behaviour were the C57, BDF_1 and DBF_1, where over 90% showed mount and intromission responses on every test. The CBF_1 and DBA(M) showed mount and intromission responses of over 65%, followed by the Balb, CBA and DBA(F), with mount and intromission responses of over 50%. The ejaculation frequencies were greatest for the BDF_1 and DBF_1 (over 80%), followed by the CBF_1, C57 and DBA(M) (over 60%), and the lowest frequencies were shown by the Balb's, CBA's and DBA(F) at between 4 and 37%. Mount latency was shortest in the BDF_1 and DBF_1 hybrids, at less than 2 minutes, and greatest in the Balb and DBA(F) at over 6 minutes. Similarly, the shortest ejaculation latency was shown by the BDF_1 and DBF_1 (less than 8 minutes), and the longest latencies were shown by the Balb and CBA at over 17 minutes.

Relations between testosterone levels and sexual behaviour

There were no correlations between plasma testosterone levels and behaviour within any of the strains. However, across the strains, the mean plasma testosterone levels

Table 3.2.

Sexual behaviour measures for eight lines of the house mouse (Mus musculus)

Strain	N	Average % tests with: Mount	Intro.	Ejac	Mount Latency Ln. (mins)	Ejac. Latency (mins)
Balb/c	15	60	51	23	1.84(6.3)	21.7
SE ±		14	14	6	0.18	3.0
CBA/H	15	61	54	4	1.80(6.1)	17.7
SE ±		3	2	2	0.34	4.7
DBA/2J(F)	16	66	62	37	2.10(8.2)	10.0
SE ±		7	7	8	0.18	2.0
CBF_1	14	73	67	60	1.41(4.1)	9.3
SE ±		8	10	12	0.10	1.9
C57Bl/6	15	96	91	62	0.99(2.7)	14.8
SE ±		2	2	3	0.18	1.7
DBA/2J(M)	14	85	80	67	1.41(4.1)	9.9
SE ±		2	2	3	0.18	2.2
BDF_1	16	100	100	96	0.59(1.8)	5.1
SE ±		-	-	1	0.10	0.9
DBF_1	8	97	92	80	0.59(1.8)	7.6
SE ±		3	3	10	0.18	1.7

Analysis of Variance :

'F' values	9.8	8.6	8.9	7.1	7.0
df =	7,105	7,105	7,105	7,98	7,78
p <	0.001	0.001	0.001	0.001	0.001

were negatively correlated with all the measures of sexual behaviour recorded, as shown in Table 3.3. Two of these relationships, those between testosterone and the average percentage of tests with mounting and with mount latency, are shown graphically in Figures 3.1 and 3.2.

3.2.4 Discussion

The wide variation in plasma testosterone levels between individuals within strains agrees with the data of Bartke, Steele, Musto and Caldwell (1973) who found levels ranging from less than 1 ng/ml to over 20 ng/ml, between individual mice. Further work by Bartke and Dalterio (1975) found that this variation in testosterone levels between individuals could also be demonstrated within single individuals which were bled on more than one occasion. They proposed that testosterone was secreted in a 'pulsatile' fashion from the testes, resulting in episodic peaks of plasma testosterone. Such a pulsatile secretion has also been proposed for the rat (Bartke et al, 1973), but is not restricted to rodent species. Katongole, Naftolin and Short (1974) report that in the ram, blood samples taken from individuals at hourly intervals reveal plasma testosterone levels fluctuating over a wide range of 3-28 ng/ml.

Such temporal variation within individuals in the levels of plasma testosterone poses problems for the assessment of overall or average levels. Ideally several samples from each individual should be taken, and

Table 3.3

Pearson correlation coefficiants between strain mean testosterone levels and sexual behaviour.

Testosterone levels (Ln) v.		r=	p ≤
Average % tests with:	Mounting	-0.83	0.01
	Intromitting	-0.73	0.05
	Ejaculating	-0.85	0.01
	Mount Latency	0.89	0.01
	Ejaculation Latency	0.71	0.05
		(df = 6)	

The correlations of testosterone with mount and ejaculation latencies were calculated using the logarithmic transform for both behaviours.

Figure 3.1

The relation between strain levels of testosterone and sexual behaviour in the house mouse.(Mus musculus)

a) Average percentage tests with mounting

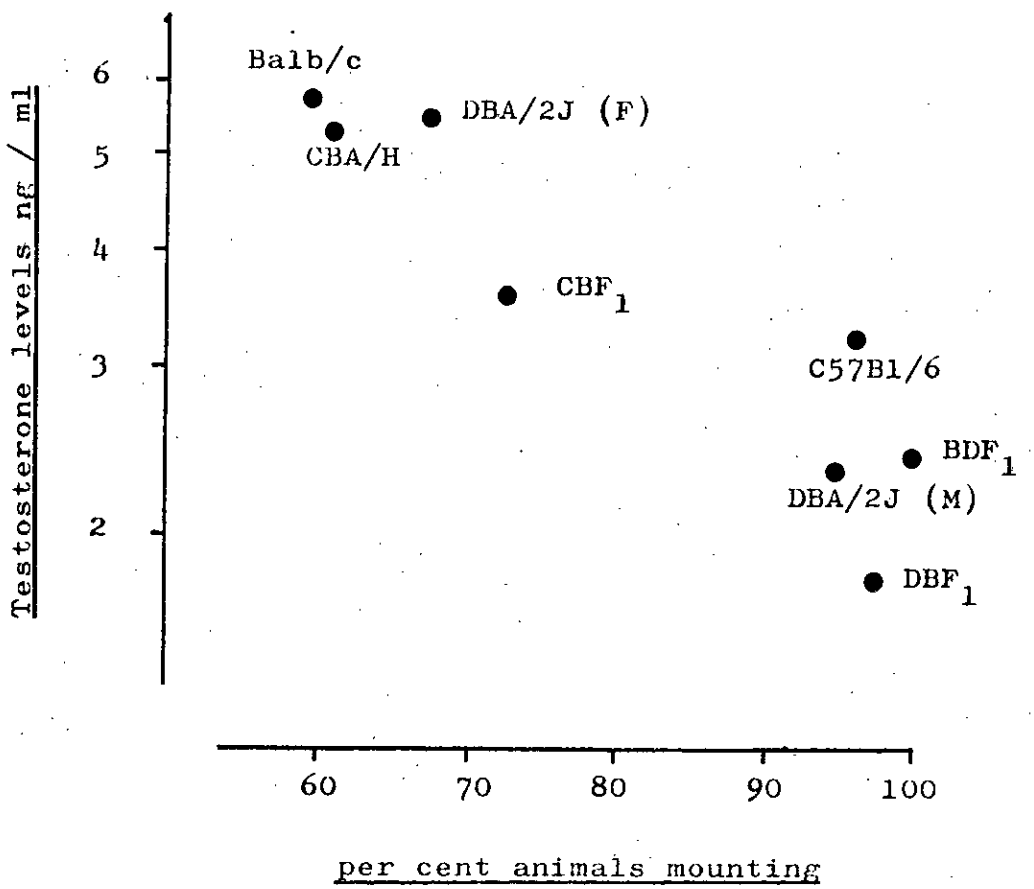
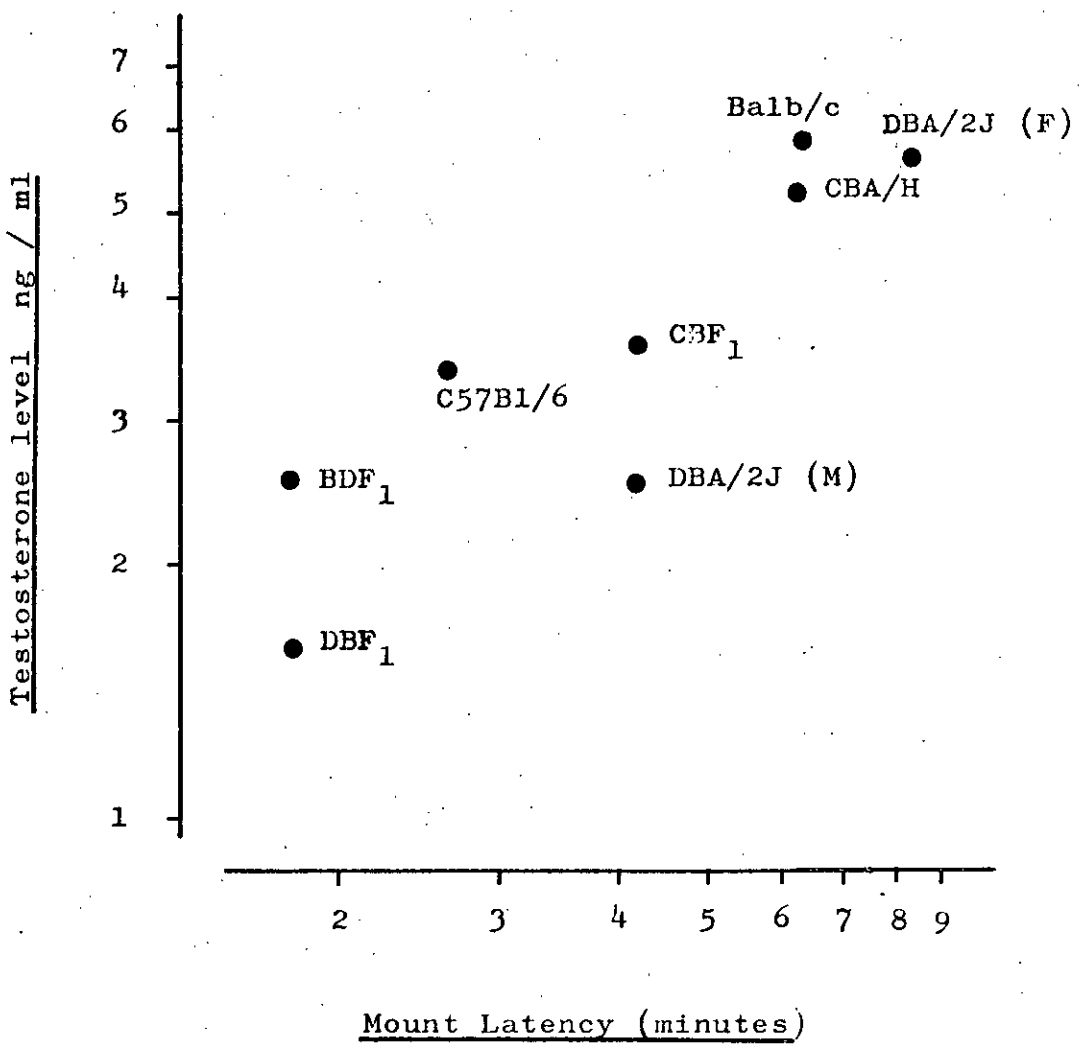


Figure 3.2

The relation between strain levels of testosterone and sexual behaviour in the house mouse. (Mus musculus)

b) Mount Latency



the average level determined from the samples. However, such a strategy was not found feasible in the mouse, and we need an alternative solution to this problem. Since the experiment described involved the use of inbred lines of mice, it may be, since these animals are isogenic, that the sampling of a group of these animals is effectively the same as the multiple sampling of an individual. Thus, I would argue that the strain mean level of testosterone is a measure which does reflect average levels of testosterone, and which can usefully be examined in relation to the behavioural characteristics of the strain. Such a pulsatile pattern of testosterone release may well account for the observation that no significant correlations between testosterone levels and measures of sexual behaviour could be demonstrated with strains, using data from individual mice, but that clear correlations could be demonstrated using the strain mean levels of testosterone.

The measures of sexual behaviour reported here agree with the findings of McGill (1962), who examined some of the strains (Balb, C57, DBA) under a similar test procedure. The strains of mice in which a large proportion of animals showed mount, intromission and ejaculation responses had lower mean testosterone levels than the strains which showed little copulatory

behaviour under the test conditions. Similarly, strains with short mount and ejaculation latencies had lower plasma testosterone levels than strains with long mount and ejaculation latencies. The observation that plasma testosterone levels were correlated with all the measures of sexual behaviour, suggests it to be involved in several aspects of sexual behaviour, and may for example be involved in the 'AM' and 'CM' as described by Beach (1956). That is, that testosterone levels may be important in the mechanisms relating to the initiation of sexual behaviour (as measured by mount frequency and latency), and to mechanisms relating to the execution of the behaviour (ejaculation frequency and latency). However, it must be pointed out that the ejaculation frequency is directly related to mount frequency (since only animals which have mounted can ejaculate), and therefore the correlation between plasma testosterone and ejaculation frequency may be partially an artifact.

The mechanisms whereby such a negative correlation between plasma testosterone levels and sexual behaviour might operate are at this stage obscure, and before discussing possible interpretations of the results, the next two experiments will attempt to verify this finding. Experiment 2 investigates whether the negative correlation was a behaviourally mediated effect, and Experiment 3 examines two F_2 populations, to ascertain whether the relationship was due to a chance association of the two measures.

3.3. Experiment 2

An investigation of plasma testosterone levels in sexually inexperienced strains of the house mouse.

3.3.1. Introduction

Experiment 1 found that, across strains of mice, levels of plasma testosterone were inversely correlated with measures of sexual behaviour. A simple, and rather trivial, explanation of such a finding may be that copulation itself has effects on plasma testosterone levels. For example, ejaculation may result in a depression of testosterone levels for several days following the behavioural tests, an effect which might produce the reported negative correlation between measures of sexual behaviour and testosterone levels. There is some evidence for such effects from data in the rat. Bliss, Frischat and Samuels (1972) report that males which copulate to satiation show depressed testosterone levels.

3.3.2. Method.

Sixteen animals from each of the following strains were used: Balb/c and BDF_1 (from stock bred in the Zoology Department, Edinburgh) DBA/2J(M) (M.R.C. Animal Breeding Centre, Carshalton) and C57Bl/6Fa (from the Genetics Department, University of Edinburgh). Animals were housed in groups of 8, in large cages, under conditions as previously described. Animals

were left undisturbed (apart from cage cleaning) until they were 13 weeks old when they were killed and their blood assayed for testosterone.

3.3.3. Results

The plasma testosterone levels of the four strains are shown in Table 3.4. There were significant differences between the strains - Analysis of Variance 'F' = 2.96, df = 3, 59, $p < 0.05$. Balb/c showed the highest values and BDF_1 the lowest.

3.3.4. Discussion

These results show that there are strain differences in plasma testosterone in animals with no heterosexual experience. Each of the strain values reported here is not significantly different from the value of the previous experiment, in which animals were sexually experienced. The rank order of decreasing testosterone levels for this experiment is Balb/c - DBA/2(M) - C57Bl/6 - BDF_1, the same order as found in the previous experiment. Therefore, we can reject the hypothesis that the negative correlation demonstrated between plasma testosterone and sexual behaviour was behaviourally mediated.

A second possible explanation of this negative relationship between testosterone and sexual behaviour is that the correlation is spurious, and the product of a chance association of the two measures within these

Table 3.4

Testosterone levels of four strains of the house mouse - with no heterosexual experience.

Testosterone Level	Strain			
	Balb/c	C57Bl/6	DBA/2J(M)	BDF_1
Ln	1.39	1.40	1.28	0.46
± SE	0.27	0.28	0.19	0.25
ng / ml	4.1	3.9	3.6	1.6

Analysis of Variance - 'F' = 2.96, df = 3,59, $p<0.05$

Number of animals in each strain = 16

almost totally genetically homogeneous strains. This possibility is examined in the next experiment, which describes an analysis of an F_2 derived from two of the hybrids described in Experiment I.

3.4. Experiment 3

An analysis of testosterone levels and sexual behaviour in male mice from an F_2 generation.

3.4.1. Introduction

In Experiment 1, it was shown that, across several strains of mice, a decrease in mean plasma testosterone level was associated with an increasing level of sexual responsiveness. Before analysing the underlying mechanisms of such a relationship, it is necessary to demonstrate that the finding is not merely fortuitous, due to a chance association of genes controlling plasma testosterone and genes controlling sexual behaviour.

The probability that the relationship was due to chance seems unlikely, since a large number of strains were examined. However, the strains used in the experiment were derived originally from only three major stocks - the CBA and DBA lines from one, the Balbs from another, and the C57s from a third (Staats, 1966). The remaining three lines were F_1 hybrids from combinations of the above. Therefore the possibility of a chance association between sets of genes relating to the control of testosterone and sexual behaviour is certainly not impossible.

This can be investigated by an examination of an F_2 generation, in which independent segregation of the two characters can occur. For example, Shire (1967) quotes an example of an F_2 analysis used to examine an association between body weight and activity in two strains of mice. One strain was low in body weight and very active, and the other was heavy and less active. The question was of whether body weight had a direct effect on ambulation scores. When an F_2 from the hybrid of these two strains was examined, the relation between body weight and ambulation disappeared - body weight measures were independent of ambulation scores. Therefore, the original observation that body weight and ambulation score were related was a chance association; ambulation was not being limited by body weight.

An F_2 analysis therefore involves the examination of the offspring from an F_I hybrid, from parental lines which differ in the characters to be investigated. For example, in the case described here, the F_1 hybrid would be produced from a cross between a strain with high testosterone levels and low sexual behaviour, and a strain with low testosterone levels and high sexual behaviour. On the chance association hypothesis, the F_1 hybrid therefore carries all four 'gene sets' controlling these characters, and recombination can be examined in individuals from the F_2 generation. If, in the F_2, there is no association between testosterone

levels and sexual behaviour, then the original observation was due to chance and not to some common physiological mechanism. Conversely, should the relationship hold, then this is evidence that there is some physiological mechanism which has effects on both testosterone levels and sexual behaviour, or that the two are causally associated. However, there are practical problems associated with such an experiment, since it depends on an analysis of individual animals. In Chapter 1, testosterone levels were found to be very variable between individual mice, and these differences were suggested to be due to a pulsatile release of testosterone. Thus, one estimate of plasma testosterone per mouse may not be a representative measure of testosterone production, and each mouse should ideally be serially sampled several times to give an estimate of 'overall' testosterone levels. However, attempts to do this were unsuccessful - direct methods of orbital sinus or cardiac puncture with or without anaesthesia were considered to be too stressful a procedure to yield any meaningful results, and attempts at fitting canulae in the tail were ineffective due to the collapse of the blood vessels. The experiment was therefore carried out with only one estimate of testosterone per mouse, and it was hoped that by the use of a large

number of animals, and by grouping the testosterone data into categories determined by the behaviour and determining the average levels, these problems could be overcome.

Two F_2 populations were examined - the first showed no relation between testosterone levels and sexual behaviour, and therefore a second line was examined, in which testis and seminal vesicle data was also taken as a further measure related to plasma testosterone levels.

3.4.2. Method.

The two F_2 populations examined were derived from the BDF_1 and the CBF_1 hybrids. The BDF_1 is the hybrid between a C57Bl/6Fa female and a DBA(F) male, and the CBF_1 is the hybrid between the Balb/c female, and a C57Bl/6Fa male. These parental lines were described in Experiment 1 - the DBA and Balb/c show much less sexual behaviour than the C57Bl line, under the test conditions described, and also have higher plasma testosterone levels. The F_2 progeny of these hybrids were housed in all male groups at weaning, in large cages, and at 9 weeks of age were isolated into small transparent plastic cages and maintained under conditions as previously described. Tests of sexual behaviour were started at 10 weeks, using the procedure previously described,

except that the receptive BDF_1 females were introduced into the home cage of the male, the metal food hopper of which was replaced by a clear observation lid. Five behavioural tests were given, and three days after the last test, the animals were killed and blood samples removed for assay of testosterone. The CBF_2 were also dissected and the weights of the testis and seminal vesicles (plus the coagulating gland, and expressed of the fluid contents) were recorded.

3.4.3. Results.

a) BDF_2 animals. Forty-two BDF_2 males were examined, and correlations between testosterone levels and the number of tests in which mounting, intromission and ejaculation responses occurred, and also mount and ejaculation latency, are shown in Table 3.5. There were no significant correlations between testosterone levels and any of the behavioural measures.

The data was also examined by grouping the animals into behavioural categories - for example by the number of tests in which each animal showed mount, intromission or ejaculation responses. The testosterone mean of each group was then calculated and correlated with the frequencies of behaviour. However, the data was not very variable, on average over 90% of animals mounted on every test, and over 70% intromitted which meant that the data was not spread evenly across the categories.

Table 3.5

Correlation coefficiants between testosterone levels and sexual behaviour in an F_2 generation from C57Bl/6 and DBA/2J parental lines.

Testosterone level (Ln)	r =	p<
Mount frequency	0.09	ns
Intromission frequency	-0.11	ns
Ejaculation frequency	-0.08	ns
Mount latency (Ln)	-0.20	ns
Ejaculation latency	0.08	ns

Number of animals = 42 , df = 40 .

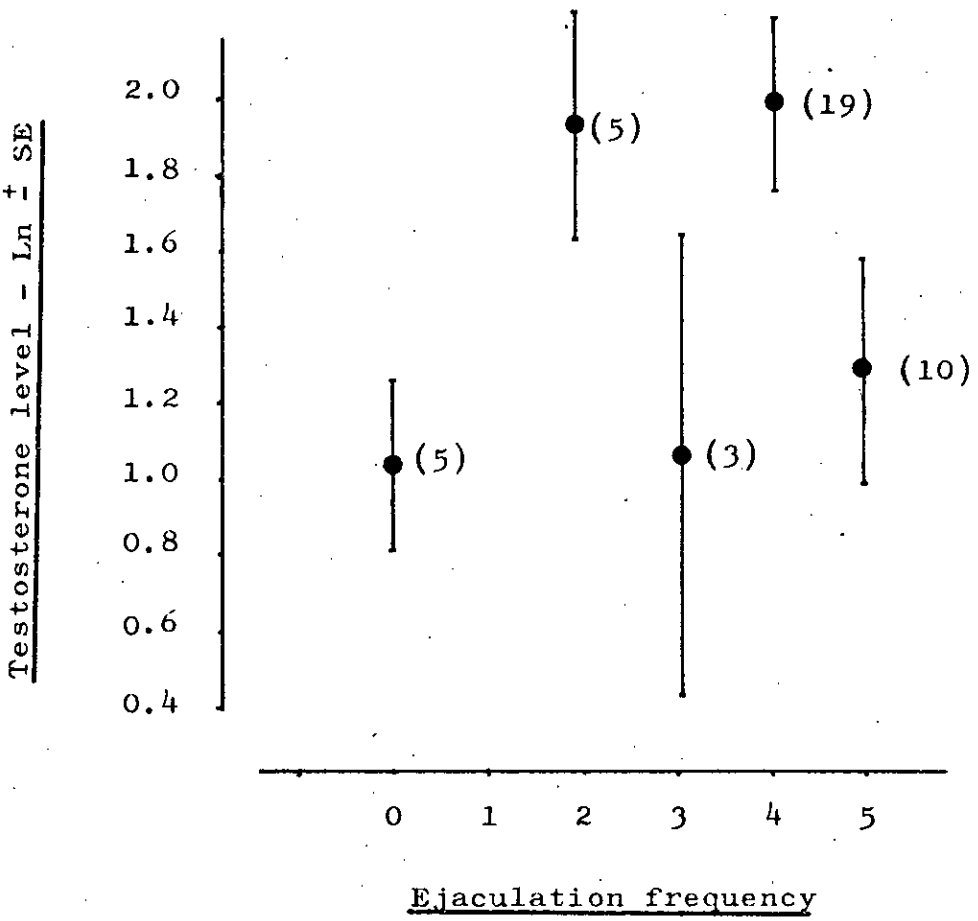
The ejaculation data was slightly more variable, with 65% of animals ejaculating per test, and this data is shown in Figure 3.3. The mean testosterone levels of the animals which fall into each frequency of ejaculation (from 0-5) is shown - but there was no significant correlation between the testosterone level and ejaculation frequency.

The mean testosterone level for the F_2 was 4.1 ng/ml, (Ln. = 1.41, SE = 0.18), a value intermediate between the values previously reported for the parental strains. The percent ($\pm$ SE) animals showing mounting was 90 $\pm$ 3, intromitting was 73 $\pm$ 6 and ejaculating was 65 $\pm$ 7. These percentages are all greater than the values reported in Experiment 1 for the parental strains. The average mount latency for the F_2 was 2.4 minutes (Ln = 0.87, SE = 0.12) and the ejaculation latency was 9.4 minutes, SE = 1.4, both of which are shorter than the values reported for the parental strains.

b) <u>CBF$_2$ animals</u>. After observing the first experimental group of this F_2, it became apparent that there were further problems of the experiment which were not forseen in the original design. One of the parental lines of the F_2 - the Balb/c - was an albino strain and therefore one quarter of the F_2 were homozygous for albinism. It was noted that the sexual behaviour of albino F_2 animals was less frequent than

Figure 3.3

The mean testosterone levels of BDF_2 animals - grouped into ejaculation frequency over the five tests.



(Numbers in () =number of animals in each group)

Ejaculation frequency has been shown because behaviour in this category showed most variability.

the pigmented animals - the percentage of animals mounting, intromitting and ejaculating was significantly different, as shown in Table 3.6. The mount latency of 'responders' was however not significantly different from the pigmented animals. It was however necessary to analyse the data from the albino and pigmented groups separately. In order to have sufficient data from the albino group, further animals were examined and the final number of pigmented and albino animals is selected and does not therefore reflect the expected Mendelian ratio.

Albino animals

Twenty albino animals were examined, and the correlation coefficients between the plasma testosterone levels and the number of tests with sexual responses, and the latencies to mount and ejaculation, are shown in Tables 3.7 and 3.8. None of these reached significance. The data was further analysed by grouping the animals according to their behaviour, as in the BDF_2 analysis, and calculating the mean testosterone levels. The only behaviour which was 'spread' across the 6 possible frequencies (0-5 inclusive) was the mount frequency, as shown in Figure 3.4, but the mean testosterone levels were not significantly correlated ($r = -0.57$, $df = 4$, $p = ns$).

Table 3.6

Differences in sexual behaviour between albino and pigmented animals in an F_2 generation derived from Balb/c and C57Bl/6 parental lines.

Behaviour (± SE)	Animals		Analysis of Variance		
	Albino	Pigment[d.]	'F'	df	p <
No. of animals	17	20			
Average percent tests with :					
a) Mounting	67 ± 7	94 ± 4	10.7	1,50	0.001
b) Intromission	44 ± 7	83 ± 6	16.3	1,50	0.001
c) Ejaculation	22 ± 6	58 ± 6	17.5	1,50	0.001
Mount latency					
(Ln ± SE)	1.94±0.19	1.49±0.11	4.7	1,44	0.05
(minutes)	(7.0)	(4.4)			
Ejaculation Latency					
(minutes)	9.4±1.8	13.9±1.4	4.3	1,38	0.05

Table 3.7

Correlation coefficiants between testosterone levels and sexual behaviour in an F_2 generation from Balb/c and C57B1/6 parental lines.

a) Albino animals — Number of animals = 20

Testosterone Levels v.	r =	df =	p <
Mount frequency	-0.15	18	ns
Intromission frequency	-0.06	18	ns
Ejaculation frequency	0.21	18	ns

b) Pigmented animals — Number of animals = 32

Testosterone Levels v.	r =	df =	p <
Mount frequency	-0.35	30	0.05
Intromission frequency	-0.40	30	0.05
Ejaculation frequency	-0.02	30	ns

Table 3.8

Correlation coefficiants between testosterone levels and mount and ejaculation latencies in an F_2 generation from Balb/c and C57Bl/6 parental lines.

a) Albino animals — Number of animals = 20

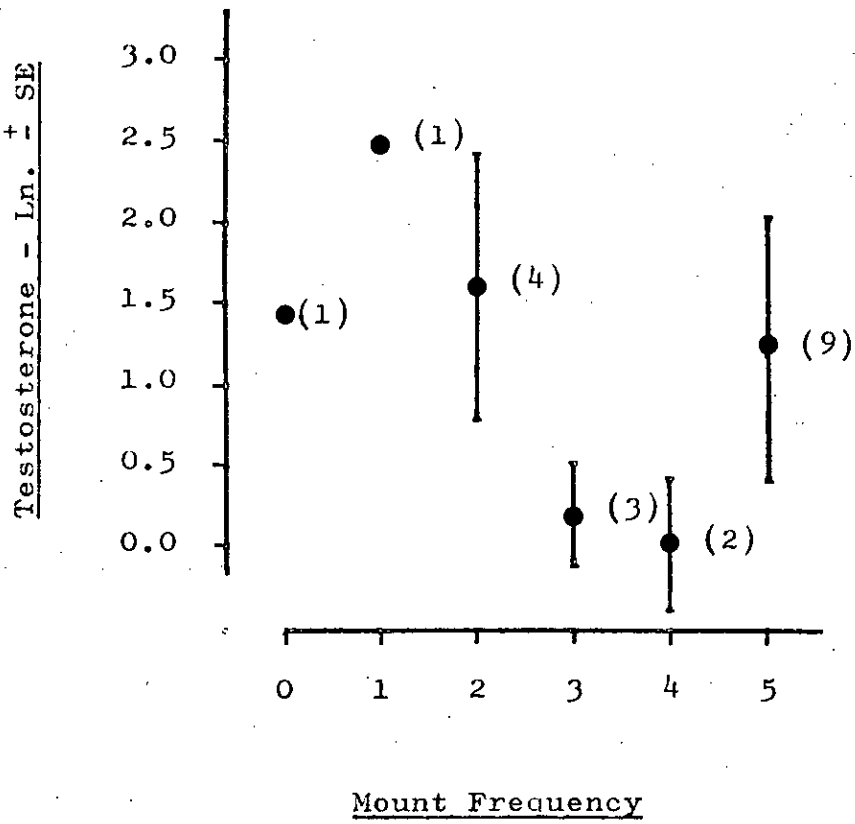
Testosterone levels (Ln) v.	r =	df =	p <
Mount latency (Ln)	0.28	19	ns
Ejaculation latency (n = 11)	-	-	-

b) Pigmented animals — Number of animals = 32

Testosterone levels (Ln) v.	r =	df =	p <
Mount latency (Ln)	0.10	30	ns
Ejaculation latency	0.13	28	ns

Figure 3.4

The mean testosterone levels of albino CBF_2 animals - grouped into mount frequency over the five tests.



(Numbers in ( ) = the numbers of animals in each frequency)

Testis and seminal vesicle weights were measured on only 11 of the mice, and there were no significant correlations with testosterone levels, or with any of the behavioural measures.

Pigmented animals

Thirty-two animals were examined, and the correlation coefficients between the number of tests in which sexual responses were shown and testosterone levels are shown in Table 3.7. The frequency of tests in which animals showed mount and intromission responses were significantly negatively correlated with plasma testosterone levels. Mount and ejaculation latencies, averaged over tests 2-4 and shown in Table 3.8, showed no significant correlations with testosterone levels.

Testis and seminal vesicle weights were measured on 25 of the mice. Both of the organ weights were positively correlated with body weight ($r = 0.74$, $df = 23$, $p < 0.001$; $r = 0.64$, $df = 23$, $p < 0.001$), and therefore were corrected to a standard body weight of 20 g, before analysis. The levels of plasma testosterone showed no significant correlations with either testis weight ($r = 0.05$, $df = 23$, $p = ns$) or seminal vesicle weight ($r = -0.025$, $df = 23$, $p = ns$). Also the testis and seminal vesicle weights were not correlated with each other ($r = -0.20$, $df = 23$, $p = ns$).

The correlations between the sexual behaviour measures and the testis and seminal vesicle weights are shown in Tables 3.9 and 3.10. The mount frequency showed a significant negative correlation with the testis weights, but none of the other measures were significantly correlated.

3.4.4. Discussion.

The aim of this experiment was to determine whether a negative correlation could be demonstrated between testosterone levels and measures of sexual behaviour in an F_2 generation, in order to ascertain whether the negative relation as found in Experiment 1 was due to a chance association of the two measurements. The first F_2 examined - the BDF_2 - showed no such correlation, but the second - the CBF_2 - did show a similar negative correlation between testosterone levels and some measures of sexual behaviour.

The failure to demonstrate any correlations in the BDF_2 suggests that the correlations found in Experiment 1 may, in fact, have been due to a chance association. However, there is evidence to suggest that this F_2 may be an atypical example. The BDF_1 genotype, from which the F_2 were derived, has very special behavioural characteristics. McGill and Manning (1976) have found that this hybrid shows a remarkable retention of sexual behaviour after

Table 3.9

Correlation coefficiants between testis weights and sexual behaviour in an F_2 generation from Balb/c and C57Bl/6 parental lines.

Testis weight[a] v.	r =	df =	p <
Mount frequency	-0.43	23	0.05
Intromission frequency	-0.35	23	ns
Ejaculation frequency	-0.17	23	ns
Mount latency (Ln)	0.19	22	ns
Ejaculation latency	-0.23	21	ns

[a]Testis weights corrected to standard body weight of 20 g .

Table 3.10

Correlation coefficiants between seminal vesicle weights and sexual behaviour in an F_2 generation from Balb/c and C57Bl/6 parental lines.

Seminal vesicle weight[a] v.	r =	df =	p <
Mount frequency	0.31	23	ns
Intromission frequency	0.33	23	ns
Ejaculation frequency	0.31	23	ns
Mount latency (Ln)	-0.07	22	ns
Ejaculation latency	-0.08	21	ns

[a]Seminal vesicle weights - expressed of fluid contents, coagulating gland still attached, and corrected to a standard body weight of 20 g .

castration, and males can retain the ejaculatory reflex for several weeks, and often several months. This behavioural retention is not a result of compensatory adrenal androgen production, since adrenalectomy has no effect on the response (Thompson, McGill, McIntosh and Manning, 1976). Therefore the BDF_1 genotype has some mechanism whereby sexual behaviour is, or can become, independent of testicular secretions. This means that in the F_2 some animals will carry the genes mediating this effect, and therefore the levels of plasma testosterone may be irrelevant to the display of sexual behaviour. In view of this, it is perhaps not surprising that a negative correlation between plasma testosterone and sexual behaviour could not be demonstrated in an F_2 derived from this hybrid.

The second F_2 examined - the CBF_2 - was derived from the CBF_1 hybrid which shows a high dependence on androgens for the display of sexual behaviour. After castration males from this hybrid lose the ejaculatory reflex rapidly, and some show no sexual responses at all (McGill and Manning, 1976).

In the CBF_2 analysis, a negative correlation could be demonstrated, although there were some problems associated with the experiment. The heterogeneity within the F_2, between the albino and pigmented mice, meant that there were unforseen factors influencing the sexual behaviour of these animals. This may have

been due to a specific effect of the albino gene on sexual behaviour, or to more general debilitating effects. There is evidence that the albino gene can have specific effects on behaviour - for example Tyler (1970) has found that in a heterogeneous population from an 8 way cross, animals homozygous for albinism are slower in learning a runway task than pigmented animals. However that the depression of sexual behaviour may have been due to a generalised effect is suggested by the observation that many of the albino animals in this experiment died before weaning, and those that survived were often low in body weight and had a 'staring' coat. It may be that the parental Balb/c strain has genes which in part compensate for the effects of albinism, and that these become dissociated in the F_2 generation.

Within the albino F_2, no significant correlations between testosterone levels and sexual behaviour measures were found. This was perhaps to have been expected since only twenty animals were examined, which appeared to be in poor condition.

In the pigmented animals, of this F_2, testosterone levels were negatively correlated with both the number of tests in which animals showed mounting responses and the number of tests with intromission responses.

The failure to demonstrate any correlations with the other measures could have been due to an insufficiency of data, or because testosterone is not in fact related to these other behavioural measures.

The testis and seminal vesicle weights were not significantly correlated with the plasma testosterone levels. However, using a larger number of animals, Eleftheriou and Lucas (1974) report that, in C57Bl/6J and DBA/2J mice of mixed ages, there is a positive correlation between plasma testosterone and testis weight, but no correlation with seminal vesicle weight. Therefore the fact that my results show testis weight to be negatively correlated with the frequency of mounting, supports the observation that testosterone levels were negatively correlated with this behaviour.

This experiment has demonstrated that testosterone levels, in one F_2 line, are inversely related to the occurrence of mounting and intromission responses, but not necessarily to the latencies to mount and ejaculation or ejaculation frequency. Therefore, for two of the measures of sexual behaviour, there is evidence that the negative correlations, found across the lines in Experiment 1, were not due to a chance association of genes controlling hormone production and genes controlling sexual behaviour. Whether the other behaviours are in fact related is open to question - it may be that the relation found across the strains

between testosterone and ejaculation frequency was because only those mice that mount can go on to ejaculate, and hence a correlation with percentage mounting may result in a correlation with percentage ejaculating. However, it might perhaps be expected that such an effect might also operate in the pigmented CBF_2 examined here. It may be that plasma testosterone levels are related more to the probability that sexual behaviour will occur, as measured by mounts and intromissions, rather than to the way it proceeds and finishes, as measured by ejaculations. In terms of Beach's model of rodent sexual behaviour that is to say testosterone levels may be related more to the Arousal Mechanism (AM) than to the Copulatory Mechanism (CM).

3.5. GENERAL DISCUSSION

The experiments described in this chapter have shown plasma levels of testosterone to be negatively related to measures of sexual behaviour, both across strains of mice, and between individuals from an F_2 generation. In Experiment I testosterone levels were inversely related to all the measures recorded, that is to factors involved in both the initiation of sexual behaviour (the arousal mechanism - AM) such as the mount latency and frequency, and to measures related to the execution and completion of the behaviour (the copulatory mechanism - CM) such as the ejaculation latency and frequency. However, in the F_2 analysis, only factors related to the AM could be shown to be related to plasma testosterone levels. This suggests that the negative correlations found in Experiment I between testosterone and 'CM' measures may have been due to chance; and that in fact testosterone levels are only involved in the mechanisms associated with sexual arousal.

In the introduction to this chapter, we discussed work suggesting that the elements of sexual behaviour may be separately controlled, and may have differential dependencies on androgens. Work on the rat shows that the elements of behaviour associated with the AM are not affected in the same way after castration as elements associated with the CM. In the first 2-3 weeks after castration, Davidson (1966) found that the intromission latency showed a significant increase, whereas the

ejaculation latency decreased. Thus, castration impaired the AM, but facilitated the CM, although only temporarily - ejaculation latencies increased in subsequent weeks, probably because of changes in peripheral feedback as described by Beach and Levinson (1950). The AM might therefore be said to be more 'androgen dependent' than the CM, since it shows impaired function after castration.

Brain lesion studies also suggest that testosterone may be more involved in the AM than the CM. Lesions in the pre-optic anterior hypothalamic area (PO-AH) result in a reduction or complete cessation of sexual behaviour in male rats (Lisk 1968, Larsson and Heimer 1964), an effect not related to changes in circulating androgen levels (Heimer and Larsson 1966/7). In rats, lesions in this area result in a significantly increased mount latency, but no effects on intromission frequency or ejaculation latency (Chen & Bliss, 1974) - that is PO-AH lesions impair the AM, but not the CM. After castration, Kierniesky and Gerall (1973) report that in the rat, implants of testosterone propionate into the medial PO-AH have no effects on ejaculation latency, but significantly decrease the mount latency. Control implants in other brain areas have no behavioural effects, suggesting that testosterone is necessary for the normal function of the PO-AH in controlling the AM, but is not necessary for the CM.

In the house mouse there is little evidence to support the hypothesis that the AM is more androgen

dependent than the CM. In fact, some work on the effects of castration would seem to suggest quite the opposite. Manning and Thompson (1976) examined the post-castrational sexual behaviour of BDF_1 hybrid males, and found that mount and intromission latencies were not affected, but that the ejaculation latency was significantly increased. This is in contrast to Davidson's (1966) results with the rat, and suggests that the CM is more dependent on androgens than the AM. However, this experiment used animals with considerable sexual experience, and made comparisons of pre- and post-operational performance, such that there are problems of assessing the influence of surgical trauma on these measures. The effects of castration on sexual behaviour of several strains of mice, including the BDF_1 hybrid, are described in Chapter Five of this thesis and the results were found to be in essential agreement with Davidson (1966). Compared to sham operated controls, the mount latencies of castrates showed a significant increase, whereas the ejaculation latency showed a slight, non-significant decrease. These results, plus the failure to demonstrate a correlation between plasma testosterone levels and measures associated with the CM in an F_2 generation, suggest that the factors associated with the AM are in fact more androgen dependent than is the CM.

How might this negative relationship between plasma testosterone levels and the incidence of sexual responses be interpreted? Unfortunately a knowledge of plasma testosterone levels is not sufficient to allow any predictions, regarding, for example, the output of the hormone from the testis, or of whether gonadotrophin levels are higher in some strains than in others. It is important to remember that the plasma level of testosterone is the product of two processes - one, the rate of output from the testis, and two, the rate of clearance from the blood-stream. There may be strain differences in metabolic clearance from the plasma - and it is perfectly possible that the low testosterone strains such as BDF_1 may in fact release more testosterone from the testes than a high testosterone strain such as the Balb/c. However, to facilitate discussion, we shall for the moment ignore these problems, and shall assume that the metabolic clearance between the strains is the same. If so, then there is a hypothesis which may explain this apparent anomally of high plasma testosterone levels being associated with low levels of sexual activity.

Plasma levels of testosterone are controlled by the secretion of gonadotrophins from the pituitary, in particular luteinizing hormone (LH) (Hall, 1970), which has effects on the Leydig cells in the testis. Testosterone and LH interact together in a negative

feedback loop, such that rising titers of testosterone inhibit the release of LH, either directly or via the hypothalamus. Similarly, falling titers of testosterone result in the release of LH from the pituitary. Thus, there is a balance maintained between LH and testosterone which results in a constant 'average' plasma testosterone level. This balance point may be defined in terms of the sensitivity of the hypothalamo-pituitary axis to the testosterone levels - thus strains of mice with high plasma testosterone may have a lower sensitivity, such that the LH feedback loop is 'switched off' only by relatively high levels of plasma testosterone. Such differences are known to exist in pre- and post-pubertal male rats - for example Negro-Vilar, Ojeda and McCann (1973) found that the doses of testosterone propionate (TP) necessary to suppress plasma LH and FSH levels vary according to the age of the animal. Thus in 15 day old rats, 10 μgTP/100 g body weight was sufficient to suppress LH, but at 58 days of age this dose was no longer effective, suggesting that the sensitivity of the hypothalamic pituitary unit to the negative feedback of testosterone declines during sexual development. It may be that the hypothalamic structures which are thought to mediate sexual behaviour, such as the preoptic area, are similarly insensitive to the effects of testosterone, resulting in the poor sexual behaviour that is characteristic of for example Balb's

and CBA's. To examine this possibility further, the experiments described in Chapter Five investigate the effects of testosterone injections on morphological and behavioural parameters to determine whether the strains differ in 'responsiveness' to testosterone. If the hypothesis is correct, then it is expected that the dose of testosterone necessary to maintain measures of sexual behaviour, at the level of the intact animal, will parallel the levels of testosterone which are characteristic for that strain. In other words, that a strain with high plasma testosterone levels 'needs' that much testosterone for the display of sexual behaviour.

A second hypothesis, which might explain this negative correlation, concerns the levels of testosterone binding proteins in the plasma. It may be that high testosterone strains, such as Balb/c and CBA/H have elevated levels of binding proteins, such that testosterone function is impaired at the site of negative feedback control, and also at brain regions mediating sexual behaviour. That is, that the testosterone may be 'locked' in a protein complex, such that gonadotrophins are released in high levels, resulting in a high testicular release of the hormone. This possibility was examined by comparing the binding properties of plasma of several strains, and is described in Appendix 2, but was not found to be an important variable - "high testosterone"

strains do not have increased levels of testosterone binding proteins.

We must also consider the increasing amount of evidence showing that plasma levels of testosterone can undergo rapid, transient increases in response to copulatory stimuli. For example, in the male rat Kamel, Mock, Wright and Frankel (1975) found that testosterone, LH and prolactin were all elevated after a sexual encounter. Such increases may be important behaviourally, and the changes in testosterone may control aspects of sexual behaviour. Similar changes may also occur in male mice, and it may be that 'low' plasma testosterone strains show a greater, more rapid responsiveness than 'high' testosterone strains. This aspect is examined in Chapter Four, where changes in testosterone in response to sexual stimulation are measured, and discussed with reference to differences in sexual behaviour.

A further consideration is that the effects of testosterone are not restricted to the adult animal. Testosterone has effects perinatally during 'critical periods' of development, and influences the differentiation of both sexual morphology and behaviour (Ward, 1974). Thus, female mice treated with testosterone soon after birth show an enhanced masculine response potential as adults (Manning and McGill, 1974), and male mice show ejaculatory responses at an earlier age than oil injected controls (Campbell and McGill, 1970).

The strains of mice described in the preceeding experiments may differ in the extent to which this perinatal function is effective, and there may be influences of both the prenatal maternal environment, and the post-natal testosterone pulse. One of the sources of behavioural variability between the strains may be the product of differences in the levels of hormones of maternal and sibling origin during gestation. For example, Clemens and Coniglio (1971) have shown that the adult behaviour of rats is effected by the position that the individual occupies relative to siblings in the uterine horns, and female rats with males on 'both sides' in utero show differences in behaviour to females adjacent to other females only. Secondly, the neonatal pulse of testosterone, as described in the rat by Resko, Feder and Goy (1968), and in the mouse by Berger, Jean-Faucher, de Turckheim, Vessière et Jean (1975), may differ in magnitude and temporal spacing between the strains. To determine whether the strain differences in plasma testosterone and sexual behaviour are the product of differences in neonatal androgens, an attempt was made to 'standardise' the neonatal environment by administering doses of testosterone propionate on day 4 to males from several strains. The plasma testosterone levels and sexual behaviour measures were then examined in the adults, and the results are described in Chapter Six.

CHAPTER FOUR

ACUTE CHANGES IN PLASMA TESTOSTERONE LEVELS AND THEIR RELATION TO SEXUAL BEHAVIOUR

4.1 GENERAL INTRODUCTION

The experiments described in Chapter 3 have shown that plasma testosterone levels were negatively correlated with measurements of sexual behaviour across strains of mice. However, there was a great deal of variation in levels between individuals, an observation thought to be the result of a pulsatile secretion pattern of the hormone. The behavioural effects of such transient changes in testosterone were not considered, and the 'average' strain levels were used in the analysis. Such a procedure has an underlying assumption that these pulses of testosterone were not important behaviourally, and that the effects of the hormone are of a sustained nature, not affected by these transient pulses. Indeed, work on the decline of sexual behaviour after castration, and its restoration with injections of testosterone, would seem to support this view. For example, in the house mouse, the elements of sexual behaviour do not disappear immediately after castration, but gradually decline over a period of weeks (McGill and Manning, 1976), and restoration of the behaviour with testosterone injections may take several weeks before full behavioural potential is re-established (Luttge, Hall and Wallis, 1974). However, there is now an increasing amount of evidence that there are large changes in plasma testosterone

levels, before, during and after copulation, and it may be that these changes are related to the initiation of sexual behaviour.

Increases in plasma testosterone following copulation have been reported for many species - for example in the bull (Katongole; Naftolin and Short, 1971; Smith, Mongkopunya, Hafs, Convey and Oxender, 1973); the rhesus monkey (Rose, Gordon and Bernstein, 1972); the rabbit (Saginor and Horton, 1968; Haltmeyer and Eik-Nes 1969; Hilliard, Pang, Penardi and Sawyer, 1975); and the rat (Purvis and Haynes, 1974; Kamel, Mock, Wright and Frankel, 1975). These increased levels of testosterone are probably produced in response to increased gonadotrophins, rather than for example to changes in blood-flow to the testes, since in many of these animals increased plasma levels of gonadotrophins can also be detected. For example, in the bull, Katongole et al found increased LH levels immediately prior to copulation, and similarly in the rat, Mock et al found increased levels of LH and prolactin after copulation.

The nature of stimulus involved in these changes is not fully understood - in some animals hormone changes can be detected before physical contact actually occurs, but in others copulation itself seems to be important. For example, in the bull, Katongole et al report that the sight of a cow being led to the bull was sufficient

a stimulus for increased levels of LH to be detected, and in the hamster, the smell of the female was found to result in the pulse of plasma testosterone in the male (Macrides, Bartke, Fernandez and D'Angelo, 1974). In the rat, increases in plasma testosterone can even occur in response to inanimate, environmental stimuli normally associated with access to receptive females and copulation: a male placed in a testing arena in which a receptive female is usually placed, can result in increased testosterone levels before the female is put in the arena (Kamel et al, 1975). Thus in some species, changes in plasma LH and testosterone are not necessarily dependent on specific stimuli, or to sexual contact itself (although copulation may enhance the response), and can be produced before behavioural changes are apparent.

Since these changes in hormone levels can be demonstrated before copulation, these increased levels may have effects on behaviour, either directly by the action of testosterone on neural areas mediating behaviour, or indirectly via changes in gonadotrophins, and gonadotrophin releasing factors in the brain. If these changes are important in determining the probability of copulation, then there are several predictions we might make regarding the time-course

and incidence of such events. Firstly, these changes would be expected to occur before the initiation of sexual behaviour, and also they should be elicited by stimuli without physical contact between male and female being allowed. Secondly, in the course of a single test with a group of animals, we might expect animals showing sexual behaviour to have higher testosterone levels than animals not showing any sexual behaviour.

The experiments to be described in this Chapter investigate whether there are such increases in plasma testosterone in response to the presence of oestrous females (in the house mouse), and also whether the incidence of such 'acute' changes in testosterone can be related to differences in behaviour.

4.2. EXPERIMENT 4

The effects of oestrous female proximity on plasma testosterone levels in several strains of mice

4.2.1. Introduction

In the general introduction, we discussed work showing that short-term (acute) increases in LH and testosterone levels occur in many male mammals in response to sexual stimuli. This experiment investigates whether such changes occur in response to olfactory contact with oestrous females, and whether there are strain differences in this response which might relate to the strain differences in sexual behaviour, as described in Experiment 1.

The strains of mice used were chosen on the basis of differences in their frequency of mounting behaviour - five lines were examined, two low frequency mounters, Balb/c and CBA/H, and medium DBA/2J and two high responders, the C57Bl/6Fa and the hybrid BDF_1. It was decided to use animals with some sexual experience since the results of Experiment 1 revealed that mount and ejaculation latencies were longer in the first test than in subsequent tests, suggesting that the first encounter with a receptive female may be partly a 'familiarization' situation.

4.2.2. Method

All mice used in the experiment were obtained from stock bred in the laboratory, and were housed in groups of 8-10 in large cages, under the conditions previously described. At 10 weeks of age the mice were given a sexual experience test which consisted of each male being placed in a small cage with a receptive BDF_1 female for one hour. Behavioural measurements were not recorded, and males were then returned to their home cage.

At 11 weeks the mice were exposed to oestrous BDF_1 females for 15 minutes, and blood samples removed. Small, clean cages were divided by a double wire-mesh grill, through which air could freely pass, but which would not allow contact between male and female. A receptive female, injected the previous evening with 100 µg estradiol benzoate, was placed in one half of the cage, and a clean plastic lid was placed on top. After 5 minutes a male was placed on the other side of the mesh, and left undisturbed for 15 minutes. The male was then removed, and exsanguinated as previously described, within one minute. As experimental controls, male mice were placed in the divided cage as before, but no female occupied the other half of the cage. It was decided not to use anoestrous females because mice will attempt to mount such females (Macrides, Bartke and Dalterio, 1975)

suggesting that 'general' female odours are sufficient to stimulate sexual behaviour, and therefore may have effects on testosterone levels.

All the tests were conducted in the room in which the mice were normally housed, and which contained females all the time. The mice were therefore used to a 'background' female smell, but were not used to such close proximity with oestrous females as was found in the tests.

Differences between experimental and control groups within each strain were examined using the student 't' test, with the data transformed to logarithms to normalise the distribution.

4.2.3. Results

The plasma testosterone levels of the five strains examined are shown in Table 4.1. In the Balb and CBA strains, there were no significant changes in testosterone levels between experimental and control animals. The DBA and C57 strains both showed significant increases in the plasma testosterone in the experimental groups (student 't' test - 't' = 2.7, df = 14, $p < 0.02$, 't' = 2.8, df = 18 $p < 0.02$).

In the BDF_1's high values of over 7 ng/ml were found for both experimental and control groups. Earlier measurements, reported in Experiment I, found mean plasma levels of less than 3 ng/ml. It was thought

Table 4.1

The influence of the proximity of oestrous female mice on plasma testosterone levels , in several strains of the male house mouse.

a) Balb/c strain.

		Testosterone level		
	N	Ln	± SE	ng / ml
With female	20	1.42	0.31	4.2
No female	20	1.51	0.26	4.5
Student "t" test -		ns		

b) C57Bl/6 strain.

		Testosterone level		
	N	Ln	± SE	ng / ml
With female	10	1.12	0.32	3.1
No female	10	-0.11	0.20	0.9
Student "t" test -		$t_{(18)} = 2.8$, $p<0.02$		

c) CBA/H strain.

	N	Testosterone level Ln	± SE	ng / ml
With female	19	1.60	0.24	4.9
No female	19	1.34	0.27	3.8

Student "t" test - ns

d) BDF_1 strain.

	N	Testosterone level Ln	± SE	ng / ml
With female	16	2.00	0.19	7.4
No female	16	2.17	0.18	8.7

Student "t" test - ns

e) DBA/2J strain.

	N	Testosterone level Ln	± SE	ng / ml
With female	8	2.63	0.37	13.9
No female	8	1.22	0.36	3.4

Student "t" test - $t'_{(14)} = 2.7$, $p < 0.02$

that perhaps this strain was showing the "anticipatory" effects, as described by Kamel et al (1975) for the rat. Although these mice were only given one sexual encounter prior to the experiment, it may be that this was sufficient for an association between the small test cages and copulation to be established. A second group of BDF_1's was therefore examined, in which the mice were sexually inexperienced. Plasma testosterone levels are shown in Table 4.2 - again both experimental and control animals showed high levels of over 6 ng/ml, but the experimental group showed levels of over 11 ng/ml, significantly higher than controls (student 't' test, 't' = 1.7, df = 14 $p < 0.05$, one-tailed since direction of change predicted).

4.2.4. Discussion

The results show that plasma testosterone levels do change in male mice in response to oestrous female proximity, and that these changes are most marked in strains which generally show a high degree of sexual responsiveness.

The Balb's and CBA's both of which showed the lowest levels of sexual behaviour in Experiment 1, did not show an increased level of plasma testosterone. The 'medium' and 'high' sexual behaviour strains - the DBA, C57 and BDF_1 - all showed increases in

Table 4.2

The influence of the proximity of oestrous female mice on plasma testosterone levels , in sexually inexperienced BDF_1 mice.

	N	Testosterone level		
		Ln.	± SE	ng / ml
With female	8	2.47	0.20	11.8
No female	8	1.88	0.29	6.6

Student 't' test- 't'=1.7, df=14, $p < 0.05$

(one-tailed)

testosterone levels compared to controls.

However there were two anomalies to these results. Firstly, when compared to the testosterone levels measured in Experiment 1, both control and experimental values for C57's were less than previously described. 'Basal' levels from Experiment I were reported as over 3 ng/ml, whereas the experimental group of this experiment also had levels of over 3 ng/ml, but controls were less than 1 ng/ml. It might be argued that in fact control values showed a depression of testosterone, rather than that experimental animals showed elevated testosterone levels. However, animals used in this experiment came from stock bred in the Zoology Department, Edinburgh, whereas the previous experiment used animals bred in the Genetics Department, Edinburgh. It may well be that environmental conditions differ sufficiently between the breeding colonies (for example - Genetics use breeding cages which have perforated metal lids, which allow very little light into the cage), to account for these differences in testosterone levels. Secondly, the BDF_1 hybrid mice all showed greater plasma testosterone levels than those previously reported. Using sexually experienced animals, both experimental and control group values were greater than 7 ng/ml, whereas 'basal' levels from Experiment I were measured at less than 3 ng/ml.

Inexperienced animals also showed high levels in both groups (over 6 ng/ml), although here the experimental group did show greater levels than controls (over 11 ng/ml). It may be that this strain is exceptionally sensitive to female odour, and that control animals could detect oestrous females in adjacent experimental cages - although this seems unlikely since they were continuously exposed to generalised female odours from the room in which they were housed. A more likely explanation is that this strain shows increases in plasma testosterone in response to a variety of stimuli, and removal from the home-cage groups, and isolation for 15 minutes, in the controls, may be sufficient a stimulus for these changes to occur. These responses are however heightened in the presence of oestrous female mice.

The failure to demonstrate changes in testosterone levels in the Balb and CBA strains may be the product of several factors - perhaps these mice need a greater exposure time to the females or need direct genital sniffing before the olfactory cues can be detected. Alternatively, the plasma testosterone of these strains may be already at maximal levels, and there may be changes in plasma gonadotrophin levels which were not measured in this experiment, but which correspond to the changes in LH which are inferred in the other strains.

Increased levels of plasma testosterone in male house mice have also been reported in other social situations. Macrides, Bartke and Dalterio (1975) report that random-bred male mice exposed to "strange," i.e., unfamiliar, females, show increased levels of plasma testosterone after 30-60 minutes contact. 'Basal' levels of 15 ng/ml, in previously isolated animals; and 'basal' levels of 8 ng/ml in group housed animals (with females) increased to over 23 ng/ml in both groups. These values are considerably higher than those from the present experiment because mean values were computed arithmetically, but the increases are of the same order. The females used here were of unknown oestral state, and it might be argued that the testosterone changes were related to a general 'social' stimulus, rather than a specifically 'sexual' stimulus as outlined in the introduction. However Macrides et al report that the mice repeatedly attempted to mount the females, despite their unreceptive state, showing that they perceived the stimulus as sexual, and secondly exposure of the animals to 'strange' males did not have any significant effects on plasma testosterone.

Are these increases in plasma testosterone important in determining the probability of sexual behaviour? The observation that strains which show little sexual behaviour, are also those which do not

show an increase suggests that they may be important. If this is so, then it would be expected that in a group of mice which have been tested for sexual responses, the 'responders' should have higher testosterone levels than 'non-responders', an aspect which is examined in the next experiment.

4.3. EXPERIMENT 5

Levels of plasma testosterone in several strains of mice after a 30 minute encounter with a receptive female

4.3.1. Introduction

The previous experiment has shown that strains of mice, which normally show a high incidence of sexual behaviour, show increased levels of plasma testosterone after exposure to oestrous female mice. Strains of mice which are generally unresponsive do not show these changes. To determine whether these increases are behaviourally important, this experiment examines whether, in a group of mice, the animals which show sexual responses are those with increased plasma testosterone levels. It may be that the increased plasma testosterone is a generalised response shown by all animals, and not related to the incidence of sexual behaviour.

4.3.2. Method

The experiment examined four of the strains as previously described - the 'low' responding Balb/c, the 'medium' responding DBA/2J and the 'high' responding C57Bl/6Fa and the hybrid BDF_1. All mice were obtained from stock bred in the laboratory, and were housed in groups of 8-10 in large cages, under conditions as previously described. At 11 weeks of age, the mice were given a sexual experience test, as described in the previous experiment.

At 12 weeks of age the mice were given a sexual behaviour test, using the procedure previously described, except males were given only one BDF_1 receptive female, and the test was terminated after 30 minutes, when the males were removed and blood samples taken as previously described. Females were not changed at 10 minute intervals, as in Experiment I, because here we wish to examine the "non-responders" to determine whether there have been testosterone changes, as well as the "responders". The test time was limited to 30 minutes only, because testosterone may decline rapidly after a 'surge', and changes may go undetected if samples are not removed relatively quickly. Further, if a mouse ejaculated during the test, he was removed and blood samples taken immediately, as it was thought there might be secondary changes in plasma testosterone associated with this response.

4.3.3. Results

The mean testosterone levels for the strains are shown in Table 4.3. There were significant strain differences (Analysis of variance $F = 4.9$, $df = 3, 113$, $p < 0.01$), with C57 showing the lowest values of 2.8 ng/ml. The behaviour measures are shown in Table 4.4, which shows the percentage of animals showing no sexual responses, and also the percentage of animals showing mounts, intromissions and ejaculation.

Table 4.3

Levels of plasma testosterone , in several strains of the house mouse (Mus musculus), after 30 minutes exposure to oestrous female mice.[1]

a) All animals.

Strain	No. of animals	Testosterone levels Ln ± SE		ng / ml
Balb/c	52	1.86	0.18	6.4
C57Bl/6	29	1.03	0.18	2.8
BDF_1	15	1.81	0.26	6.1
DBA/2J(F)	11	1.76	0.26	5.8

Analysis of Variance - "F" = 4.9 , $p < 0.01$

1) Animals ejaculating before 30 minutes were killed and blood samples taken.

Table 4.4

Sexual behaviour measures for several strains of the house mouse, in a 30 minute behavioural test.

Behaviour is expressed as the percent animals showing each behavioural category.

		Sexual behaviour			
Strain	N	No sex behav%	% Mount	% Intromit	% Ejaculate
Balb/c	52	42	58	49	11
C57B1/6	29	17	83	83	53
BDF_1	15	20	80	80	76
DBA/2J	11	31	69	56	37

N = number of animals

The mean levels of testosterone of animals showing no sexual responses, and of animals showing at least mounting responses are shown in Table 4.5. In all the strains, the mean plasma testosterone levels of animals showing no sexual responses were lower than those of animals showing at least mounting responses. In two of the strains, Balb/c and C57Bl, this difference was significant (Student 't' test - 't' = 2.1, df = 50 $p < 0.05$; 't' = 3.3 df = 27 $p < 0.05$, using correction for heterogeneity of variance).

The relation between the behavioural levels reached in the test of no response, mount, intromission or ejaculation and plasma testosterone levels were further examined in the Balb/c strain, where over 50 animals were examined. The mean plasma testosterone level of animals in each behavioural category was determined, and are shown graphically in Figure 4.1. Using the non-parametric Spearman-Rank correlation coefficient, the 'levels of behaviour' reached were positively correlated with plasma testosterone. The individual data points for this relationship are shown in Figure 4.2, and show that a high plasma testosterone level was <u>not necessarily</u> associated with high behaviour, but that low plasma testosterone levels were associated with a low level of behaviour. The other strains were not analysed in this way because of the smaller sample sizes, and because the data was not so evenly distributed.

Table 4.5

Levels of plasma testosterone, in several strains of the house mouse (Mus musculus), after 30 minutes exposure to female mice in oestrous.

b) A comparison of testosterone levels of animals which show no sexual behaviour, with those of animals which show at least mounting behaviour.

Balb/c strain

		Testosterone levels		
	N.	Ln.	± SE	ng / ml
No sexual behaviour	22	1.54	0.27	4.6
Sexual behaviour	30	2.11	0.11	8.2

Student "t" test - "t" = 2.1 , $p < 0.05$

C57Bl/6 strain

		Testosterone levels		
	N.	Ln.	± SE	ng / ml
No sexual behaviour	6	0.39	0.33	1.4
Sexual behaviour	23	1.16	0.15	3.2

Student "t" test - "t" = 3.34 , $p < 0.05$

Table 4.5 cont.

BDF_1 strain

		Testosterone levels		
	N.	Ln.	± SE	ng / ml
No sexual behaviour	3	1.07	0.34	2.9
Sexual behaviour	12	2.00	0.26	7.4

Student "t" test - n.s.

DBA/2J(F) strain

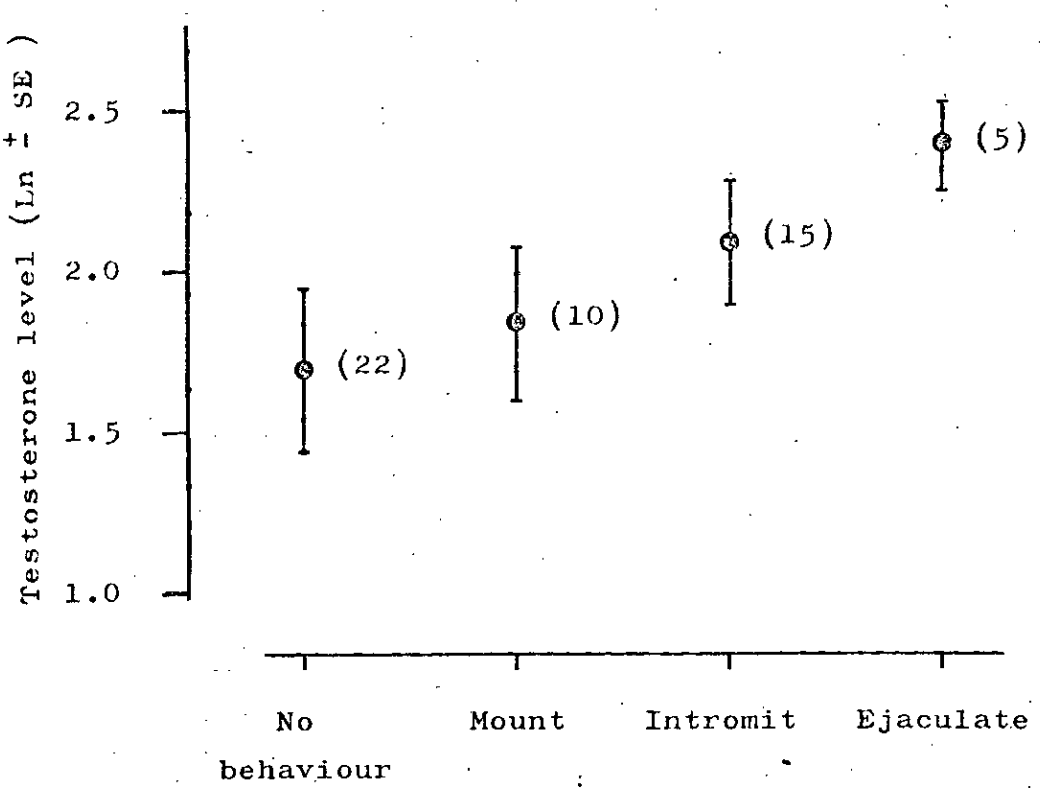
		Testosterone levels		
	N.	Ln.	± SE	ng / ml
No sexual behaviour	5	1.38	0.54	4.0
Sexual behaviour	11	1.92	0.27	6.8

Student "t" test - n.s.

Figure 4.1

Levels of plasma testosterone in male Balb/c mice after 30 minutes exposure to female mice in oestrous.

Mean testosterone levels are grouped according to the maximum level of behaviour shown by each animal. Animals ejaculating before 30 minutes were killed, and blood samples taken.

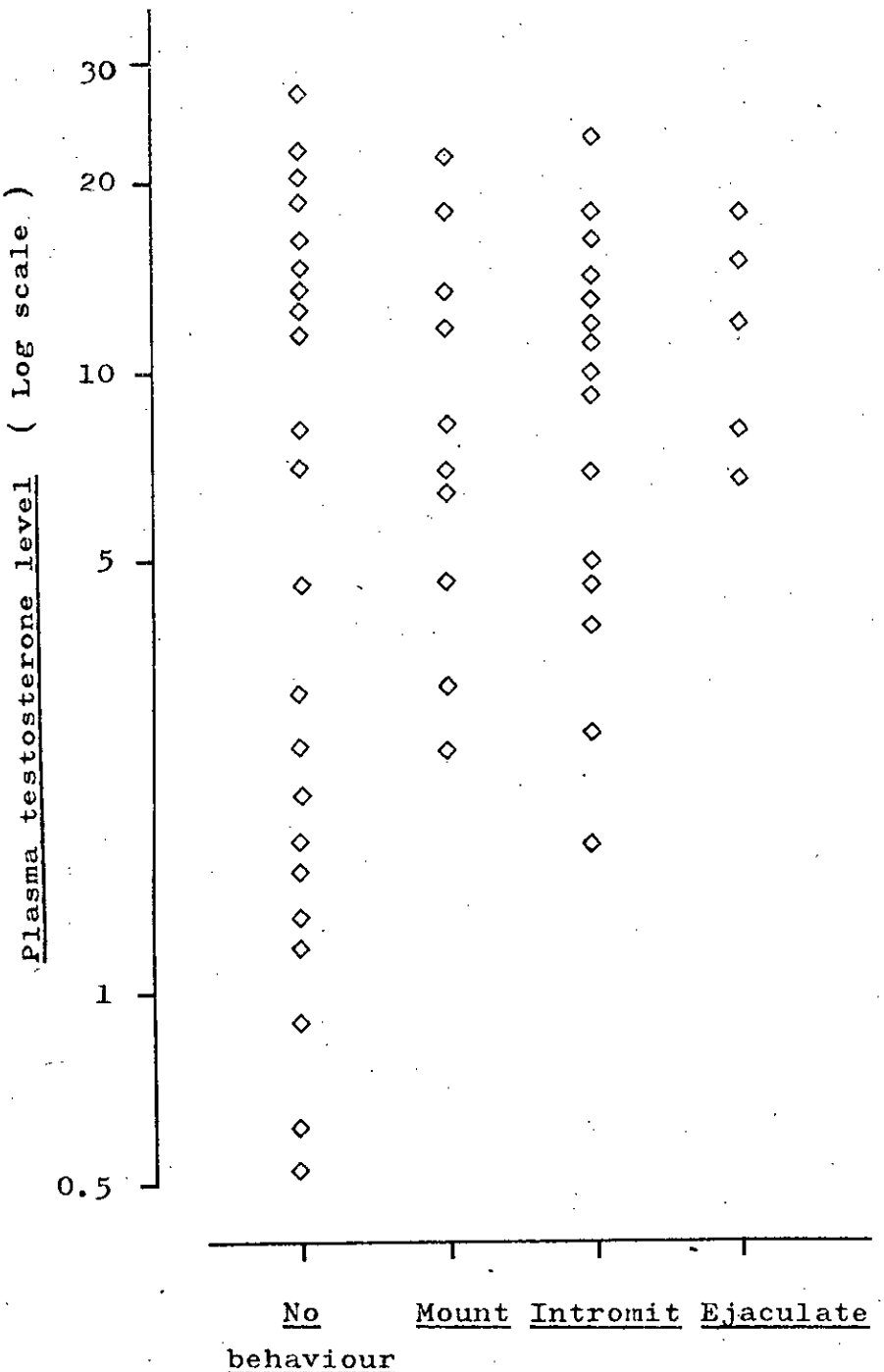


Numbers in ( ) = number of animals.

Figure 4.2

Levels of plasma testosterone in individual Balb/c mice after 30 minutes exposure to oestrous females.

The testosterone levels are grouped according to the behaviour shown in the test.



Within each strain the relationship between mount and ejaculation latency and testosterone levels were determined, and are shown in Table 4.6. In all of the strains, mount latency showed a significant negative correlation with plasma testosterone levels, using data logarithmically transformed. There were no significant correlations between ejaculation latency and testosterone levels in any of the strains.

4.3.4. Discussion

Analysis of plasma testosterone within strains showed levels to be higher in animals showing sexual responses than in those not showing sexual responses. Further, the latency to mounting was inversely correlated to plasma testosterone - animals which mounted quickly had higher testosterone than those mounting later in the test. However, ejaculation latency showed no correlations with plasma testosterone levels.

Within each strain, animals which showed sexual responses of at least mounting had higher testosterone levels than animals showing no sexual responses - these differences were significant in the Balb and C57 strains, the other strains failing to reach significance due to the smaller number of animals examined. In the Balb/c strain, further analysis revealed that testosterone levels were also positively related to the level of behaviour shown, thus animals which intromitted and ejaculated had increased testosterone over animals which only mounted.

Table 4.6

Pearson correlation coefficiants between plasma testosterone levels and mount and ejaculation latencies in the male house mouse(Mus musculus).

Animals were killed and blood samples taken either at ejaculation or after 30 minutes exposure to oestrous female mice.

a) Mount latency

	Strain			
Statistic	Balb/c	C57Bl/6	BDF_1	DBA/2J
r =	-0.33	-0.44	-0.73	-0.58
n =	30	23	12	11
p <	0.05	0.05	0.05	0.05

b) Ejaculation latency

	Strain			
Statistic	Balb/c	C57Bl/6	BDF_1	DBA/2J
r =	0.30	-0.35	-0.13	0.32
n =	5	16	11	6
p <	ns	ns	ns	ns

In all the strains, mount latency was inversely related to plasma testosterone levels, but ejaculation latency showed no such correlations. This suggests that testosterone levels are more related to factors involved in the initiation of sexual behaviour, rather than to factors involved in the 'execution' and termination of the behaviour. The apparent relationship between increased plasma testosterone and percentage ejaculation in the Balb's was probably an artifact - only those animals which mounted early in the test, and therefore had high testosterone, had sufficient time in which to show the ejaculatory response. Thus, in terms of Beach's model of rodent sexual behaviour (1956), plasma testosterone may be associated more with the arousal mechanism than with the copulatory mechanism.

Although these results support the idea that increased testosterone levels may be important in the initiation of sexual behaviour, there are several problems which must be further examined. Firstly, we must examine whether testosterone increased before the initiation of copulation, or whether animals with already high levels were those that showed sexual responses. Although this seems unlikely in view of the 'teaser' experiment described earlier, it must be noted that an effect could not be demonstrated in the high 'basal' testosterone strains such as Balb/c and CBA. Whether

testosterone 'rises' prior to copulation, or whether animals with already high testosterone levels are those which show sexual responses is difficult to determine, and may well be a combination of both factors. After the introduction of a receptive female, those animals with low plasma testosterone levels may increase their levels, and those animals with already high levels may maintain them for the duration of copulation. In the preceeding teaser experiment, it was found that strains of mice with low 'basal' testosterone levels, as determined in Experiment I, showed elevated levels after 15 minutes exposure to an oestrous female, but that strains with high basal testosterone levels did not show such increases. Thus, in BDF_1, C57 and DBA, where testosterone levels significantly increased under 'teaser' conditions, it seems likely that the testosterone increases seen after the test were related to the incidence of sexual behaviour; but that in the Balb strain it was those animals with already high testosterone levels that showed sexual responses. The failure to detect increases in plasma testosterone in the Balb strain was also confirmed in this experiment, the level of testosterone after 30 minutes' exposure to a receptive female was 6.4 ng/ml, a value not significantly different from the 'teaser' level of 4.2 ng/ml, and the 'basal' levels, from Experiment I, of 5.8 ng/ml. A similar phenomenon has been reported in the rabbit (Hilliard et al, 1975), where if testosterone

levels were low preceeding copulation they tended to rise, but if already nigh they did not show further increases. Similarly, in the bull (Katongole et al, 1971), sexual stimuli result in a large pulse of LH, which if testosterone levels were low, results in a release of the hormone, but if testosterone was already high has no further effect.

Secondly, since all blood samples were removed after copulation, it may be that the positive correlation between plasma testosterone and measures of sexual behaviour was a secondary effect, due to the release of testosterone in response to copulatory stimuli, in particular intromissions and ejaculation. Although the previous 'teaser' experiment demonstrated that testosterone levels could increase without physical contact between male and female, it may be that this 'pulse' declines rapidly, and the changes seen in the present experiment may be secondary responses to other stimuli. This question would be simply answered if it were possible to take continuous blood samples from the mice, but as this was not possible, a second approach was adopted, which involved removing blood samples from the mice when particular behavioural criteria had been reached. That is, to keep the behaviour as a constant, and examine the testosterone levels at several stages as is described in the next experiment.

4.4. EXPERIMENT 6

The levels of plasma testosterone at different stages of copulation in three strains of the house mouse

4.4.1. Introduction

The experiment aims to determine whether testosterone levels change during copulation, and in particular whether they increase after intromissions and ejaculation. The previous experiment has demonstrated that immediately after sexual behaviour, mice have elevated testosterone levels, which positively correlate with the amount of sexual behaviour shown. This experiment examines whether this correlation was the result of copulatory stimuli having direct effects on plasma testosterone levels.

One way of determining whether copulation has effects on testosterone levels is to measure the levels in blood samples removed from the animal as soon as the behaviour is shown. If these levels are high, then we know that the increases in the levels occur before any direct effects of genital stimulation can have effect. Analysis of blood samples removed at later stages of copulation will show whether copulatory stimuli have 'additive' effects - perhaps ejaculation results in a pulse of testosterone.

For this type of experiment, it is important to assign the animals to be measured at each stage of

behaviour before the experiment begins so that, for example, 'fast' copulators are not inadvertently selected for particular groups. We know from the previous experiment that animals which start to copulate quickly have higher testosterone than animals slow to initiate behaviour and care must be taken to ensure these animals are not, for example, grouped in the ejaculation category.

The four stages of copulation used here were (i) the first mount, (ii) the end of the second bout of intromission, (iii) ejaculation and (iv) 30 minutes after ejaculation.

4.4.2. Method

Balb/c, C57Bl/6Fa and BDF_1 male mice, from stock bred in the laboratory, were housed in groups of 8-10 in large cages, under conditions as previously described. At 10 weeks of age, all animals were given a sexual experience test, and were placed with receptive BDF_1 females in single cages for one hour.

At 11 weeks, the animals were placed with receptive BDF_1 females, under conditions as previously described, in single cages. Each male was assigned to one of the 4 behavioural categories, and as soon as criterion was reached, the male was killed and blood samples removed for assay of testosterone.

Animals not reaching mount and intromission criteria within 30 minutes of the test, or which did

not ejaculate after 30 minutes of intromission were eliminated from the experiment.

4.4.3. Results

The plasma testosterone levels at the different stages of copulation, for each of the strains, are shown in Table 4.7. There were no significant differences within the Balb's and C57's for each stage of behaviour, but the BDF_1's showed significantly higher levels at mount and intromission than at ejaculation, and 30 minutes after ejaculation (Analysis of Variance F = 8.7, df = 3, 29 $p < 0.01$). The strain means were averaged across the corresponding behavioural categories, and the 'overall' changes in testosterone for the three strains are shown in Table 4.8 - there was a decline in testosterone levels from mounting to 30 minutes after ejaculation.

It is also of interest to compare the testosterone levels between strains at corresponding behavioural categories. At 'mounting' there were significant differences between the strains (Analysis of Variance 'F' = 12.2, df = 2, 17 $p < 0.01$), and all strain levels were significantly different from one another (Student Neuman-Keuls a posteriori test). BDF_1 showed the highest testosterone level of 16.5 ng/ml, followed by Balb/c with 7.8 ng/ml, and C57 showed the lowest levels

Table 4.7

Levels of plasma testosterone, in samples removed at different stages of copulation , in several strains of the house mouse.

Animals were allowed to copulate until reaching a pre-assigned criterion of sexual behaviour, after which they were killed and blood samples taken. The criteria used were - the first mount (Mount) ,the second intromission bout (Intro) , ejaculation (Ejac) and 30 minutes after ejaculation (Ejac-30).

a) Balb/c strain.

Testosterone	Behaviour			
	Mount	Intro	Ejac	Ejac-30
No. of animals	7	10	10	4
Mean - Ln	2.06	2.00	2.20	1.01
SE	0.27	0.33	0.24	0.50
ng/ml	7.8	7.4	9.1	2.7

Analysis of Variance - $"F"_{(3,27)} = 1.88$, p = ns

b) C57Bl/6 strain.

Testosterone	Behaviour			
	Mount	Intro	Ejac	Ejac-30
No. of animals	6	7	10	6
Mean - Ln.	1.22	0.27	0.98	0.88
SE	0.22	0.34	0.35	0.11
ng/ml	3.4	1.3	2.7	2.4

Analysis of Variance - $"F"_{(3, 25)} = 1.64$, p = ns

c) BDF_1 strain.

Testosterone	Behaviour			
	Mount	Intro	Ejac	Ejac-30
No. of animals	7	8	10	8
Mean - Ln.	2.80	2.63	1.50	1.51
SE	0.17	0.20	0.27	0.24
ng/ml	16.5	13.9	4.5	4.5

Analysis of Variance - $"F"_{(3,29)} = 8.74$ $p < 0.01$

Table 4.8

Overall plasma testosterone levels for Balb/c, C57Bl/6 and BDF_1 male mice, at different stages of copulation.

Means were calculated from the mean plasma testosterone levels for each of the strains, at each behavioural category, as shown in Table 4.5 .

Behaviour	Testosterone level		
	Ln	± SE	ng / ml
Mount	2.03	0.46	7.6
Intro.	1.63	0.71	5.1
Ejac.	1.57	0.35	4.8
Ejac-30	1.12	0.19	3.1

of 3.4 ng/ml. Similarly, at intromission there were overall strain differences ($F = 14.8$, df = 2, 22 $p < 0.01$), the highest values were shown by BDF_1 (13.9 ng/ml) and Balb (7.4 ng/ml) and C57 was significantly lower at 1.3 ng/ml (student Newman-Keuls test). At ejaculation there were also strain differences ($F = 4.4$ df = 2, 27 $p < 0.05$), with Balb/c showing the highest values (9.1 ng/ml), and the lowest by C57 (2.7 ng/ml) (Newman Keuls test). BDF_1 was intermediate at 4.5 ng/ml, and not significantly different from either C57 or Balb. Thirty minutes after ejaculation there were no significant differences between the strains ($F = 1.7$ df = 2, 15 p = ns).

4.4.4. Discussion

These results show that testosterone levels do not increase during copulation, but show a general decline which continues after ejaculation. The highest plasma testosterone levels were found at the initiation of mounting (over 7 ng/ml), and the lowest values were seen 30 minutes after ejaculation (3 ng/ml). There were strain differences in testosterone levels at mount, intromission and ejaculation, although not at 30 minutes after ejaculation.

The finding that testosterone levels did not increase during copulation agrees the findings of

Kamel et al (1975), who found that in the rat, levels of testosterone were as high after one intromission bout as after ejaculation, but that both levels were greater than the 'resting' levels of animals not exposed to receptive females. They also measured LH levels, and their data suggests that levels reached a peak after less than five minutes contact with the female, and then began to decline, although not significantly.

In the previous experiment of this chapter, it was found that mice 'spontaneously' copulating had higher testosterone levels than non-copulators, and it was suggested that this may have been due to stimulatory effects of copulation itself on testosterone release. However, the results from this experiment refute this hypothesis since plasma levels of testosterone were at their highest at the initiation of the behaviour, and declined rather than increased as behaviour progressed. Thus, the rises in testosterone levels (or the already high levels) of the 'copulators' of the previous experiment must have occurred before the initiation of sexual behaviour, and might therefore be involved in the mechanisms which mediate sexual behaviour. In fact, the decline in testosterone levels found to occur during copulation would have partially 'masked' the relation between testosterone and sexual behaviour reported in the previous experiment.

At the initiation of mounting responses there were large strain differences in plasma testosterone, with BDF_1 showing highest levels, followed by Balb/c and the C57's showing lowest levels. This is a very different situation from the 'basal' levels as reported in Experiment 1, where the Balb's showed higher testosterone levels than BDF_1 and C57. Thus the negative correlation described in Experiment 1, between plasma testosterone levels and measures of sexual behaviour, breaks down when levels are measured at the time of behaviour itself, and no simple relationship can be substituted.

Thirty minutes after ejaculation, there were no significant differences in plasma testosterone between the strains, and levels were lower than those found at mounting and intromission. In part, this may have been because fewer animals were examined (in particular Balb/c), but the data does suggest that all the strains were converging towards uniformly low levels of testosterone after ejaculation. This is of interest because after ejaculation, mice show a 'refractory' period, during which sexual responses cannot be elicited, and which may last for hours or even several days (McGill and Blight, 1963b). The results of this experiment suggest that the refractory period may be related to low post-ejaculatory testosterone levels.

4.5. GENERAL DISCUSSION

To summarise, the three experiments described in this Chapter have demonstrated that:-

1) Close proximity of oestrous female mice results in increased levels of plasma testosterone in males, in particular from strains with low 'basal' testosterone levels, and a high probability of showing sexual behaviour.

2) Blood samples removed immediately after a sexual behaviour test show that the incidence of these changes in plasma testosterone is related to the incidence of sexual behaviour such that within strains, plasma testosterone levels were higher in 'copulators' than 'non-copulators'; there were correlations between testosterone levels and the mount latency, but none with the ejaculation latency.

3) Blood samples removed at particular stages of sexual behaviour showed testosterone levels to be greatest at the initiation of mounting responses and to decline during copulation.

These observations suggest that increases in testosterone levels (or in some cases already high levels) are directly involved in the mechanisms which control the initiation of sexual behaviour, but not necessarily with those involved in the execution of the behaviour, in particular with mechanisms mediating the ejaculatory response.

However, it is important to note that changes in the levels of testosterone are the product of a sequence of neuro-endocrine events, and any of the preceeding links may be important in the control of sexual behaviour. Thus a correlation between testosterone levels and measures of behaviour, implies that there is also a correlation between other hormones, such as the pituitary trophic hormones (e.g., LH, FSH) and hypothalamic releasing factors (e.g., LH-RF). In fact there is some evidence, largely from work in birds, showing that the gonadotrophins can be important in behavioural control. In the weaver bird (Quelea quelea) seasonal changes in agonistic behaviour correlate closely with changes in plumage colouration, but not with testis size (Butterfield and Crook, 1968). The plumage is considered to be largely under the control of circulating gonadotrophins, and they suggest that the tendency to respond aggressively is largely controlled by pituitary gonadotrophins in this species. Further, administration of testosterone propionate to low ranking Quelea males does not change their social rank, but injections of LH (of mammalian origin) can change social status, within an hour, through improved performance in agonistic encounters (Crook and Butterfield 1968). Similarly, in Starlings (Sturnus vulgaris) castration, or administration of TP, has no effect on

the social rank of an individual from a group (Davis, 1957), but administration of LH (of mammalian origin) can increase the aggression of low ranking males within 10-15 minutes such that they change in social rank (Mathewson, 1961). In the rabbit adrenocorticotrophic hormone (ACTH) has also been reported to have behavioural effects. Bertolini, Vergoni, Gessa and Ferrari (1969) injected male rabbits with synthetic β-ACTH into the lateral ventricles and found that after 30 minutes the animals were "sexually excited", showing penis erection and ejaculation. They found no such effects in castrates, or in animals pretreated with the androgen inhibitor cyproterone acetate. It is interesting that in female rabbits, intraventricular injections of ACTH result in increased levels of LH (Baldwin, Haun and Sawyer, 1974), and it may be that the sexual excitatory effects seen in males are in fact related to changes in gonadotrophins. Thus, pituitary hormones are implicated as being important in sexual and aggressive behaviours, but their role is not necessarily absolute, for as Pfaff (1970) has shown, hypophysectomised castrated rats still show increased sexual behaviour after injections of testosterone.

The hypothalamic releasing factors, such as LH-RF, may also have some behavioural effects. In this context the observations of Moss and McCann (1973) on the

effects of LH-RF on the induction of lordotic responses in female rats are very relevant. They found that injections of LH-RF resulted in lordosis responses within half an hour in 50 percent of animals, and in over 90 percent after 2 hours. Other releasing factors, such as thyrotrophin, had no effects on lordosis responses. There may be similar effects in male rats, and since as described by Kamel et al (1973) there are increases in plasma LH immediately prior to copulation, it may be that the LH-RF, which is presumably also released into brain areas, has direct effects on male sexual behaviour.

To summarise, the relationship found between increased levels of plasma testosterone and sexual behaviour may be the product of several, not necessarily exclusive, neuro-endocrine events. The increased release of androgens, gonadotrophins and hypothalamic-releasing factors may all have some effect on the mechanisms which control sexual behaviour. It is unlikely that the occurrence of sexual behaviour is the product of a single neuro-endocrine signal, but rather the product of a pattern of many stimuli, of which some, as these experiments have shown, may be normonal.

CHAPTER FIVE

THE EFFECTS OF CASTRATION AND TESTOSTERONE PROPIONATE THERAPY ON SEMINAL VESICLE WEIGHTS AND MEASURES OF SEXUAL BEHAVIOUR

5.1. INTRODUCTION

In Chapter Three, plasma testosterone levels across several strains of mice were examined, and it was found that strains which normally show a high incidence of sexual behaviour had lower testosterone levels than strains with a low incidence of sexual behaviour. It was suggested from these results that the strains of mice may differ in their sensitivity to testosterone, and that sexually responsive, low testosterone strains had a heightened responsivity at the hypothalamopituitary axis, mediating the 'feedback loop', and also at brain areas controlling sexual behaviour. However, this inference has an underlying assumption which may not be warranted - that the levels of testosterone measured in the plasma reflect the testosterone requirements for the display of sexual behaviour. For example, that the strains of mice with high testosterone levels "need" these levels for normal sexual behaviour. However, it may be that the strains of mice with high plasma testosterone have, in fact, an excess of hormone over that necessary for the display of sexual behaviour. The high testosterone levels may be related to some other phenomenon concerned with other aspects of fertility, for example the maintenance of peripheral androgen dependent structures, such as the seminal vesicles.

This problem of whether plasma testosterone was only coincidentally related to behaviour has been partially investigated in Chapter Three. An F_2 analysis was carried out, and the results supported the suggestion that testosterone levels were closely related to sexual behaviour, but positive results were in fact only found in one of the two F_2 populations examined. A further examination of the role of plasma testosterone would seem necessary, and the experiment to be described in this Chapter investigates the effects of injections of testosterone into castrate males of three of the strains previously described.

There is evidence from studies in both the rat and the rabbit, that in fact testosterone may be in excess of levels required for the display of sexual behaviour. In the castrate rat, the dose of injected testosterone propionate which will maintain ejaculatory behaviour is lower than that necessary to maintain the weights of the seminal vesicles and the prostate at 'intact control' levels (Davidson, Johnston, Bloch, Smith and Weick, 1970). This suggests, as the authors point out that... "the normal circulating amount of androgen is greater than the amount necessary for the maintenance of sexual behaviour." Similarly, in the rabbit, the doses of testosterone necessary to restore sexual behaviour to precastrational levels are less than the doses required to restore the seminal fructose

concentration (an androgen sensitive response) to levels comparable to those of intact controls (Ågmo, 1974). Thus, in both of these species, plasma testosterone levels may be in excess of those necessary for sexual behaviour.

One of the questions which this experiment examines therefore, is whether a similar phenomenon occurs in the mouse, in particular in those strains with high plasma testosterone levels. To determine this, the doses of testosterone which maintain sexual behaviour are compared with those that maintain the weights of the seminal vesicles. The minimum dose of testosterone which maintains the seminal vesicles at the level of intact controls, may be equivalent to normal levels of testosterone. By determining whether the levels necessary for behavioural maintenance are less, or greater than, these levels, we can determine whether 'normal basal' plasma testosterone levels are in excess of those necessary for sexual behaviour.

These levels of testosterone which maintain seminal vesicle and behavioural parameters can also be compared across the strains. In this way we can determine whether the strains differ in 'responsiveness' to testosterone. Thus a strain in which sexual behaviour can be maintained with a low dose of testosterone might be considered more androgen responsive than a strain which needs a high dose of testosterone.

In Chapter Four, the plasma testosterone levels of several of the strains of mice, examined in Chapter Three, were re-examined under different circumstances. Here, plasma samples were removed when the animals were near females, or before and during copulation. Under these conditions, it was found that the plasma testosterone levels were very different from the unstimulated 'basal' levels previously measured. In some of the strains, there were large increases in testosterone levels, and these increases were related to some aspects of sexual behaviour. For example, animals with high testosterone levels showed shorter mount latencies than animals with lower testosterone levels. It was postulated that perhaps rises in testosterone prior to responding sexually might be important in controlling some aspects of the behaviour, in particular the mount latency. If these changes in testosterone are important in behavioural terms, then we might expect to be able to demonstrate a dose response curve between injected testosterone and aspects of sexual behaviour.

In the rat, a dose-response relationship between testosterone propionate and measures of sexual behaviour has been described by Beach and Holz-Tucker (1949). Sexually experienced male rats were castrated and 48 hours later different groups were given 0, 25, 50, 100 or 500 µg of Testosterone Propionate respectively every

day for 9 weeks. It was found that 50 µg TP/day maintained, that lower doses increased, and that higher doses decreased the mount latency. Daily injections of 50 µg TP or more maintained, or even increased the proportion of rats displaying intromissions. It was found that it takes more androgen to stimulate ejaculatory responses, than to stimulate mounts or intromissions, and that ejaculation latency was decreased with increasing testosterone dose. However, the behavioural comparisons made were between pre- and post-castrational tests, rather than to sham-operated controls, and it is possible that the increased behaviour observed may in part have been due to the increased sexual experience of the rats.

In the castrate rabbit, it has been reported by Ågmo and Kihlström (1974) that there is no dose response curve between testosterone and quantitative measures such as the mount latency, although this study examined the restoration, rather than the maintenance of sexual behaviour. Similarly, in the hybrid CD2F$_1$ mouse (Balb x DBA), Champlin, Blight and McGill (1968) found that although 32 µg TP injected every day would restore the behaviour to normal levels, increasing the dose to 1024 ug TP/day had no further effect, and measures like mount latency could not be significantly decreased.

The experiment to be described here investigates whether dose-response curves can be demonstrated in strains of the mouse, and also whether behaviour can be increased above normal levels, as Beach and Holz-Tucker (1949) could do in the rat.

To summarise, Experiment 8 examines three main questions:-

1) Do the 'basal' plasma testosterone levels, as measured earlier, reflect the "behavioural needs" of the strain, or is, for example, testosterone in excess of that necessary for the display of sexual behaviour?

2) Do the strains differ in responsiveness to testosterone in terms of maintenance of peripheral structures such as the seminal vesicles?

3) Is there a dose-response relationship between testosterone and measures of sexual behaviour, and can behaviour be increased over 'normal' levels?

5.2. Method

Balb/c, C57Bl/6Fa and BDF_1 male mice, from stock bred in the laboratory, were housed singly, at 11-13 weeks, in small clear plastic cages, and maintained under conditions as previously described. All males were housed overnight with receptive BDF_1 females on two occasions, to ensure that they were sexually experienced before castration. The females were checked for copulatory plugs the following morning, and only males which were known to have ejaculated at least once were subsequently used in the experiment.

At 12-14 weeks of age all males were castrated, or sham-operated under ether anaesthesia. The testes, plus associated fat bodies, were removed via a mid-scrotal incision, which was then sutured with a wound clip (Clay-Adams, New Jersey - 9 mm Autoclip). Sham operated animals were anaesthetized, the scrotal sac opened, left for one minute, and then the wound sealed with an autoclip.

Hormone replacement injections were given every day - the first injection being given while the animal was still under anaesthesia after castration or sham castration. The testosterone propionate (TP) (Sigma Ltd: Δ^4: Androsten-17-OL-3-One-Propionate) was dissolved in Arachis oil:ethyl oleate, 90:10 vv, and injected in 0.02 ml under the skin at the 'scruff' of the neck. There were six experimental groups of 8-10

mice for each of the strains. These were:

1) Sham operants + vehicle only injections
2) Castrates + vehicle only injections
3) Castrates + 6.2 µg TP "
4) Castrates + 12.5 µg TP "
5) Castrates + 25 µg TP "
6) Castrates + 50 µg TP "

The mice were injected for 8 days, (castration = day 1), and tested for sexual behaviour on days 5 and 9. The testing procedure was as previously described. After the test on day 9, the mice were weighed, killed and the seminal vesicles (plus coagulating glands) were removed, expressed of fluid contents and weighed.

Statistical Analysis

To compare the doses of injected TP which have effects on different parameters, we need some way of estimating the 'effective' doses of TP with some indicators of central tendency and error factors of measurement. Methods described in the literature compare the doses of testosterone which result in parameter values not significantly different from those of intact controls (e.g., Davidson et al, 1970; Bartke, 1974). However this method only gives a crude estimate of the TP dose necessary, and gives no information as to the variation in responsiveness of individuals. A more

effective method of analysis may be to determine the regression equation between the dose of TP and the response, and to calculate the effective doses of TP from this equation using the known response levels from the control animals.

Therefore the correlation coefficient between the doses of testosterone used (individual values) and the response in question was calculated using transformations as necessary in order to linearise the data. The logarithmic transform was found to be necessary for all testosterone values, and therefore in order to be able to include the oil, i.e. 0 dose levels, the data was actually transformed as Ln (Dose + 1). If the correlation was significant at $p < 0.05$ then the equation of the line was determined. From this equation, the doses of testosterone which were equivalent to the levels of the measured parameter in the intact control animals was calculated. Individual values from the controls were used, and the calculated levels of testosterone were expressed as the mean $\pm$ standard error. The equivalent levels of testosterone for the parameters were then compared using Analysis of Variance and Student Newman-Keuls a posteriori tests.

If the correlation between testosterone dose and the behaviour was not significant, the data was examined by ANOVAR to determine whether there were any significant differences between the groups receiving different doses of testosterone propionate.

5.3. Results

5.3.1. Seminal Vesicle Weights.

The dose response curves between seminal weights and TP dose are shown in Figure 5.1. There were no significant differences between the body weights of the strains (Sham controls - $F = 0.54$, $df = 2$, p = ns, body weights between 25-26 g), and therefore the seminal vesicle data was not corrected to a standard body weight. There were no significant differences between the seminal vesicles of the three strains in the 'sham' operant controls, but in the castrate, oil treated (0.TP) group there were significant differences ($F = 7.5$, $df = 2, 24$ $p < 0.01$), with the lowest value being shown by Balb/c (Student-Newman Keuls test $p < 0.05$). The Balb/c strain showed a significantly greater decrease in seminal vesicle weight after castration - BDF_1 and C57 fed by 48 and 52% respectively, but Balb/c fell by 70%, significantly greater than both BDF_1 and C57. (Linear combination of means - Snedecor and Cochran (1967 p. 269) - C57 x BDF_1, 't' = 0.097 ns; C57 x Balb 't' = 3.839 $p < 0.005$, BDF_1 x Balb 't' = 4.172 $p < 0.001$.)

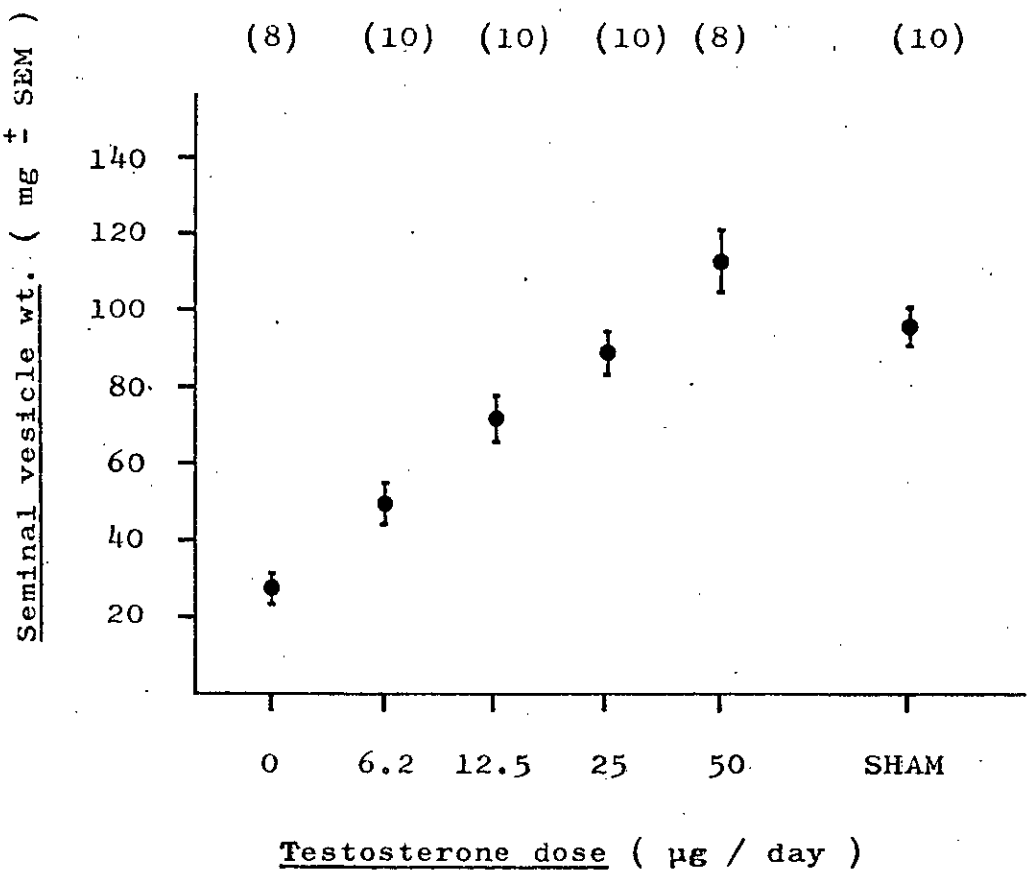
Examination of the dose-response curves (Fig. 5.1) suggests that one strain, BDF_1, shows a ceiling effect of response, at doses of TP greater than 12.5 µg/day. There were no significant differences between the

Figure 5.1

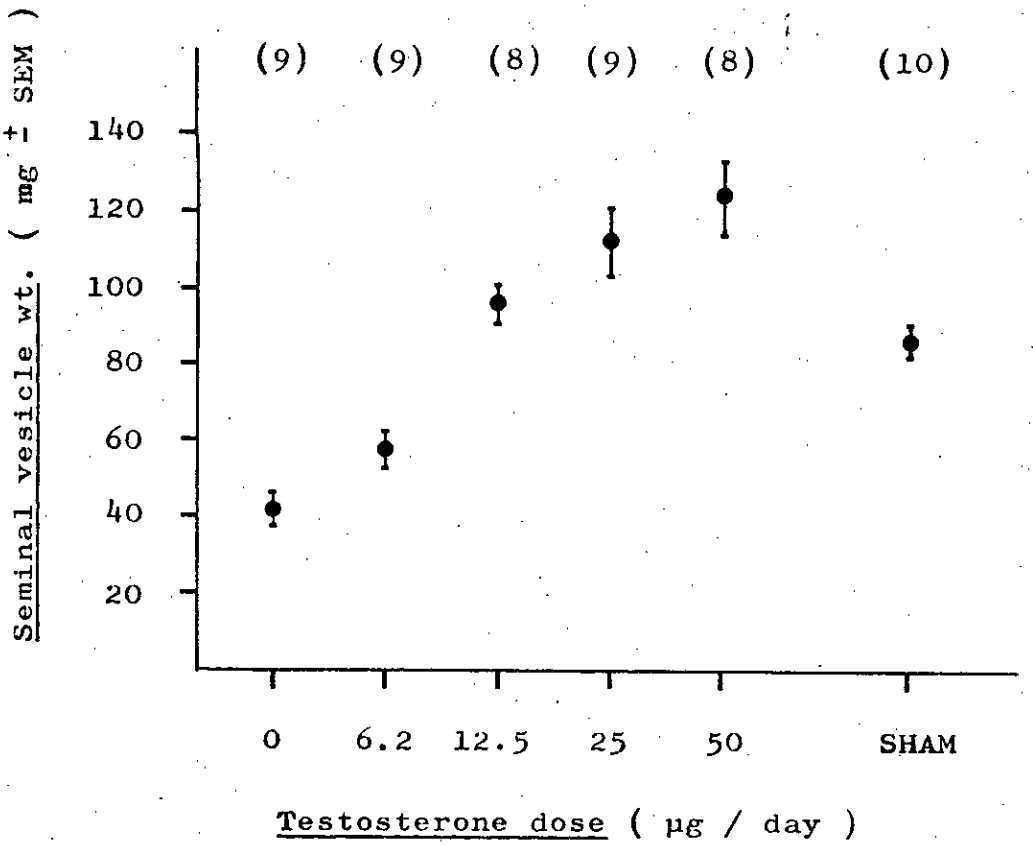
Mean wet weights of seminal vesicles (plus coagulating glands) from castrate mice after 8 daily injections of testosterone propionate.

The numbers in ( ) = the number of mice in each group

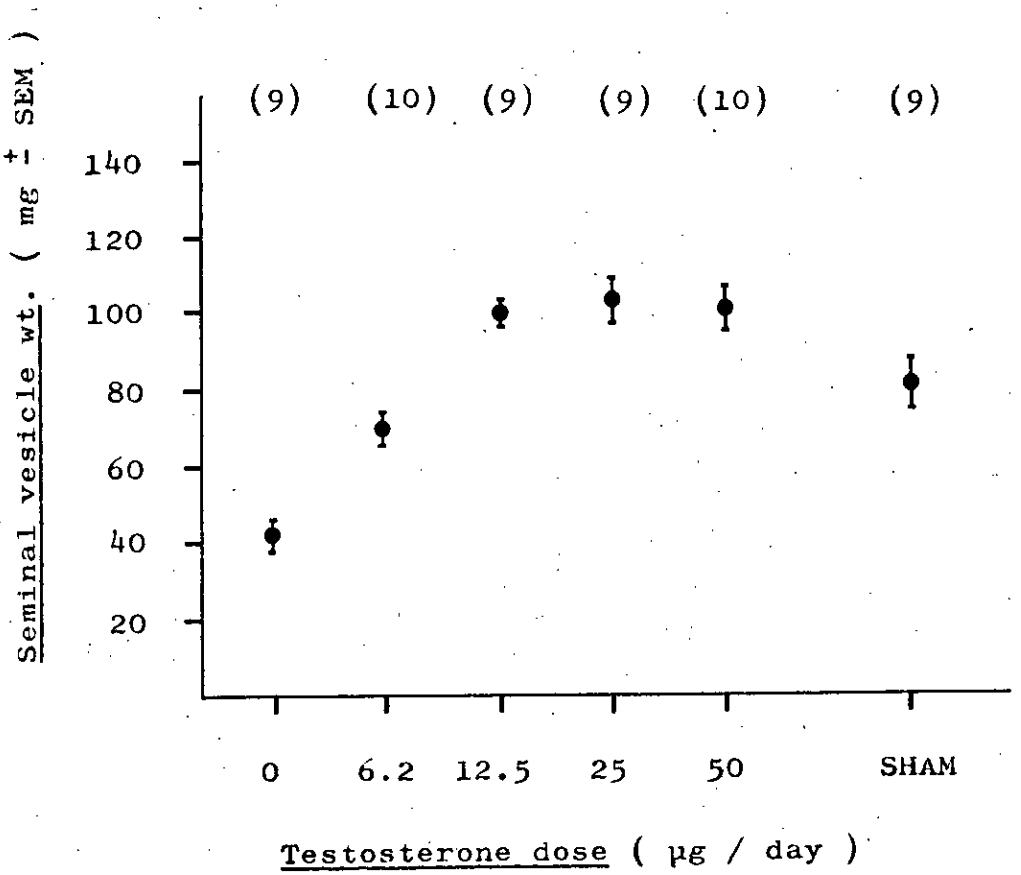
a) Balb/c strain



b) C57Bl/6 strain



c) BDF_1 hybrid



seminal vesicle weights at TP doses of 12.5, 25 and 50 µg/day, and therefore the correlations were calculated only from the 0, 6.2 and 12.5 µg/day doses. The correlation coefficients and regression equations were calculated as shown in Table 5.1, using the testosterone dose transformed to logarithms. In order to be able to include the oil controls (i.e., 0 µg TP) the data was actually transformed as Ln (Dose +1). Individual data points for each mouse were used, and all correlations were significant at $p < 0.001$. The regression equations were then calculated, using transformations as for the correlation coefficient, and from this equation was calculated the doses of TP which would maintain the seminal vesicles at the 'sham' levels. That is, each value of 'sham' seminal weight (y) was interpolated in the equation, and the dose of TP (x) calculated. The doses of TP were then expressed as logarithmic mean $\pm$ standard error. The doses for the three strains are shown in Table 5.2 - Balb/c needed significantly greater doses of TP (over 30 µg/day) than either C57 or BDF_1 (both less than 10 µg/day) (Analysis of Variance $F = 11.07$, $df = 2, 26$ $p < 0.01$, Student Newman Keuls - Balb highest dose over C57 and BDF_1 $p < 0.05$).

Table 5.1

Correlation coefficients and regression equations of seminal vesicle (plus coagulating gland) weights and injected doses of Testosterone Propionate in castrate mice.

Strain	Correlation coefficiant			Regression line	
	r =	df =	p <	a =	b =
Balb/c	0.90	44	0.001	19.454	21.913
C57Bl/6Fa	0.87	43	0.001	33.909	21.192
BDF_1	0.91	26	0.001	39.535	20.445

Equation : $y = a + bx$, where y = seminal vesicle weight, x = dose of TP (Ln + 1).

Seminal vesicle weights were examined in animals receiving 0, 6.2, 12.5, 25 and 50 µg TP / day for Balb/c and C57Bl/Fa, but only 0, 6.2 and 12.5 µg TP / day for BDF_1 mice.
All TP doses were logarithmically transformed.

Table 5.2

Equivalent doses of Testosterone Propionate (TP µg / day) necessary to maintain the observed seminal vesicle weights of sham castrated animals; as calculated from the regression of seminal vesicle weights of castrate mice on injected doses of Testosterone Propionate.

Strain	Dose Testosterone Propionate / day		
	Ln	± SE	(µg)
Balb/c	3.49	0.16	(32)
C57Bl/6Fa	2.32	0.12	(9)
BDF_1 hybrid	2.28	0.21	(9)

Analysis of Variance $'F'_{(2,26)} = 11.07$, $p < 0.01$

5.3.2. Sexual Behaviour Measures

The dose response relationships between the mount, intromission and ejaculation frequencies and doses of TP for the three strains are shown in figures 5.2, 5.3 and 5.4. The group frequencies were calculated by taking the mean of the number of tests in which each mouse showed the behaviour at least once - thus for example a group average of '2' shows that all mice showed the behaviour at least once in both tests.

Mount Frequency

There were no significant differences between the mount frequencies of the three strains in the 'sham' operant controls ($F = 1.70$ df = 2, 27, p = ns), and in the oil treated castrates ($F = 1.59$ df = 2, 23, p = ns). The three strains all showed decreases in mount frequency in the castrated oil treated groups, although these reached significance only in the Balbs (Student 't' test 't' = 2.41 df = 16 $p < 0.05$) and the BDF_1's ('t' = 2.69 df = 9 $p < 0.05$).

The correlation coefficients and regression equations between mount frequency and TP dose are shown in Table 5.3, using TP dose transformations as described above. Correlations were significant in only the C57's and BDF_1's, and regression equations were calculated as before. The levels of TP which would maintain the mount frequency at 'sham' levels were

Figure 5.2

Average mount frequency of castrate mice receiving different doses of Testosterone Propionate.

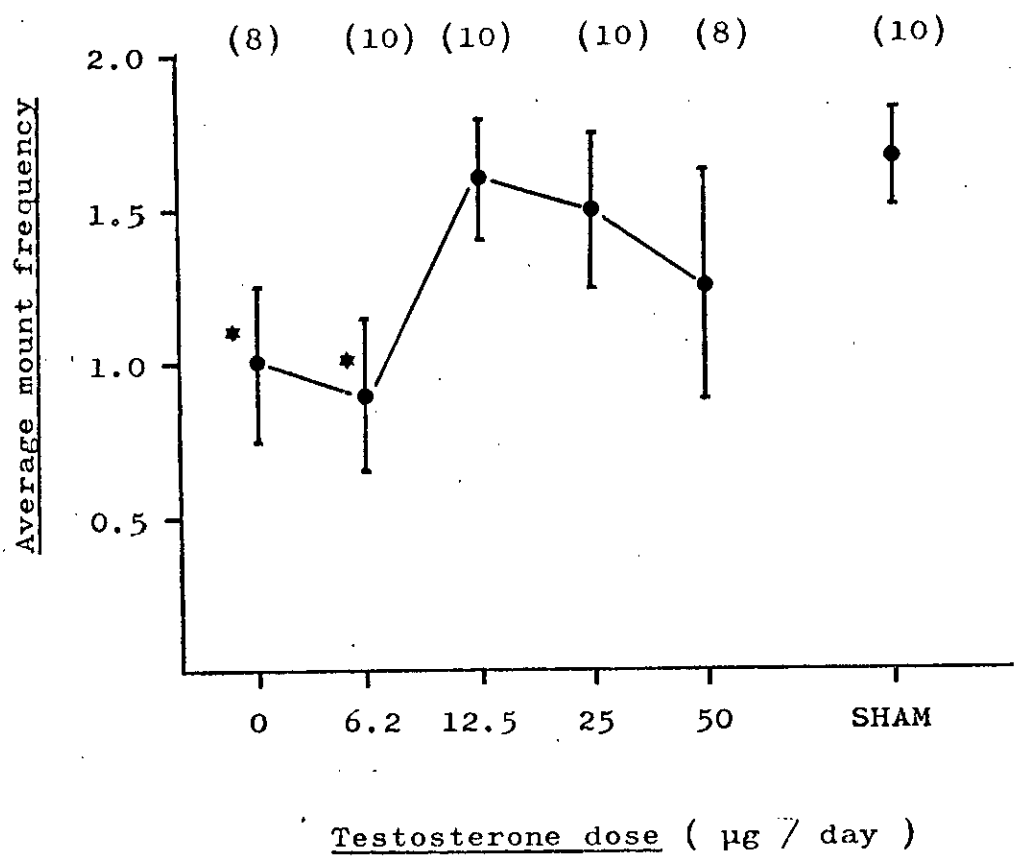
Mice were castrated on day 1 of the experiment, injected daily with TP and tested for sexual responses on days 5 & 9. The number of tests (0,1 or 2) in which each mouse mounted at least once was determined and the group average expressed as mean ± Standard Error.

The 'SHAM' group were sham castrated and given daily injections of oil.

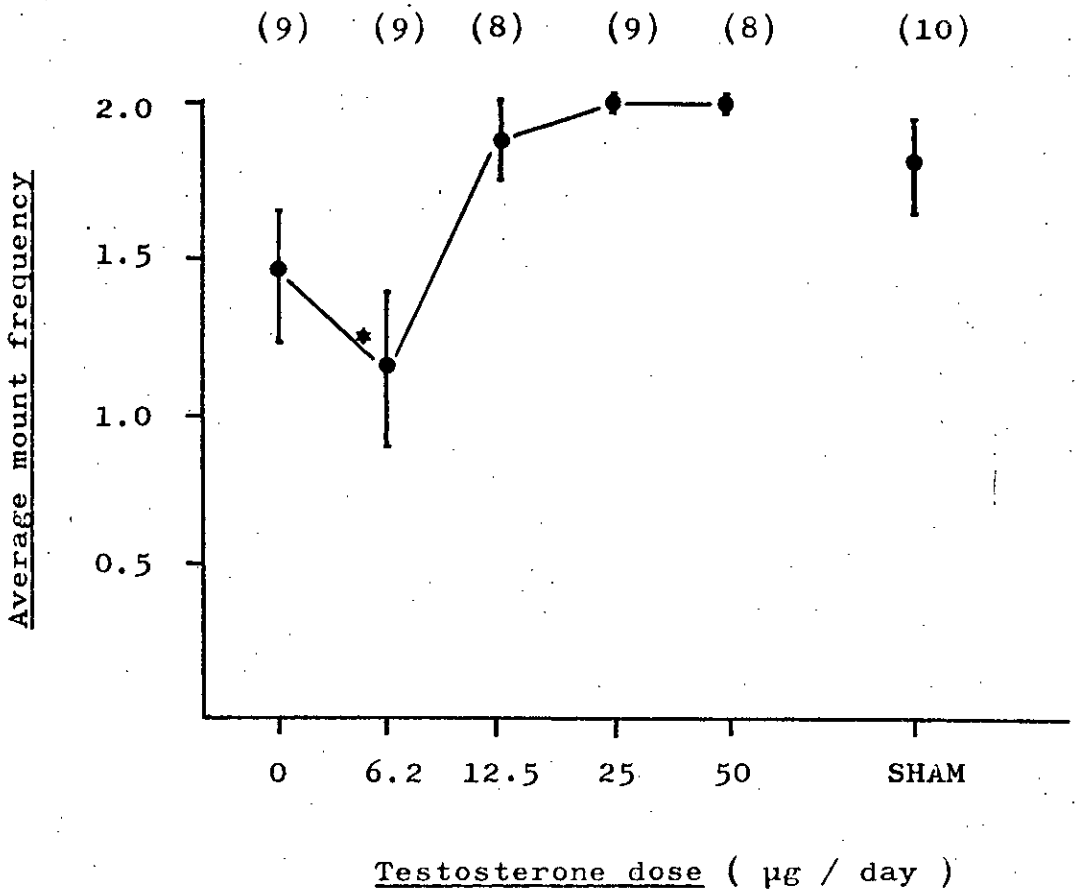
The numbers in ( ) = the number of mice in each group.

(* group significantly different from SHAM controls)

a) Balb/c strain



b) C57Bl/6Fa strain



c) BDF_1 hybrid

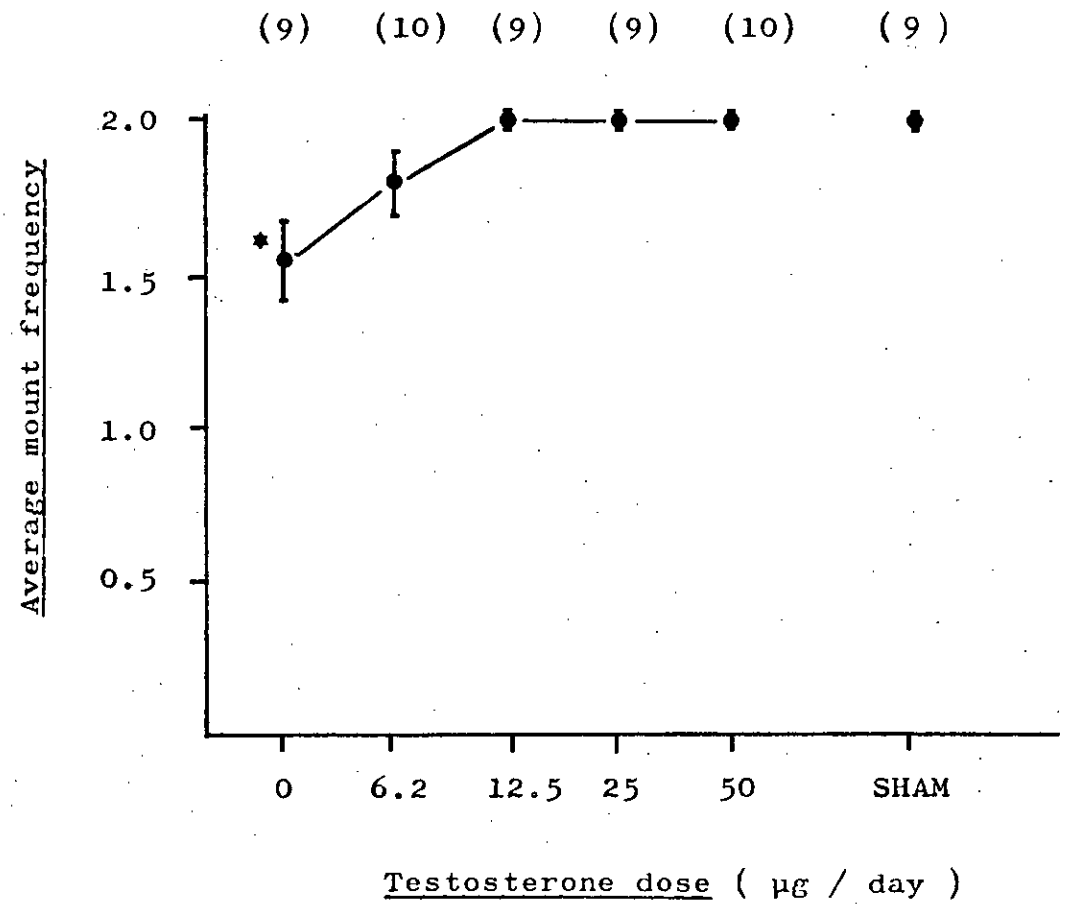


Figure 5.3

Average intromission frequency of castrate mice receiving different doses of Testosterone Propionate.

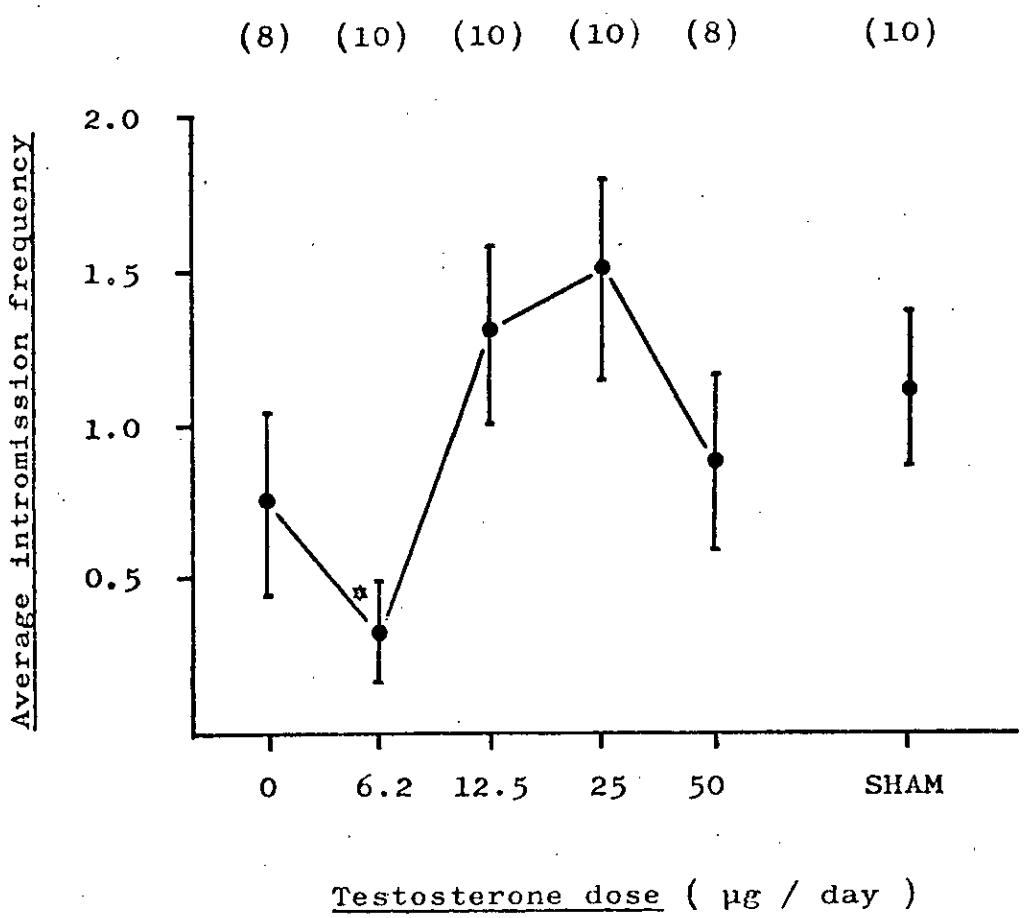
Mice were castrated on day 1 of the experiment, injected daily with TP and tested for sexual responses on days 5 & 9. The number of tests (0,1 or 2) in which each mouse intromitted at least once was determined and the group average expressed as mean $\pm$ Standard Error.

The 'SHAM' group were sham castrated and given daily injections of oil.

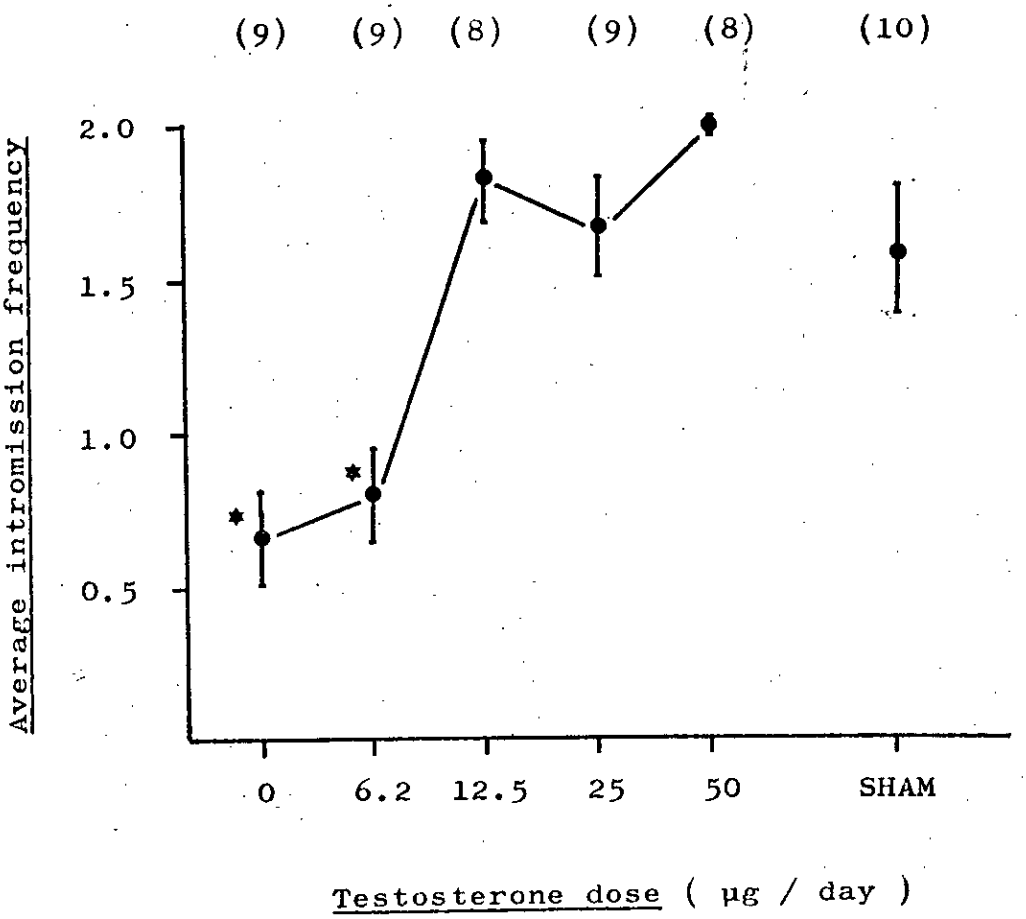
The numbers in ( ) = the number of animals in each group.

(* group significantly different from SHAM controls)

a) Balb/c strain



b) C57Bl/6Fa strain



c) BDF_1 hybrid

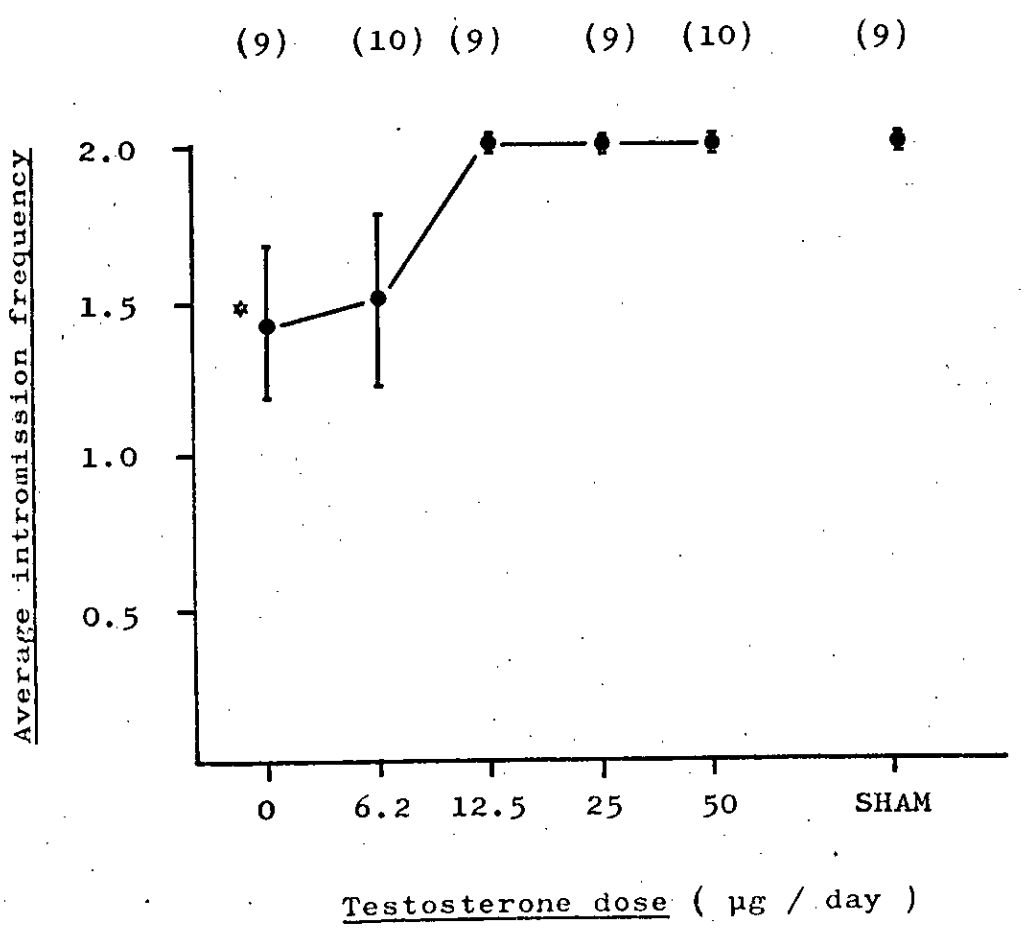


Figure 5.4

Average ejaculation frequency of castrate mice receiving different doses of Testosterone Propionate.

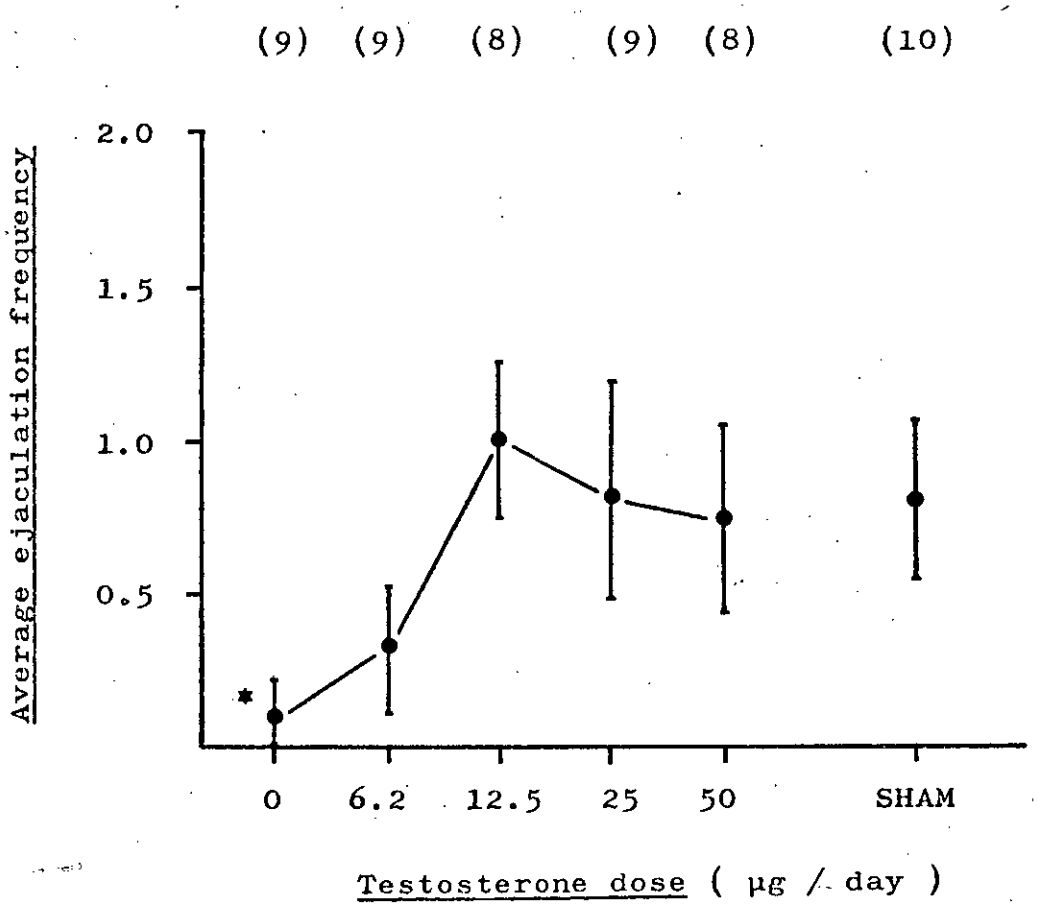
Mice were castrated on day 1 of the experiment, injected daily with TP and tested for sexual responses on days 5 &9. The number of tests (0,1 or 2) in which each mouse ejaculated was determined and the group average expressed as mean ± Standard Error.

The 'SHAM' group were sham castrated and given daily injections of oil.

The numbers in ( ) = the number of mice in each group.

(* group significantly different from SHAM controls)

a) C57Bl/6Fa strain



b) BDF_1 hybrid

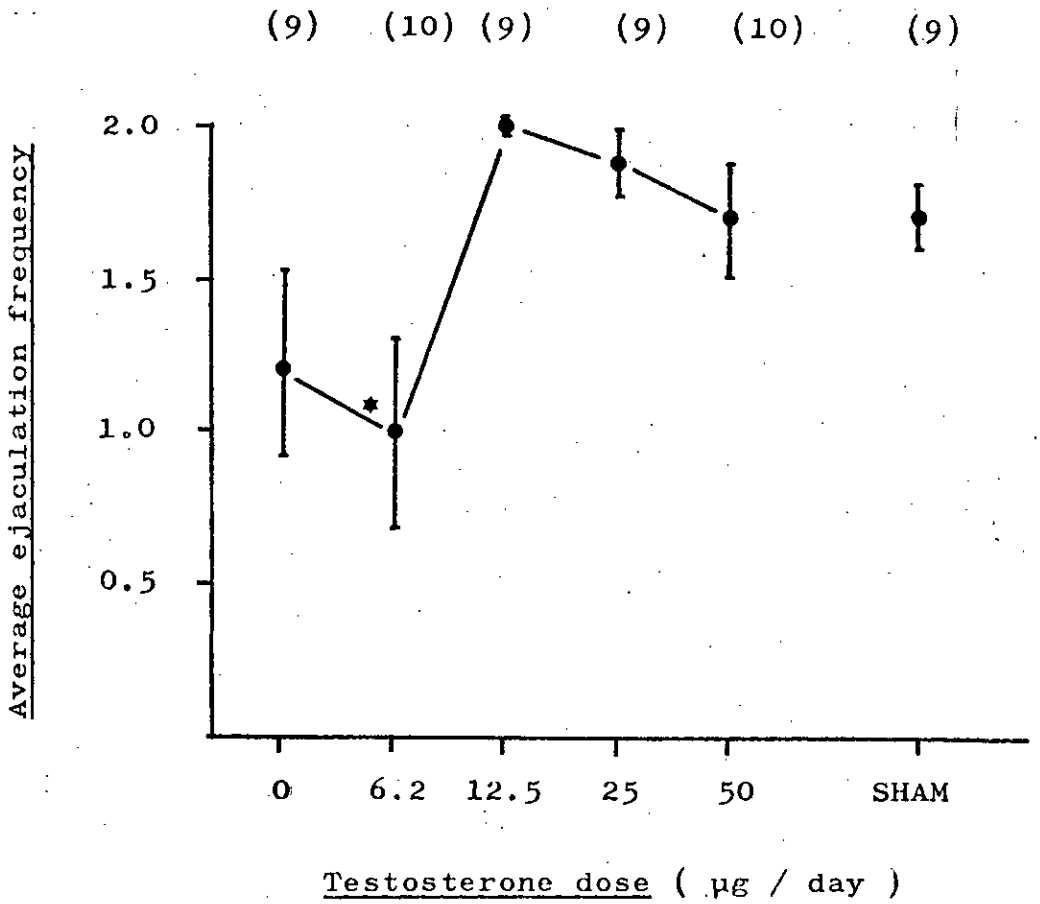


Table 5.3

Correlation coefficients and regression equations of mount, intromission and ejaculation frequencies and injected doses of Testosterone Propionate in castrate mice.

Strain	Correlation coefficient			Regression line	
	r =	df =	p <	a =	b =
				y = a + bx	
Balb/c					
Mount frequency	0.19	44	ns	-	-
$Intr^n$ frequency	0.16	44	ns	-	-
$Ejac^n$ frequency	-	-	-	-	-
C57Bl/6Fa					
Mount frequency	0.41	41	0.01	1.265	0.178
$Intr^n$ frequency	0.68	41	0.001	0.543	0.359
$Ejac^n$ frequency	0.37	41	0.05	0.123	0.200
BDF_1 hybrid					
Mount frequency	0.59	35	0.001	1.488	0.164
$Intr^n$ frequency	0.28	35	ns	-	-
$Ejac^n$ frequency	0.36	35	0.05	1.063	0.237

Behaviour frequencies were examined in animals receiving 0, 6.2, 12.5, 25, &50 µg TP / day in Balbs and C57s, and 0, 6.2, 12.5, &25 µg TP / day in BDF_1s. All correlations between behaviour frequencies and testosterone dose were carried out on data from individuals of each group. Testosterone doses were logarithmically transformed.

interpolated from the equations, and are shown in Table 5.4. Both strain TP doses were estimated at greater than 19 µg/day, with no significant difference between the two strains. Analysis of Variance of the Balb groups showed there to be no significant differences between TP-treated groups ($F = 1.34$ df = 4, 41).

Intromission frequency

There were significant differences between the intromission frequencies of the three strains in the 'sham' operant controls ($F = 4.36$ df = 2, 27 $p < 0.05$, with Balb/c showing the lowest IF - Student Newman Keuls $p < 0.05$), but not in the oil treated castrates ($F = 3.18$ df = 2, 23, p = ns), although the 'p' value approached significance. The three strains all showed a decreased intromission frequency in the castrate oil treated groups, although these only reached significance in the C57's ($t = 3.23$, df = 17 $p < 0.005$) and the BDF's ($t = 2.56$ df = 17 $p < 0.02$).

The correlation coefficients and regression equations between intromission frequency and TP dose are shown in Table 5.3, using transformations as before. The correlation was only significant for the C57's, and the dose of TP equivalent to the 'shams', shown in Table 5.4, was over 18 µg/day. Analysis of Variance of the Balb and BDF_1's showed there to be significant differences within the TP treated groups ($F = 3.35$

Table 5.4

Equivalent doses of Testosterone Propionate (TP µg / day) necessary to maintain the observed mount, intromission and ejaculation frequencies of sham castrated animals, as calculated from the regressions of behaviour frequencies of castrate mice on injected doses of Testosterone Propionat

Strain	Dose Testosterone Propionate / day		
	Mount Frequency	Intromiss^{n} Frequency	Ejaculat^{n} Frequency
Balb/c			
Ln ± SE (µg)	-	-	-
C57Bl/6Fa			
Ln ± SE (µg)	3.01 ± 0.75 (19)	2.94 ± 0.62 (18)	3.62 ± 0.69 (36)
BDF_1 hybrid			
Ln ± SE (µg)	3.12 ± 0.00 (22)	-	2.60 ± 0.69 (13)
Analysis of Variance	ns	-	ns

df = 4, 41 $p < 0.05$; $F = 2.66$ df = 4, 43 $p < 0.05$). Thus, in these strains there were dosage effects of TP, but they were not linear, or readily transformed to linear relationships. However inspection of the dose-response graph for BDF_1, shows that TP doses of 12.5 µg/day were sufficient to maintain the intromission frequency.

Ejaculation frequency

There were significant differences between the ejaculation frequencies of the three strains in the 'sham' operant controls ($F = 16.55$ df = 2, 27 $p < 0.01$) all strains were different from one another (Student Newman Keuls $p < 0.05$), and also strain differences in the oil treated castrates ($F = 7.36$, df = 2, 23, $p < 0.01$, with BDF_1 showing the highest EF $p < 0.05$ S.N.K.). The castrate-oil treated groups were not significantly different from the 'shams' in the Balb/c's and BDF_1's but EF was significantly reduced in the C57's ($t = 3.14$ df = 18 $p < 0.01$). The correlation coefficients and regression equations are shown in Table 5.3 - only C57's and BDF_1 showed significant correlations. The doses of TP equivalent to 'sham' control levels of ejaculation frequencies are shown in Table 5.4 - the levels for C57 was over 36 µg TP/day and for BDF_1 was over 12 µg TP/day, but these values were not significantly different.

Mount latency

The dose-response relationships between mount latency and TP are shown in Figure 5.5. There were significant differences between the strains in the 'sham' controls ($F = 5.32$ df = 2, 26 $p < 0.05$) and in the oil treated castrates ($F = 8.54$ df = 2, 20, $p < 0.01$). The three strains all showed an increased mount latency in oil treated castrate groups, but these were significant only in the BDF_1's ($t = 2.14$ df = 16 $p < 0.05$) and the Balb's ($t = 2.17$ df = 14 $p < 0.05$).

The correlation coefficients and regression equations are shown in Table 5.5. Mount latency was significantly correlated in all three strains, using the transformations indicated in the table, and the TP doses equivalent to the mount latency shown by the 'shams' was determined from the regression lines, and are shown in Table 5.6. All strains showed levels of over 17 µg TP/day, and BDF_1 showed the highest at 39 µg TP/day - but there were no significant differences between the strains ('F' = 0.17 df = 2, 26, p = ns).

Intromission latency

The dose response curves between intromission latency and dose of TP are shown in Figure 5.6. There were no significant differences in the 'sham' groups between the strains ($F = 2.05$ df = 2, 22, p = ns). In the oil treated castrate groups there were no significant differences ($F = 1.58$ df = 2, 15, p = ns).

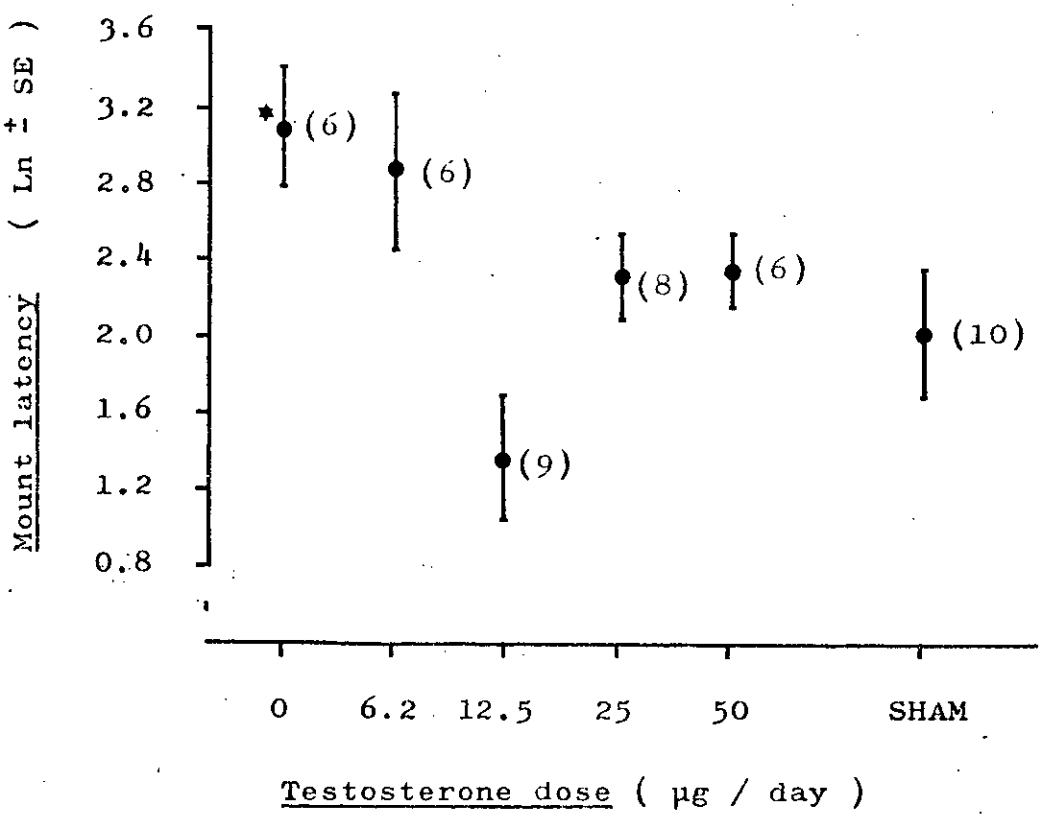
Figure 5.5

Mean mount latency of castrate mice receiving different doses of Testosterone Propionate (TP).

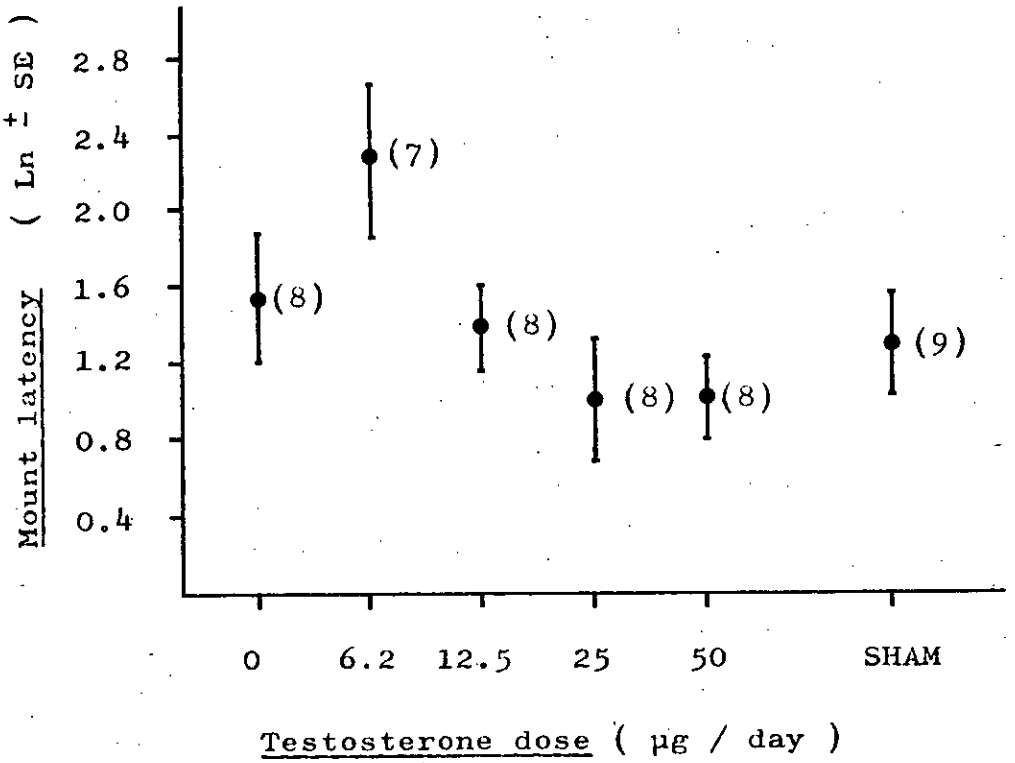
Mice were castrated on day 1 of the experiment, injected daily with TP and tested for sexual responses on days 5 and 9. Latencies for each mouse were averaged over both tests, and the group average determined using the data logarithmically transformed. The means are shown as Ln ± SE, and the numbers in ( ) show the number of animals mounting at least once over the two tests.

(* group significantly different from SHAM controls)

a) Balb/c strain



b) C57Bl/6Fa strain



c) BDF_1 hybrid

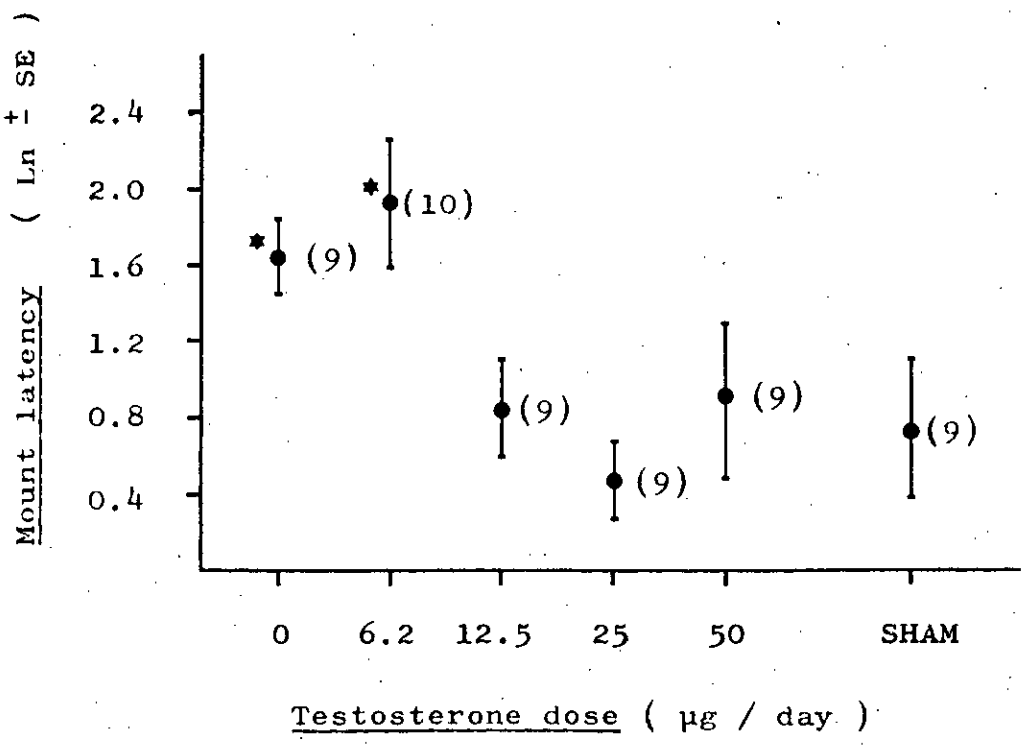


Table 5.5

Correlation coefficiants and regression equations of mount intromission and ejaculation latencies and injected doses of Testosterone Propionate in castrate mice.

Strain	Correlation coefficiant			Regression line	
	r =	df =	p <	a = y = a + bx	b =
Balb/c					
Mount latency	-0.56	33	0.001	26.851	-5.152
Intr^{n} latency	-0.41	25	0.05	3.102	-0.229
Ejac^{n} latency	-0.21	7	ns	-	-
C57Bl/6Fa					
Mount latency	-0.33	37	0.05	1.753	-0.197
Intr^{n} latency	-0.34	34	0.05	14.986	-2.362
Ejac^{n} latency	0.39	17	ns	-	-
BDF_1 hybrid					
Mount latency	-0.61	54	0.001	2.302	-0.425
Intr^{n} latency	-0.55	41	0.001	2.118	-0.323
Ejac^{n} latency	0.10	39	ns	-	-

Behaviour latencies were examined in animals receiving 0, 6.2, 12.5, 25 and 50 µg TP / day. All correlations between latencies and testosterone dose were carried out on data from individuals of each group. Testosterone doses were logarithmically transformed, as was the mount latency of C57 and BDF_1, and the intromission latency of BDF_1. The remaining latencies were not transfomed.

Table 5.6

Equivalent doses of Testosterone Propionate (TP µg / day) necessary to maintain the observed mount and intromission latencies of sham castrated animals, as calculated from the regressions of the latencies of castrate mice on injected doses of Testosterone Propionate.

Strain	Dose Testosterone Propionate / day	
	Mount Latency	Intromission Latency
Balb/c		
Ln ± SE (µg)	2.88 ± 0.75 (17)	4.79 ± 0.58 (119)
C57Bl/6Fa		
Ln ± SE (µg)	3.11 ± 0.49 (21)	3.64 ± 0.78 (37)
BDF_1 hybrid		
Ln ± SE (µg)	3.68 ± 0.69 (39)	2.86 ± 0.80 (17)
Analysis of Variance	F = 0.17 df = 2,26 p ns	F = 1.15 df = 2,22 p ns

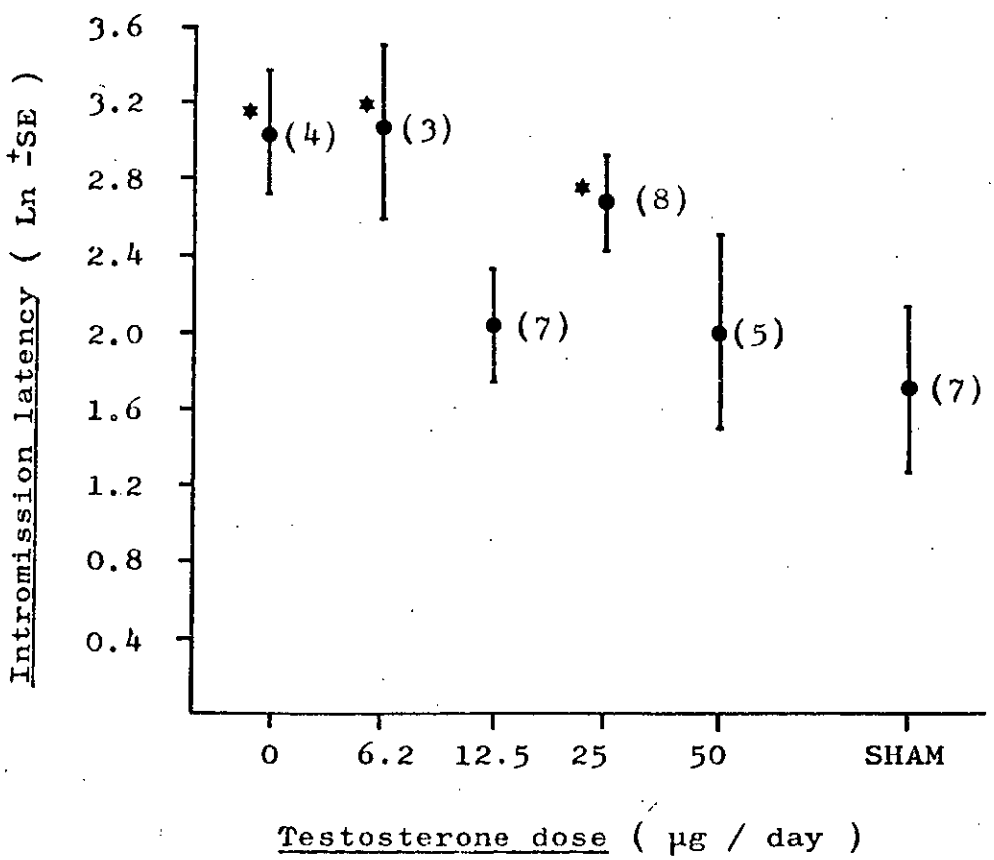
Figure 5.6

Mean intromission latency of castrate mice receiving different doses of Testosterone Propionate (TP).

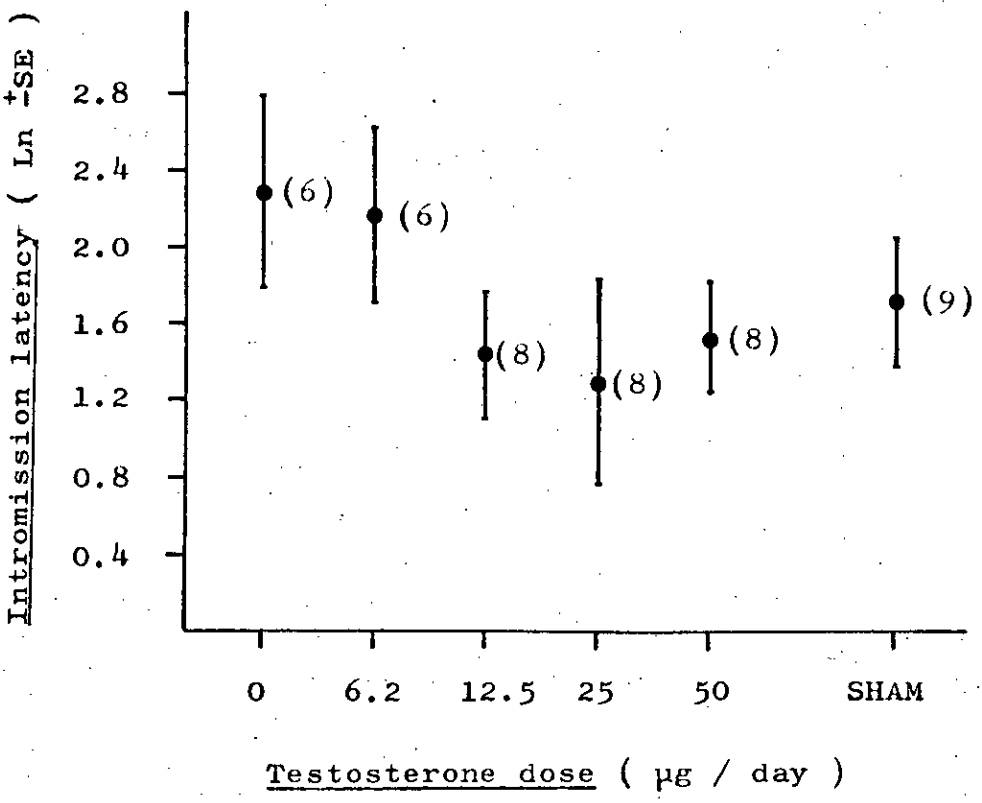
Mice were castrated on day 1 of the experiment, injected daily with TP and tested for sexual responses on days 5 & 9. Latencies for each mouse were averaged over both tests, and the group average determined using the data logarithmically transformed. The means are shown as Ln ± SE (minutes), and the numbers in ( ) show the number of animals intromitting at least once over the two tests.

(* group significantly different from SHAM controls)

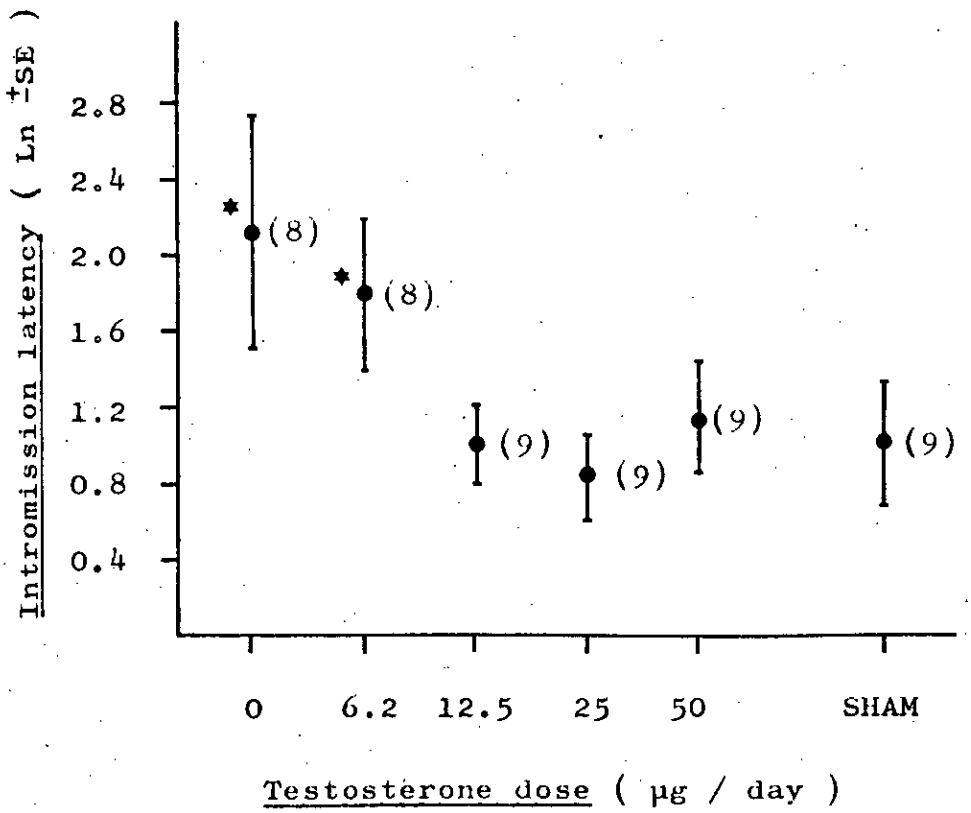
a) Balb/c strain



b) C57Bl/6Fa strain



c) BDF_1 hybrid



The three strains all showed an increased intromission latency in the oil treated castrate groups, but these were only significant in the Balb's ('t' = 2.42 df = 9 $p < 0.05$) and the BDF_1's ('t' = 2.78 df = 15 $p < 0.02$).

The correlation coefficients and regression equations are shown in Table 5.5, intromission latency and TP dose were correlated in all the strains, using the transformations as indicated. The doses of TP equivalent to 'sham' levels of intromission latency are shown in Table 5.6 - all strain TP doses were calculated as being greater than 17 µg/day, with Balb/c showing the greatest level at 119 µg - but these were not however significantly different ($F = 1.15$ df = 2, 22, p = ns).

Ejaculation latency

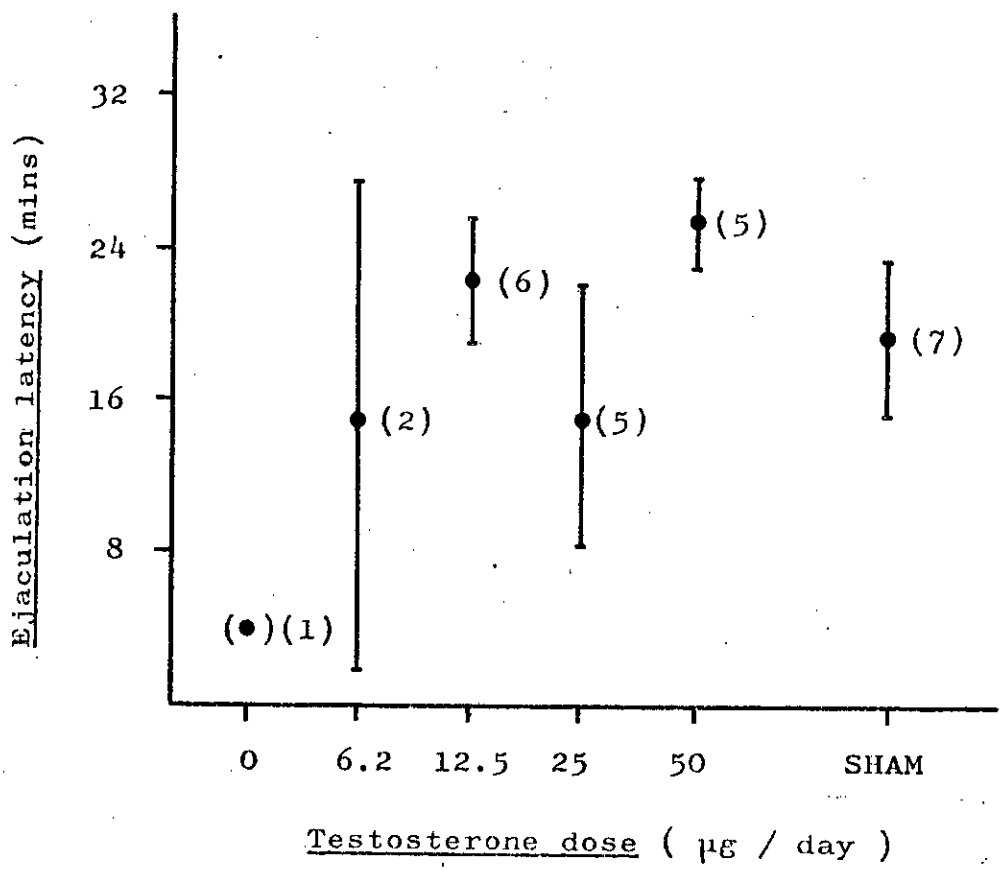
The dose-response curves between ejaculation latency and dose of TP are shown in Figure 5.7. No data has been shown for the Balb's, since only 11 ejaculations were seen in all the groups, and these appear to have been randomly distributed throughout the groups. Analysis of Variance of the C57 and BDF_1 'sham' controls showed there to be no significant differences between the strains ($F = 1.51$ df = 1, 17, p = ns). Comparison in the castrate group was not possible since only one C57 ejaculated. The castrate BDF_1's showed no significant difference in ejaculation latency from the sham group ('t' = 1.31 df = 13 p = ns).

Figure 5.7

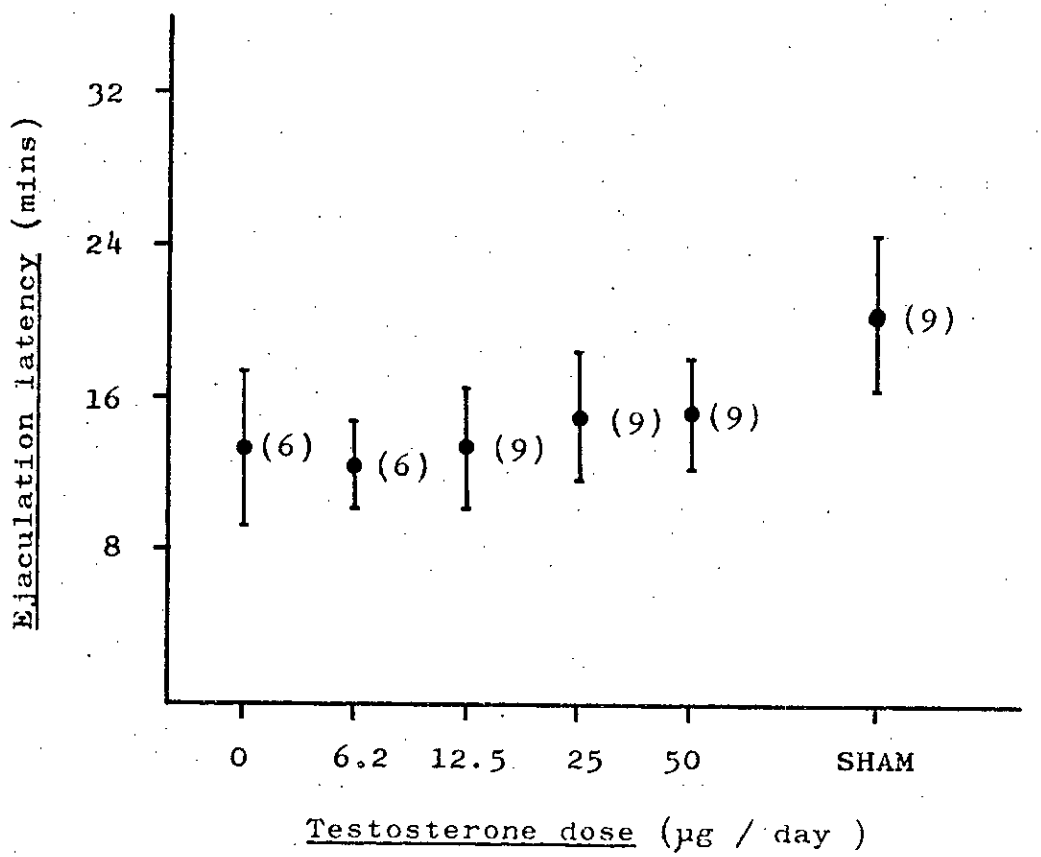
Mean ejaculation latency of castrate mice receiving different doses of Testosterone Propionate (TP).

Mice were castrated on day 1 of the experiment, injected daily with TP and tested for sexual responses on days 5 & 9. Latencies for each mouse were averaged over both tests, and the group average determined and expressed as mean ± Standard Error. The numbers in ( ) show the number of animals intromitting at least once over the two tests.

a) C57Bl/6Fa strain.



b) BDF_1 hybrid



The correlation coefficients between ejaculation latency and dose of TP are shown in Table 5.5 - none of the correlations were significant. Analysis of Variance showed there to be no significant differences between the TP-treated groups in any of the strains. (Balb-'F' = 2.89 df = 1, 6; C57-'F' = 1.51 df = 4, 14; BDF_1-'F' = 0.09 df = 3, 26.)

5.3.3. Comparison of TP doses necessary to maintain seminal vesicle weights and those necessary for maintenance of measures of sexual behaviour.

The doses of TP calculated as being necessary to maintain seminal vesicle weights, and those necessary to maintain measures of sexual behaviour were compared within each strain using the Student 't' test. Where variances between groups were significantly different, the degrees of freedom used in estimating the probability value were halved, as described in Snedecor and Cochran (1967).

Mount frequency

In both strains in which the comparisons were possible (C57 and BDF_1) both TP values for mount frequency maintenance were greater than for seminal vesicle weight, but the difference reached significance

in only the BDF_1's; (C57 - 't' = 1.14 df = 18, BDF_1 - 't' = 4.18 df = 8 $p < 0.01$). Since the C57's and BDF_1's did not show significant differences in the TP doses required to maintain the seminal vesicles, or mount frequency,- the data for the two strains was pooled - and the overall comparison between doses for seminal vesicles and behaviour compared. The dose of TP for maintenance of behaviour was then found to be significantly greater than that required for seminal vesicle maintenance (TP for seminal vesicles = 2.20 $\pm$ 0.11, TP for mount frequency = 3.06 $\pm$ 0.36; 't' = 2.21 df = 37 $p < 0.05$).

Intromission frequency

The comparison between TP doses equivalent to seminal vesicle weight and intromission frequency was only possible in the C57 - where the equivalent dose for intromission frequency was greater than that for seminal vesicle weight, but this did not reach significance (t = 0.93 df = 18 p = ns).

Ejaculation frequency

In both strains where the comparison was possible (C57 and BDF_1), the doses of TP which would maintain ejaculation frequency were greater than those for the seminal vesicles, but these failed to reach significance. The data was not pooled between the strains because the ejaculation frequencies of the 'shams' were significantly different.

Mount latency

Comparisons of TP maintenance doses for seminal vesicle weight and mount latency were possible for all three strains. In Balb/c the equivalent dose for mount latency was less than that for seminal vesicle weight, but not significantly so. In C57's and BDF_1's the doses of TP were greater than for seminal vesicle weight, but these did not reach significance ($t = 1.29$ $df = 18$, $t = 1.77$ $df = 16$). However pooling the data from the two strains resulted in the maintenance dose of TP for mount latency being significantly greater than for seminal vesicle weight (TP for seminal vesicles = 2.20 ± 0.11, TP for mount latency = 3.25 ± 0.45; $t = 2.24$, $df = 18$ $p < 0.05$).

Intromission latency

Comparison of TP maintenance doses for seminal vesicle weight and intromission latency were possible for all three strains. In Balb/c, the equivalent TP dose for intromission latency was significantly greater than that for seminal vesicle weight maintenance ($t = 2.55$ $df = 7$, $p < 0.05$). In C57's and BDF_1's doses for intromission latency maintenance were greater than for seminal vesicle weight maintenance, but these were not significant ($t = 1.08$ $df = 17$, $t = 1.31$ $df = 16$). However, the pooled data from the two strains did reach significance (TP for seminal vesicles = 2.17 ± 0.11, TP for intromission latency = 3.48 ± 0.57; $t = 2.24$ $df = 17$ $p < 0.05$).

Ejaculation latency

No comparisons were possible, since the doses of injected TP were not significantly correlated with ejaculation latency in any of the strains.

5.4. Discussion

To summarise the results, it was found that in all strains seminal vesicle weight and measures of sexual behaviour were reduced after castration, but could be maintained at 'normal' levels with injections of TP. The effective doses of TP for the maintenance of seminal vesicle weights at control levels were significantly different between the strains, but those required to maintain sexual behaviour were not. In all strains, the doses of TP required for seminal vesicle weight maintenance were lower than those required to maintain measures of sexual behaviour.

The maintenance doses of TP for seminal vesicle weight were significantly different between the strains - Balb's needed over 30 µg/day, whereas C57's and BDF_1's needed less than 10 µg/day. It may be that this difference is partially an artefact resulting from the differential rates of decline of the seminal vesicles in the absence of androgens between the strains. The Balb's showed the largest fall in seminal vesicle weights after castration, and it may be that the inability of comparatively low TP doses to maintain seminal vesicle weights may have been because these structures regress more rapidly in this strain than

the other two. One way of overcoming this problem might be to examine the dose response relationships of the doses of TP necessary to restore the seminal vesicles after they have fully declined. However this approach also has problems, since the doses of TP required to restore any hormone dependent process following castration are almost always higher than the doses necessary to maintain the process (Davidson and Levine, 1972). This suggests, as Davidson et al (1970) point out, that tissues which respond to androgens may show some form of regression following the removal of the appropriate stimulating hormone, and therefore the 'restoration' dose of a hormone may bear little relation to the 'maintenance' dose. However, Bartke (1974) has shown that there are strain differences in the doses of TP which will restore seminal vesicle weights in DBA/2J and C57Bl/10J mice. He castrated mice from both strains, and two weeks later started replacement injections of TP which continued for two further weeks. Animals were injected on alternate days, and then seminal vesicles removed and weighed. The restoration dose for DBA/2J was 118 µg TP (equal to 59 µg TP/day), and for C57Bl/10J was 58 µg TP (equal to 29 µg TP/day). These values are higher than the values reported in the present experiment, and may reflect further strain differences, or more likely

the phenomenon of increased 'restoration' doses as described for the rat (Davidson et al, 1970).

Measures of sexual behaviour were reduced after castration, with some strains showing greater decrements in behaviour than others. There were decreases in mount (Balb, BDF_1), intromission (C57, BDF_1) and ejaculation (C57) frequencies; and increases in mount (Balb, BDF_1) and intromission (Balb, BDF_1) latencies. There were however, no significant changes in ejaculation latency. Considering the behavioural results as a whole, it was apparent that the Balb/c strain was most affected by castration, followed by the C57's, with BDF_1 showing the greatest retention of sexual behaviour. This result agrees with that of McGill and Manning (1976) who found the same rank order of behavioural retention in the strains. However, Manning and Thompson (1976) found that mount and intromission latencies of the BDF_1 strain were unaffected by castration, whereas in this experiment they were found to be increased. This difference is probably due to the fact that Manning and Thompson compared post-castrational performance with pre-castrational performance, and also gave animals more pre-castration sexual experience. They also found an increase in ejaculation latency, which was not found in any of the strains described here, but again comparisons were made between pre-

and post- castrational behaviour, and animals were sexually more experienced. By the time of castration they recorded ejaculation latencies of less than 5 minutes - considerably lower than any of the latencies recorded here. They observed animals for a longer period after castration, and in long-term castrates an increasing ejaculation latency might be expected, since there is evidence to show that peripheral structures mediating peripheral sensory feedback decline after castration. For example, in the rat, Beach and Levinson (1950) have shown that after castration there is a reduction in the sensory papillae of the penis, resulting in reduced stimulation during intromission.

All of the post-castrational changes in sexual behaviour recorded in this experiment could be prevented by doses of TP, and there was a dose-response relationship between TP and most measures of sexual behaviour. In no case however could the sexual behaviour be increased beyond the levels of sexual behaviour shown by oil-injected sham operated controls.

There were significant correlations between dose of TP and mount (C57, BDF_1), intromission (C57) and ejaculation (C57, BDF_1) frequencies; and in all the strains for mount and intromission latencies. These results are in agreement with data for the rat (Beach and Holz-Tucker, 1949). However, in the rat

it was also found that the dose of TP required to maintain ejaculation frequency was greater than that required for maintenance of mount and intromission frequency, a result not found in the present experiment. Analysis of Variance of doses of TP required to maintain each measure of sexual behaviour were not significantly different in any of the strains. This difference in TP requirements between the rat and mouse probably relates to differences in the pattern of sexual behaviour - a male rat ejaculates many times in one short test session, whereas a mouse ejaculates only once and thereafter may show no more sexual responses for periods of hours or days (McGill and Blight, 1963b).

The failure to demonstrate an increased level of sexual performance in TP-injected animals also contrasts with data from the rat. Beach and Holz-Tucker (1949) reported that doses of TP greater than 50 µg/day decreased mount latency and increased intromission frequency beyond levels recorded in pre-castration tests. No such effects were found in this experiment, and as pointed out earlier, the increased level of behaviour found in the rat may be a product of increased experience. Champlin, Blight and McGill (1963) also report that in the CDF_1 mouse (a hybrid between Balb/c females and DBA/2J males)

doses of 32 µg TP/day were sufficient to maintain normal sexual behaviour, but that increasing the TP dose to over 1 mg/day had no further effects.

In all of the strains, the doses of TP necessary to maintain measures of sexual behaviour were greater than the doses for maintenance of the seminal vesicles. This is in direct contrast to the rat, where the seminal vesicles required more TP than did behaviour (Davidson et al, 1970). If the doses of TP which maintain seminal vesicle weights at control levels are considered as being equivalent to normal circulating androgen levels, then for the mouse the 'basal' androgen levels may be lower than those necessary for behaviour. This deficit could provide a functional explanation for the acute changes in plasma testosterone, which occur immediately prior to copulation, as described in Chapter 4.

In the introduction, I posed three questions which, it was hoped, would be answered by this experiment. The first was whether the 'basal' plasma testosterone levels, as measured in Experiment 1, reflected the behavioural needs of the strains. That is, whether the 'high' plasma testosterone Balb/c strain 'needed' more testosterone than the 'low' plasma testosterone C57 and BDF_1 strains. Apparently they do not for there were no significant differences between the strains

in the doses of TP required to maintain sexual behaviour. Further, it was necessary to give higher doses of TP to maintain behaviour than to maintain seminal vesicle weights, suggesting that in these three strains 'basal' plasma testosterone levels are below levels required for sexual behaviour. The second question posed was whether there were strain differences in responsiveness to TP as measured by the maintenance of the seminal vesicles. The results suggested that the C57's and BDF_1's were more responsive than the Balb's - but as previously discussed there were some problems of interpreting the results. Question 3 asked whether dose-response relationships could be demonstrated between TP and measures of sexual behaviour, and whether behaviour could be boosted beyond 'normal' levels. There were clear dose-response relationships for all measures of sexual behaviour, apart from ejaculation latency; but behaviour could not be increased above control levels.

How do these results relate to the findings of the previous experiments? In Chapter 3, it was demonstrated that there was a negative correlation between plasma testosterone levels and measures of sexual behaviour. It was suggested that this negative relationship may have been the result of different sensitivities to testosterone, of both the

hypothalamo-pituitary axis, and of brain areas mediating sexual behaviour. However, the results from the present experiment suggest this is not the case - although no estimations of the hypothalamo-pituitary axis responsiveness to testosterone were made, the estimations of behavioural responsiveness to androgens showed there were no strain differences. Thus sexual behaviour from the high 'basal' testosterone Balb/c strain can be maintained with the same doses of TP as the low 'basal' testosterone C57 and BDF_1 strains.

Chapter 4 examined 'acute' changes in testosterone, and found that testosterone levels increased prior to and during copulation. It was suggested that these increases in plasma testosterone might be important in raising responsiveness and indeed there was found to be a correlation between plasma testosterone and the mount latency.

We might expect to find then, as here, that there would be a dose-response relationship between injected doses of TP and mount latency. In Chapter 4, it was also found that acute changes in testosterone levels were not correlated with ejaculation latency, and this independence from circulating testosterone was confirmed in this experiment where there were no significant differences in ejaculation latency between the TP treated groups within each of the strains.

CHAPTER SIX

THE INFLUENCE OF NEONATAL INJECTIONS OF TESTOSTERONE PROPIONATE ON SEXUAL BEHAVIOUR AND PLASMA TESTOSTERONE LEVELS

6.1. INTRODUCTION

The experiments described in the preceeding Chapters have concentrated on an examination of the role of plasma testosterone levels in regulating aspects of the sexual behaviour of adult males. However, we now know that the effects of testosterone are not restricted to the adult, and that during perinatal periods of development testosterone has permanent 'organisational' effects on morphology, physiology and behaviour (Ward, 1974). Much of this field of research has been concerned with the control of the differentiation of masculine and feminine potentials, and it has concentrated on the effects of perinatal androgens on genetic females. From such work it is clear that there are 'critical periods' during which androgens interact with a succession of responsive tissues, resulting in either further growth and development or a regression. For example, as reviewed by De Moor, Verhoeven and Heyns (1973), in the rat, there are clusters of androgen related developmental events. For example, the first 'urogenital cluster' occurs prenatally, and during this period androgens are necessary for the development of the Wolffian ducts, urogenital sinus and scrotal structures, and a second 'brain cluster' occurs neonatally, when androgens determine the gonadotrophin release pattern, and also aspects of

sexual behaviour. Many mammalian structures are initially undetermined - and can develop in either male or female directions, the controlling factor being the presence or absence of androgens at particular developmental stages.

If androgens can have such pronounced effects on sexual differentiation, it might be expected that manipulations of androgens in the developing male would have effects on the sexual behaviour of the adult. If the administration of androgen to genetic females can increase the probability of male-like sexual behaviour, would the manipulation of androgen levels in males during the appropriate developmental period result in quantitative differences in male behaviour?

In the rat, the major testicular and plasma androgen during the neonatal period is testosterone (Resko, Feder and Goy, 1968), suggesting that this is the hormone involved in these developmental processes. If male rats are castrated, before or on the fifth day after birth, they show decrements in sexual behaviour when given replacement testosterone as adults. Although mounting and intromission responses occur, the ejaculation response is absent (Hart, 1968). If castration is delayed beyond day 13 however, males

respond completely normally when given testosterone replacement therapy (Larsson, 1967). Attempts to increase the incidence of male sexual behaviour, by neonatal injections of testosterone in the rat, have not however been successful, and such procedures have no effects on the proportions of animals mounting and intromitting (Whalen, 1964) or ejaculating (Feder, 1967).

To my knowledge, the influence of neonatal castration on the development of male sexual behaviour in the house mouse has not been investigated. There have however been some studies of such treatment on aggressive behaviour. Peters, Bronson and Whitsett (1973) castrated C57Bl/6 mice at different ages after birth, and found that castration before day 6 significantly reduced the incidence of fighting in testosterone treated adults. As with the rat, the androgen necessary during this period is most likely to be testosterone, since this is the major testicular androgen during the neonatal period (Berger et al, 1975), and testosterone replacement injections will prevent this decrease in aggressive behaviour of neonatally castrated mice (Bronson and Desjardins, 1969).

Attempts to influence adult sexual behaviour by supplementing neonatal androgens with injections of testosterone into intact-male mice have however been

described, and two studies have shown that there are significant effects. Work by Campbell and McGill (1970) demonstrated that in the DBA/2J strain, injections of 100 µg testosterone propionate between 2-20 hours after birth resulted in the adults showing ejaculatory responses at an earlier age than oil treated controls. There were however no qualitative differences in the mating pattern of these animals. A study by Vale, Ray and Vale (1974) examined a further three strains of mice and found significant increases in sexual behaviour in animals injected on day 3 with a large dose (1 mg) of testosterone propionate. Males from the Balb/c strain showed an increase in the proportion of animals mounting, and C57Bl/6 males showed an increase in the proportion of animals ejaculating. The third strain 'A' showed no differences between control and experimental groups.

Thus, in contrast to the rat, neonatal injections of testosterone given to intact male mice can have effects on the display of sexual behaviour when the animals become adult. The mechanisms of these effects are obscure but may be related to the observations of Bronson and Desjardins (1968), and Vale, Ray and Vale (1974) that neonatally androgenized male mice have smaller testis and seminal vesicle weights than oil injected controls. This suggests that there may be

permanent changes in the basal levels of hormones secreted by these animals. In Experiment 1, it was demonstrated that across strains of mice, it is those strains with low plasma testosterone which show most sexual behaviour. It may be that the decrease in testis weights found in androgenised male mice results in a decreased androgen secretion which may in some way be related to the increased levels of sexual behaviour.

These speculations lead us to the hypothesis that variations in androgen levels in early life may modify both adult testosterone secretion and sexual behaviour, and may account for the striking strain differences in hormone levels and sexual behaviour reported earlier in this thesis. In order to test this hypothesis, an attempt was made to 'standardise' the neonatal levels of circulating testosterone, by injecting animals from several strains with a single dose of testosterone propionate on day 4 after birth. If the hypothesis is correct, then we expect there to be a subsequent convergence of hormonal and behavioural parameters, such that for example, an increased level of sexual behaviour might be associated with decreased plasma testosterone levels.

6.2. METHOD

The four genotypes used in the experiment were the 'low' responding Balb/c and DBA/2J strains, the 'medium' responding C57Bl/6Fa and the 'high' responding BDF_1 hybrid. Breeding pairs, from stock bred in the Department of Zoology, University of Edinburgh, were established and whole litters injected with either hormone or oily vehicle. At first, attempts were made to inject litters on day 1, but due to the very high mortality rate, this was changed to day 4 (birth = day 1). Whole litters of the strains were subcutaneously injected with 100 µg testosterone propionate (Sigma Ltd. - Δ^4 - Androsten-17 - ol-3-one-propionate, dissolved in 0.02 ml Arachis oil:Benzyl benzoate 95:5, v:v) and control litters were injected with the vehicle only. At 4 weeks, the litters were weaned and the males housed in groups of 2-6 until they were aged 8-9 weeks when they were isolated into small cages. The sexual behaviour tests were begun at 10 weeks - 5 tests were given using the procedure previously described, except that the receptive BDF_1 female was introduced in to the home cage of the male.

At 13 weeks of age, the mice were weighed and blood samples taken for determination of plasma testosterone. The testes and seminal vesicles (plus the coagulating gland and expressed of fluid contents) were also removed and weighed.

6.3. RESULTS

In the C57's and BDF_1's all animals in the TP and vehicle treated groups showed mount, intromission and ejaculation responses at least once in the 5 tests. In the DBA strain all testosterone treated animals showed mount, intromission and ejaculation responses, but in the vehicle injected animals, one failed to show intromission, and two did not ejaculate. In the Balb's, although all animals showed mount and intromission responses, in the TP treated group 10 of the 12 mice ejaculated at least once, but only 5 of the 12 control mice did so (Fisher Exact test $p < 0.05$). The mount, intromission and ejaculation frequencies, and mount and ejaculation latencies are shown in Table 6.1. The behaviour frequencies were calculated from the number of tests in which the behaviours were shown for each mouse, and the average over the 5 tests calculated and expressed as a percentage. The mount and ejaculation latencies were calculated similarly, but using data from tests 2-5 only, and using the logarithmic transform for mount latency data. In all the strains the TP treated groups showed a greater than or equal frequency of mount, intromission and ejaculation responses, compared to controls, but these differences were not significant, within any of the

Table 6.1

Sexual behaviour measures of four strains of mice treated with 100 µg Testosterone Propionate on day 4 after birth.

a) Balb/c strain

Sexual behaviour (± SE)	Treatment	
	vehicle	TP
Percent.tests/mouse with:	(N = 12)	(N = 12)
Mounting	78 ± 6	84 ± 6
Intromission	52 ± 6	68 ± 10
Ejaculation	16 ± 6	34 ± 8
Mount latency (Ln. ± SE)	2.2 ± 0.2	2.1 ± 0.2
(minutes)	(9.0)	(7.9)
Ejaculation latency(minutes)	24.3 ± 3.5	24.7 ± 4.3

b) C57Bl/6 strain

Sexual behaviour (± SE)	Treatment	
	vehicle	TP
Percent.tests/mouse with:	(N = 11)	(N = 11)
Mounting	94 ± 2	100 ± -
Intromision	92 ± 2	100 ± -
Ejaculation	78 ± 6	84 ± 6
Mount latency (Ln. ± SE)	1.0 ± 0.2	0.3 ± 0.1
(minutes)	(2.8)	(1.4)
Ejaculation latency(minutes)	15.3 ± 1.4	15.3 ± 1.6

c) BDF_1 strain

Sexual behaviour (± SE)	Treatment	
	vehicle	TP
Percent.tests/mouse with:	(N = 11)	(N = 12)
Mounting	100 ± -	100 ± -
Intromission	100 ± -	100 ± -
Ejaculation	84 ± 4	84 ± 4
Mount latency (Ln. ± SE)	0.1 ± 0.1	0.3 ± 0.2
(minutes)	(1.1)	(1.3)
Ejaculation latency(minutes)	7.1 ± 1.4	10.4 ± 1.6

d) DBA/2J strain

Sexual behaviour (± SE)	Treatment	
	vehicle	TP
Percent.tests/mouse with:	(N = 6)	(N = 6)
Mounting	76 ± 10	94 ± 6
Intromission	66 ± 12	94 ± 6
Ejaculation	20 ± 8	50 ± 12
Mount latency (Ln ± SE)	2.0 ± 0.3	1.5 ± 0.2
(minutes)	(7.2)	(4.6)
Ejaculation latency(minutes)	23.8 ± 4.2	17.2 ± 4.1

strains. However, across the strains, the TP treated animals showed a significant increase in behaviour frequency (Sign Test N = 9 x = 0 $p < 0.02$).

There were no significant differences in the mount latencies between TP treated and control animals in the Balb's, DBA's and BDF_1's, but the C57 strain showed a significantly reduced latency in the TP treated group (Student 't' test 't' = 3.2 df = 9 $p < 0.01$) corrected for heterogeneity of variance. There were no significant differences in ejaculation latency between TP treated and control animals in any of the strains.

The body, testis and seminal vesicle weights for the four strains are shown in Table 6.2. Organ weights were all corrected to a standard body weight of 20g to enable comparisons within and between strains to be made. In the C57's, body weight was significantly increased in the treated group (Student 't' test 't' = 2.01, df = 20 $p < 0.05$), but there was no change in body weight in the other strains. In all strains, the testis weights showed a significant decrease in the TP treated groups compared to controls (Student 't' test: Balb/c 't' = 2.6 df = 22 $p < 0.02$; DBA/2 't' = 2.15 df = 10 $p < 0.05$; C57Bl/6Fa 't' = 3.9, df = 20 $p < 0.001$; BDF_1 't' = 6.0 df = 21 $p < 0.001$).

Table 6.2

Body, testis and seminal vesicle weights of four strains of mice treated with 100 µg Testosterone Propionate (TP) on day 4 after birth.

(Testis and seminal vesicles all corrected to 20 g body weight.)

a) Balb/c strain

Weights ± SE	Treatment	
	vehicle	TP
	N = 12	N = 12
Body (g)	23.6 ± 0.6	23.5 ± 0.5
Testis (mg)	154 ± 4	127 ± 9
Seminal vesicles (mg)	61 ± 3	65 ± 2

b) C57Bl/6 strain

Weights ± SE	Treatment	
	vehicle	TP
	N = 11	N = 11
Body (g)	21.0 ± 1.9	24.1 ± 0.6
Testis (mg)	156 ± 3	134 ± 4
Seminal vesicles (mg)	70 ± 5	73 ± 4

c) BDF_1 strain

Weights ± SE		Treatment: Vehicle	Treatment: TP
		N = 11	N = 12
Body	(g)	25.2 ± 0.4	25.0 ± 0.3
Testis	(mg)	174 ± 2	155 ± 3
Seminal vesicles	(mg)	70 ± 4	59 ± 4

d) DBA/2J strain

Weights ± SE		Treatment: Vehicle	Treatment: TP
		N = 6	N = 6
Body	(g)	24.2 ± 0.3	24.5 ± 0.2
Testis	(mg)	186 ± 4	170 ± 7
Seminal vesicles	(mg)	60 ± 4	56 ± 8

There were no changes in seminal vesicle weights within any of the strains.

Plasma testosterone levels are shown in Table 6.3 - the Balb's and C57's showed no significant changes in this measure, but the BDF_1 hybrid showed a significant elevation in testosterone level in the TP treated group. (Student 't' test 't' = 2.82, df = 21 $p < 0.02$.) No plasma testosterone data was available for the DBA strain.

To determine whether the TP treatment had any effect on the within and between strain variability, the F_{max} test (Snedecor and Cochran, 1967) which tests for heterogeneity of variance was applied to the behavioural and hormonal data. Using this method, there were found to be no significant differences in the variance of mount, intromission and ejaculation frequencies of the TP and control groups within each of the four strains. The variance of the mount latency was significantly reduced in the TP treated groups compared to controls in the Balb's ($F_{max} = 3.08$ $p < 0.05$) and the C57's ($F_{max} = 4.78$ $p < 0.01$), but was not significantly affected in the other two strains. The ejaculation latency of the C57's was significantly more variable in the TP treated group compared to controls ($F_{max} = 5.74$ $p < 0.01$), but was not affected in the other three strains. There were no significant

Table 6.3

Levels of plasma testosterone of three strains of mice treated with 100 µg Testosterone Propionate (TP) on day 4 after birth.

a) Balb/c strain

Treatment	N.	Testosterone levels		
		Ln.	± SE	ng / ml
vehicle	12	2.22	0.27	9.2
TP	12	1.48	0.34	4.4

Student 't' test - 't' = 1.67, df = 22, p < ns

b) C57Bl/6 strain

Treatment	N.	Testosterone levels		
		Ln.	± SE	ng / ml
vehicle	11	0.71	0.39	2.0
TP	11	0.92	0.41	2.5

Student 't' test - 't' = 0.82, df = 20, p < ns

c) BDF_1 strain

Treatment	N.	Testosterone levels		
		Ln.	± SE	ng / ml
vehicle	11	0.90	0.31	2.5
TP	12	1.93	0.21	6.9

Student 't' test - 't' = 2.82. df = 21, $p < 0.02$

(N = number of animals)

changes in the variability of the testosterone levels of TP treated animals compared to controls within any of the strains.

In order to assess whether there were any changes in the 'between strain' variability of behavioural and hormonal measures of TP treated animals, compared to controls, the between strain variance was calculated using the 'strain means' as shown in Table 6.1 and 6.3. Of the 5 behaviour measures taken, the between strain variance was less in the TP treated groups, than controls, but none of these reached significance (F_{max} values - MF = 2.46, IF = 2.14, EF = 2.11; ML = 1.16; EL = 1.88). However, if all the measures are grouped together and examined by the sign Test, then there was a significant reduction in the between strain variability for the TP treated group ($x = 0$, $N = 5$, $p < 0.03$). The between strain variance in plasma testosterone levels was less in the TP treated groups ($F_{max} = 2.64$) - but this was not significant. However, Analysis of Variance of individual testosterone levels from the TP treated groups revealed there to be no significant differences between the strains ('F' = 2.47 df = 2,32 $p < 0.25$), whereas in the control animals there were significant strain differences in this measure ('F' = 6.54 df = 2,31 $p < 0.001$).

6.4. DISCUSSION

These results in part support the hypothesis outlined earlier - neonatal testosterone treatment did result in a convergence of sexual behaviour measures between the strains. The adult testosterone levels, although showing a decreased interstrain variability, were however not reduced in the TP treated groups; and the BDF_1 hybrid showed increased adult plasma testosterone levels after neonatal TP treatment.

The normally low responding Balb/c strain showed an increased number of animals showing the ejaculatory response, and the C57Bl strain showed a decreased mount latency. These results are in agreement with those of Vale et al (1974), who found in addition that their similarly treated C57's showed an increased frequency of ejaculation compared to controls. The failure to find any increases in measures of sexual behaviour in the BDF_1 hybrid may partly be because the tests were not sufficiently sensitive to detect differences, but also, it seems likely that the behaviour of this hybrid is already at a maximal level. The testosterone injections had been "effective", since, as with the other strains, there was a reduction in testis weight of the treated group.

The reduction in testis weights, in the treated animals, is in agreement with the findings of Vale et

al (1974) and Bronson and Desjardins (1968). These authors also found decreases in seminal vesicle weights, a result not recorded in this experiment. This difference may be a dosage effect, since the two previous studies used a far greater neonatal dose of 1 mg TP, and also injected the animals at an earlier age.

The lack of detectable changes in the levels of testosterone in treated Balb/c and C57Bl mice may have been partially because of the small numbers of animals examined. A study in the rat by Frick, Chang and Kincl (1969) also failed to find effects of neonatal TP on adult testosterone levels, although a more recent study (Joseph and Kincl, 1974) did find a change in testicular enzymes concerned with androgen biosynthesis, which might lead to a reduction in plasma testosterone levels. However the BDF_1 hybrid of this study showed a marked <u>increase</u> in plasma testosterone in the neonatally treated group, showing TP <u>can</u> have effects on adult testosterone levels - but they do not necessarily accompany changes in sexual behaviour.

The changes in sexual behaviour and testosterone levels in the TP treated groups were such that there was a decreased between strain variability in these measures. This finding in part supports the hypothesis that differences in neonatal androgens during the neonatal period may contribute to the variance in

behaviour and hormone levels observed in adults from several strains. However, it is important to demonstrate that this neonatal treatment reproduces a 'normal' physiological event, and is not a treatment so extreme as to produce a very abnormal adult animal. Although we know that testosterone levels do increase after birth, we do not know the effects of 'boosting' this neonatal pulse from 'physiological' to what must be 'pharmacological' levels. There is some indirect evidence that this treatment does not exert its effects through 'abnormal' physiological mechanisms from the findings of the F_2 analysis described in Chapter 3. The F_2 derived from Balb/c and C57 parental lines was examined, and it was found that there was a negative correlation between testis weights and measures of sexual behaviour. Thus, the change in testis weights produced by neonatal TP, and the 'natural' variation in a mixed population are both related to measures of sexual behaviour, such that a decreased testis weight is associated with an increased level of sexual behaviour.

What are the mechanisms whereby these increases in sexual behaviour might be effected? There are of course many factors involved in the control of sexual behaviour, and several, not necessarily exclusive changes in physiology might be involved. For example

neonatal testosterone might have influences on both quantitative and qualitative aspects of adult hormone secretion, and there might be changes in the levels of adult androgens in the plasma, as found for the BDF_1 hybrid; or changes in the way the androgens are utilized such that early testosterone facilitates the formation of active metabolites (De Moor et al, 1973).

If there can be changes in adult testosterone levels, this suggests that the hypothalamo-pituitary feedback loop might be affected - perhaps by changes in sensitivity - although if this became more sensitive, we might then expect adult plasma testosterone levels to show a decrease, rather than the increase suggested by the BDF_1 results.

It has been suggested by Vale et al (1973) that the effect of neonatal testosterone is to "activate behaviourally relevant genes normally activated by endogenous hormone", and that increasing the hormone by injections of TP may activate (or suppress) genes which are normally not activated (or suppressed) in some strains. Similarly, Edwards (1968) proposed that the increased fighting behaviour shown by neonatally androgenised female mice was a result of changes in the neuronal structures involved in fighting. If this is so, then we might expect to find ultra-structural differences of relevant brain areas, as

has in fact been reported by Raisman and Field (1971) in the neonatally androgenised female rat.

A further mechanism by which these changes in behaviour might come about relates to changes in the sensitivity of peripheral structures. For example, it may be there is an increased penile spine sensitivity, leading to increased peripheral feedback, which might explain the increased incidence of ejaculation in the Balb/c strain. However, since ejaculation latency was not significantly reduced in any of the strains this seems unlikely. There is evidence from work on the rat that, in fact, changes in peripheral sensitivity are not involved. Hart (1972) examined the sexual responses of rats castrated at 4 days of age, and given either fluoxymesterone (FM) or TP for the next 7 days. FM is a synthetic steroid which was found by Beach and Westbrook (1968) to maintain the penile spines in adult castrate rats, without maintaining sexual behaviour. Thus, if the loss of the ejaculatory reflex characteristic of neonatally castrated rats, was compensated for by neonatal FM, then this would suggest that the decrement in behaviour was a result of reduced peripheral feedback. However, neonatally FM treated castrate rats, given replacement testosterone as adults still failed to show ejaculatory reflexes, even though there were no differences in penile spine

development. Thus, the differences in adult behaviour were probably not due to changes in peripheral feedback, but to differences in areas of the brain controlling the ejaculatory responses.

The injection of neonatal androgens can affect other aspects of reproductive physiology. Bronson, Whitsett and Hamilton (1972) examined the responsiveness of the seminal vesicles of mice castrated on days 2-4 after birth, and injected with either TP or oil. As adults the mice were all injected with TP, and the neonatally-TP treated animals were found to have heavier seminal vesicle weights than neonatally-oil treated animals. This suggests that the TP treated animals have a heightened responsiveness to testosterone in adulthood . It may be that other structures show a heightened responsivity to testosterone, in particular brain areas mediating sexual behaviour. An examination of the dose-response relationships, between injected testosterone and measures of sexual behaviour, in castrate, neonatally treated and control animals might be a way of approaching this question.

To summarise, neonatal injections of TP do result in increased behaviour from normally 'low responding' strains, resulting in a decreased between strain variability. This supports the hypothesis that

differences in the adult behaviour of the strains may be partially a result of differences of early androgen levels. However, although testosterone level-interstrain variability was also reduced, the levels did not change in the predicted direction, suggesting this may not be the mechanism by which the increased behaviour was effected.

CHAPTER SEVEN

FINAL DISCUSSION

7.1. FINAL DISCUSSION

The aim of this thesis has been to examine how differences in male sexual behaviour are related to differences in the levels of circulating testosterone. The approach has been to measure the hormone levels directly by RIA, in the plasma of strains of mice, which differ markedly in quantitative measures of their behaviour. However, as the series of experiments revealed, the complexities of the situation required that both the measures of behaviour analysed, and the conditions under which blood samples were removed, had to be carefully defined and controlled.

The experiments demonstrated that individual differences in sexual behaviour are related to differences in plasma testosterone levels, but in two distinct ways, which relate to specific measures of the behaviour. In the first relationship measures of sexual behaviour were found to be negatively correlated with plasma testosterone levels across strains of mice, but only when blood samples were removed in a non-sexual context.

When blood samples were removed from males which had recently been exposed to oestrous females, this negative relationship broke down. Samples from strains with normally low levels of testosterone showed increased levels, over those measured in samples taken from the

same strain in the 'non-aroused' state. Further if the relationship between an individual male and his sex performance was examined within a strain there was a positive correlation between plasma testosterone levels and measures of sexual behaviour. In both of these situations, it was measures associated with the arousal mechanism (AM), as indicated by mount frequency and latency, which were related to testosterone, but measures associated with the copulatory mechanism (CM) of ejaculation frequency and latency were not so related. In other words, 'high' copulating strains, in terms of mount frequency and latency, are characterized by having low basal levels of testosterone, which show rapid increases when the males are exposed to oestrous females. 'Low' copulating strains have high basal testosterone levels, and these do not show acute increases when the males are stimulated sexually.

In the introduction, studies on the guinea-pig and rat (Grunt and Young, 1952, 1953; Larsson, 1966), were described, which showed that males differing in their sexual behaviour, retained these differences after castration and replacement treatment with <u>equal</u> doses of TP. The authors interpreted this as indicating that differences in sexual behaviour between individuals were not related to differences in plasma testosterone.

however, as the present experiments have shown this may not be a valid conclusion - the strains of mice examined here, when castrated and treated with TP, were also found not to differ in the doses of TP necessary to maintain their pre-castrational behaviour; and yet we know that there are differences between the strains in both the basal levels and the 'lability' of plasma testosterone.

As noted earlier, we need to 'test' these correlations between testosterone and measures of sexual behaviour by injecting the hormone into the castrate animal and observing what changes in behaviour take place. If these changes correspond to the correlations observed in the intact animals, then this eliminates the possibility that the correlation was an effect of the behaviour on hormone levels, and is supportive evidence that the hormone may be a causal factor for the behaviour. A failure to demonstrate the expected relationship between the hormone and behaviour suggests that the observed correlations were not causal in nature, and that the correlation with the hormone may be a 'symptom' of some other physiological change.

Testing the correlations found between plasma testosterone and sexual behaviour is not however a simple experiment: there are problems in determining the doses

of hormone which are equivalent to 'basal' levels, and those equivalent to the 'female stimulated' levels. An approximation to the 'basal level' hormone treatment may be determined by examining the changes in the weights of the seminal vesicles - a dose of TP which maintains these at normal control weights may produce normal circulating levels of hormone. Doses above this level may be equivalent to the 'female stimulated' levels, as measured in Chapter Four.

The results described in Chapter Three showed there to be a negative correlation between basal plasma testosterone levels and measures of sexual behaviour associated with the AM. However, in castrate animals from three strains, injected with TP doses equivalent to these basal levels, the measures of sexual behaviour were not maintained. Thus, the basal plasma testosterone levels of the strains are not sufficient to maintain behaviour, and the differences in basal testosterone levels cannot be directly involved in the differences in behaviour, but may be a symptom of some other difference in endocrine physiology. In Chapter Four, there was found to be a correlation between increased levels of testosterone and measures of sexual behaviour associated with the AM, but not the CM, when blood samples were removed from sexually aroused males. This correlation was confirmed in castrate

males injected with TP, and increased doses of TP resulted in an increased frequency of, and decreased latency to, mounting responses; but had no effects at all on the frequency of, and latency to, ejaculation. This suggests that the correlations observed in Chapter Four are important in behavioural change, and that increased levels of testosterone, or some factor closely associated with testosterone levels, are involved in the mechanisms which initiate sexual behaviour.

Some of the physiological mechanisms which may be involved in these relationships have been discussed earlier. The two major mechanisms considered were firstly, that the low basal testosterone levels of high behaviour strains might be related to increased responsiveness at the hypothalamo-pituitary axis, and at hypothalamic areas mediating sexual behaviour, and secondly that the increased 'female-stimulated' levels of testosterone may have direct behavioural effects in the hypothalamus, and that the preceeding LH and LH-RF release might also be involved. To test these hypotheses we need to measure the appropriate variables directly, in properly controlled experiments, and further discussion of these points would be no more than speculation.

In conclusion, this thesis has shown that differences in male sexual behaviour are related to differences in

the levels of circulating testosterone, but that the experimental conditions, and the measures of behaviour analysed must be carefully defined. Hormones are only one of the many factors which control the display of sexual behaviour, and other factors, such as experience, can modify both behaviour and the response to hormones. They are however the most important modulators of sexual behaviour, for it is through their dual action on behaviour and physiological variables related to spermatogenesis, and maintenance of peripheral reproductive structures, that mating behaviour can be coordinated with periods of maximum fertility.

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APPENDIX I

Programme for use on Olivetti Programma 101 Computer for calculation of regression statistics of standard curve for radioimmunoassay of testosterone.

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APPENDIX II

An estimation of the comparative testosterone binding properties of plasma samples from four strains of mice

Introduction

In Chapter I, strain differences in plasma testosterone levels were described, which were related to measures of sexual behaviour. Thus, strains of mice with high testosterone levels, such as Balb/c and DBA/2J, were found to show little sexual behaviour under test conditions. An interpretation of this result might be that these high testosterone strains have plasma binding proteins which "impede" the action of testosterone at hypothalamic and pituitary areas, such that the "feedback-loop" is not activated, which may result in an excess of gonadotropnin release. Similarly, high binding protein concentrations may prevent testosterone from acting on hypothalamic areas mediating sexual behaviour. If this is the case, then we would expect the 'binding capacity' of the plasma of Balb's and DBA's to be greater than the 'binding capacity' of low testosterone strains such as C57 and BDF_1. This hypothesis can be tested by incubating plasma samples with radiolabelled testosterone, and measuring the radioactivity associated with the plasma after removal of 'free' unbound testosterone by some physical method, such as adsorption onto charcoal.

Method

Animals.

The strains of mice were obtained from stock bred in the Zoology Department, University of Edinburgh, and were housed in groups of 8-12 in large cages, under conditions as previously described. At 10-12 weeks of age, the mice were exsanguinated under ether anaesthesia. Blood from groups of three mice, within strains, was pooled, and centrifuged as previously described, and plasma removed.

Binding capacity determination.

a) Materials. Materials were obtained from the suppliers as described in Chapter 2.

Phosphate Buffered Saline (PBS). PBS was prepared by dissolving 0.86 g potassium dihydrogen phosphate, 5.69 g disodium hydrogen phosphate ($Na_2HPO_4.12H_2O$) and 5.67g sodium chloride in 1 litre of distilled water.

Charcoal solution A - for removal of endogenous steroids. Charcoal (5g) and Dextran T70 (0.5g) were added to 100 ml PBS and shaken thoroughly.

Charcoal solution B - for removal of unbound labelled testosterone. Charcoal (0.25g) and Dextran T70 (0.02g) were added to 100 ml PBS and shaken thoroughly.

1,2-^{3}H-Testosterone.

Labelled testosterone (SA 46.7 Ci/mM) was dissolved in ethanol:PBS, 1:3 v:v to give approximately 3,000 cpm in 0.1 ml.

Radioactivity measurement

Aqueous samples were dissolved in 10 ml scintillation fluid (as described in Chapter 2) and counted in a Packard 2425 counter.

Procedure

Six, 0.5 ml plasma samples from each of the strains, plus 2 tubes containing 0.5 ml PBS were incubated with 0.5 ml Charcoal solution A for 45 minutes at 40^{o}C, after mixing thoroughly on a Vortex mixer. The samples were then centrifuged at 3,000 rpm for 15 minutes, and 0.5 ml of the supernatant was pipetted into fresh tubes. This procedure removes endogenous protein bound and free steroid, without affecting protein content of the plasma (Heyns, Van Baelen and de Moor, 1967). Tritiated testosterone (0.1 ml) was then added to each tube, and after "whirlimixing" tubes were left to stand at room temperature to equilibrate for 45 minutes. (Two 0.1 ml aliquots of tritiated testosterone were also pipetted directly into scintillation vials for counting later). The tubes were then placed in an ice bath and 0.5 ml of Charcoal solution B was added to each tube. Tubes were then whirlimixed again, and after 10 minutes were centrifuged at 3,000 rpm for 10 minutes. The

TABLE A2.1

The percentage of 1,2-^{3}H-Testosterone counts which bind to plasma samples from four strains of mice

STRAIN	N	% COUNTS BOUND Mean ± SE
Balb/c	6	30.8 ± 1.2
DBA/2J	5	39.0 ± 1.6
C57Bl/6Fa	6	43.2 ± 1.4
BDF_1	6	41.7 ± 1.1
(PBS only)	2	3.0 ± 1.0

supernatant was then poured into scintillation vials, containing 10 ml scintillation fluid, and counted for 10 minutes.

Calculations

The counts per minute for each tube were expressed as a percentage of the total number of counts added (TC tubes). Strain differences in percentage binding were examined by Analysis of Variance.

Results

The percentage counts bound for the plasma samples of the four strains were shown in Table A2.1. There were strain differences in binding capacity (Analysis of variance $F = 15.9$, $df = 3, 19$ $p < 0.01$), with Balb/c showing the lowest value. Binding in the PBS samples was very low, at less than 5% of the total radioactivity added.

Discussion

Although this method does not indicate the absolute binding capacity of plasma, it has shown that the hypothesis outlined in the introduction was not correct. The high plasma testosterone levels of the Balb and DBA strains were not the result of increased plasma binding properties, in fact Balb showed the lowest binding of all the strains. As described by Hampl, Iványi and Stárka (1971) this binding capacity is probably due to a β-globular protein, which may be specific for sex-steroids. They measured binding properties in plasma from 5 strains of mice (A, C57Bl/10ScSn, B10A, B10BR and C3H/Di), and found binding capacities ranging from 5.09 ng/ml (C3H) to 6.90 ng/ml (B10A). These values do not mean that these are the maximum levels which can be carried in the blood, but that only this maximal amount can be carried as a protein-steroid complex.

ACKNOWLEDGEMENTS

Firstly, I wish to thank my supervisor Professor A.W.G. Manning for his advice and encouragement throughout the course of this work. Professor Manning also castrated the mice used in Chapter Five. I am also grateful to Dr.'s R.V. Short and C. Corker for allowing me the use of laboratory facilities in the M.R.C. Reproductive Biology Unit, and especially to Rhona Cunningham for allowing me the liberal use of hormone standards, buffers etc., required for the testosterone assay. I thank Maurice Dow for his endless patience in explaining statistics, and also for valuable discussion. I am also grateful to Douglas Scott and Lilian Neilson for maintaining the mouse colony.

This study was supported by an M.R.C. Studentship and I declare that the work presented here is entirely original and all experiments have been performed by myself.

Jennifer Batty

h3. What evidence that T may produce peripheral changes in hormone production and metabolism if given orally?

h11 What is the breeding season for the hare?
Correlation of plasma levels with testis levels?
Lincoln et al not 1976.

Very low scores of androgens and sexual behaviour in male. Experience?

h14 who proposed aromatization hypothesis?
Androstenedione..... what is aromatization?

h22 100 µg testicular homogenate to induce shrieks?!!

h26 10 mg T would not dissolve in 100 µl ethanol.

h27 Effect of ether anesthesia on LH? *

h30 Precision. How reliable is the chromatography?

h35 Is ejaculation possible after castration? Nothing to ejaculate.

h42 In which species is T bioactive, in which not?

h52 coagulating gland and seminal?

General question - sequence of killing animals? Latency anesthesia → bleeding? Have you measured an effect of ether susceptibility?

h104 Δ^4: Androstene-17 OL-3-One ..... meaning?

poor review of literature - only 130 refs! 160 refs to work of

Brown Grant. Foy, Money & Ehrhardt, Lyon, Cattanach, Short &

Clark, Swanson

Why is secretion episodic? Tfm? Prenatal vs neonatal androgen?