EARLY PREGNANCY DISRUPTION IN MICE (THE BRUCE EFFECT): THE ROLE OF SEXUAL SATIETY, CASTRATION, AND EXCRETED STEROIDS OF NOVEL MALES

By

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A Thesis

Submitted to the School of Graduate Studies

in Partial Fulfilment of the Requirements

for the Degree

Doctor of Philosophy

McMaster University

STEROIDS AND EARLY PREGNANCY DISRUPTION

Doctor of Philosophy (2000)
(Psychology)

McMaster University
Hamilton, Ontario

TITLE: Early Pregnancy Disruption in Mice (the Bruce Effect): The Role of Sexual Satiety, Castration, and Excreted Steroids of Novel Males

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NUMBER OF PAGES: 129

ABSTRACT

Early pregnancy in mammals is vulnerable to various environmental factors, one of which is exposure to novel males (the Bruce effect). It is well established that androgen-dependent chemical excretions from novel male mice prevent ovoimplantation in proximate previously-inseminated females. The chemical nature of these pheromones has not been determined. This thesis explores the role of steroids in the Bruce effect after castration and sexual satiety and also analyzes excreted steroids released by novel males.

Experiments 1 and 2 demonstrated that recent sexual experience can reduce novel males' capacity to disrupt pregnancy. Novel males were allowed to mate with stimulus females prior to exposure to inseminated females during the implantation period. Unmated males disrupted pregnancy more often than did mated males.

Experiment 3 was designed to examine the time course of males' ability to disrupt pregnancy following castration. The probability of retention of pregnancy increased as a linear function of time since castration. Castrated males continued to disrupt pregnancy during the initial weeks after surgery, but by 6 weeks, the majority of females remained pregnant. Males' excretion of testosterone and 17β-estradiol in urine, measured via ELISA procedures, diminished gradually during the weeks after castration.

In Experiments 4 and 5, the concentrations of urinary steroids from novel males were measured during exposure to inseminated females. These studies revealed that

males' urinary excretions of testosterone and 17β-estradiol correlated with females' pregnancy loss. Also, males excreted higher concentrations of 17β-estradiol but not testosterone when exposed to females on day 3 of pregnancy compared to when males were isolated.

In Experiment 6, steroids were applied to the nasal region of recently inseminated females. Nasal application of testosterone propionate did not disrupt pregnancy. However, topical application of either 17β-estradiol or 17β-estradiol benzoate completely prevented implantation at very low doses.

Results from these studies suggest that steroids play an important role in novel-male-induced pregnancy disruption. As endogenous levels of androgens are diminished, either through castration or sexual satiation, novel males' ability to disrupt pregnancy decreases. Furthermore, novel males' urine contains 17β-estradiol, the quantity of which rises during exposure to inseminated females. Also, this steroid can disrupt pregnancy through nasal application on the female. Levels of 17β-estradiol are known to be elevated in females experiencing early pregnancy disruption. Thus, 17β-estradiol may be a potential pheromone involved in the Bruce effect.

ACKNOWLEDGEMENTS

I would like to thank my supervisor, Denys deCatanzaro, for his patience, support, encouragement and guidance in my research. I would also like to thank my committee members, Ron Racine and David Rollo, for their help in preparing my thesis.

I am indebted to Cameron Muir for his help in developing the ELISA in our laboratory. I was fortunate to have worked with such a kind, insightful and resourceful individual. He made it a pleasure to go to work every day. I would like to thank Tara Allen, Marco Baptista, Arun Gupta, Miriam Hansen, Cecile Marczinski, Neena Prasad, and Melissa Stuiver for their assistance in these experiments. These people as well as Maryanne Fisher, Albert Ler and Jennifer Woodside have been great companions.

I would like to thank all the members of the Yellow Fever soccer team and Cindy Polak for making my stay at McMaster fun and helping to keep my spirits up. Special thanks goes to Marco Baptista and Joseph Kim for their support during first year.

I would like to thank my family, Lucy and Doreen Spironello and Josie and Vince Vella, for their continual support over the years. Thank you for encouraging me to achieve my goals. Last, but not least, I would like to thank my husband, Frank. Thank you for being a stable source of love and strength. Your belief in me has given me the confidence to see this through. You are my inspiration.

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ACTH	Adrenocorticotropic hormone
AOB	Accessory olfactory bulb
CV	Coefficient of variation
DHB	dehydro-*exo*-brevicomin
ELISA	Enzyme-linked immunosorbent assay
GABA	Gamma-aminobutyric acid
HPA	Hypothalamic-pituitary-adrenal
HS	Heterogeneous
MOB	Main olfactory bulb
MUP	Major urinary protein
NE	Norepinephrine
RIA	Radioimmunoassay
SBT	2-(*sec*-butyl)-4,5-dihydrothiazole

Chapter 1

General Introduction

Early Pregnancy Disruption

Successful reproduction depends upon a carefully synchronized sequence of endocrine events. In mammals, disruption in the timing and occurrence of these events by diverse environmental factors can lead to increased embryo mortality (deCatanzaro & MacNiven, 1992). Under unfavourable conditions, it may be adaptive for pregnant females to abort, postponing reproduction until more suitable times in the future. During the early stages of pregnancy, before the fertilized ova have implanted into the uterine wall, females are particularly sensitive to adverse pressures such as chronic restraint (Wiebold, Stanfield, Becker & Hillers, 1986), daily handling (Runner, 1959), overcrowding (Calhoun, 1949), high temperatures (MacFarlane, Pennycuik & Thrift, 1957) and exposure to loud noises (Zondek & Tamari, 1967). Compared to the major investment following implantation, early pregnancy is a time of low investment. Thus, the implantation period provides a convenient time in which further reproductive investment can be arrested in response to environmental demands.

Determining the physiological mechanisms underlying implantation failure may have implications for animal husbandry in optimizing the reproductive success of farm and rare animals bred in captivity. In humans, early pregnancy may be affected by

psychosocial factors (Walker, Cooney & Riggs, 1999). Spontaneous miscarriages are common in the first trimester and their incidence has been found to be correlated with anxiety and stress experienced by females (Nuckolls, Cassell & Kaplan, 1992). Therefore, research in this field may also benefit infertile women attempting to conceive.

One way in which early pregnancy can be disrupted is through exposure to novel males, discovered by Bruce (1959). If a pregnant female mouse or vole is exposed to a novel male during the first five days after mating, implantation fails and the female returns to estrus. A novel male is any strange male that did not mate with the female, and hence, is not the sire of the litter or the stud male. This stimulus has a strong effect, with approximately 70-100% of females aborting if the strange male belongs to a strain different from that of the sire (Parkes & Bruce, 1961). If the novel male belongs to the same strain as that of the sire, approximately 40% of females abort (Parkes & Bruce, 1961).

Termination of a pregnancy upon exposure to strange males is adaptive for females since males will tend to kill pups that they have not sired (Huck, Soltis & Coopersmith, 1982). Thus, it is advantageous for a female to sustain pregnancy only when the majority of her pups are likely to survive (Storey & Snow, 1990). Similarly, there are benefits for novel males, since a female that has aborted quickly becomes sexually receptive and returns to estrus, potentially enhancing the reproductive success of such males.

Currently, the physiological events underlying the Bruce effect are not fully known. One hypothesis suggests that novel males may be acting as chronic stressors that

elicit a stress response in females resulting in implantation failure (deCatanzaro & MacNiven, 1992). Other hypotheses involve the female forming a memory trace of the sire, reacting to novel male odours with a cascade of neuroendocrine events that disrupt pregnancy, or females reacting to excreted steroids and behaviour of novel males. In any event, the delicate hormonal balance required during early pregnancy may be disrupted with the presence of novel males.

Stress and Early Pregnancy Disruption

A stressor can be defined as any stimulus that disturbs or interferes with the normal physiological equilibrium of an organism, requiring its adjustment or adaptation (Hinkle, 1977). Initially, during a stereotypical stress response to various adverse or sudden novel stimuli, an acute reaction occurs known as the fight-or-flight system, which mobilizes the body to handle extreme situations such as fear and rage (Selye, 1956). Stress experienced over an extended period of time can activate the hypothalamic-pituitary-adrenal axis (HPA) which facilitates the availability of energy over more prolonged periods (Selye, 1956).

During acute stress, the sympathetic nervous system is activated and stimulates the secretion of catecholamines, norepinephrine (NE) and epinephrine, from the adrenal medulla (Marieb, 1995). These, in turn, intensify the response of the sympathetic nervous system, causing an immediate increase in arousal, heart rate, blood pressure, metabolic rate, and muscular activity. Blood is diverted from temporarily nonessential organs to the brain, heart, and skeletal muscles, thereby allowing for a fight-or-flight response. During

recovery and rest, the parasympathetic nervous system serves to counteract these effects.

During chronic stress, corticotropin-releasing hormone from the hypothalamus stimulates the release of adrenocorticotropic hormone (ACTH) from the corticotroph cells of the anterior pituitary (Marieb, 1995). ACTH acts on the adrenal cortex, stimulating the synthesis and release of glucocorticoids such as corticosterone, and in some circumstances androgens and estrogens. Glucocorticoids channel resources to the brain and muscles by mobilizing fats for energy metabolism and stimulating protein catabolism. Corticosteroids also enhance the production of epinephrine by the adrenal medulla. Adrenal steroids can target reproductive organs and the limbic system in the brain (Guyton, 1976). Thus, steroid release can influence reproduction and mood.

The involvement of stress hormones in early pregnancy disruption has been investigated. The adrenal medullary catecholamine, epinephrine, has no effect of disrupting early pregnancy (deCatanzaro & Graham, 1992; Trend & Bruce, 1989). Additionally, despite evidence that corticosterone can suppress reproductive functioning (deCatanzaro, 1987; deCatanzaro, Knipping & Gorzalka, 1981; deCatanzaro, Lee & Kerr, 1985), it does not disrupt implantation in inseminated female mice (deCatanzaro, MacNiven & Ricciuti, 1991). However, exogenous ACTH administration during pregnancy can reduce litter sizes, diminish implantation, and increase spontaneous abortions and fetal resorption (deCatanzaro, Maerz, Heaven & Wilson, 1986; Kittinger, Gutierrez-Cernosek, Cernosek & Pasley, 1980; Robson & Sharaf, 1952; Velardo, 1957; Yang, Yang & Lin, 1969).

Controversy remains over whether the HPA axis is involved in pregnancy disruption, as illustrated in adrenalectomy studies. In one study, adrenalectomized female mice had significantly more pregnancies than sham-operated female mice when exposed to a strange male (Snyder & Taggert, 1967). In another study, adrenalectomy failed to prevent pregnancy disruption (Sahu & Dominic, 1981). Therefore, it is difficult to draw any conclusions regarding the role of adrenal hormones in the disruption of pregnancy.

The role of sex steroids in early pregnancy disruption is more clearly defined. In response to stress, the ovaries or adrenal glands could secrete estrogens and androgens which would disrupt the hormonal balance between the corpus luteum and the endometrium. There is some evidence to suggest that adrenal androgens are involved. Injections of androstenedione and dehydroepiandrosterone have the capacity to disrupt early pregnancy (deCatanzaro et al., 1991; Harper, 1967a, 1967b, 1969). Nevertheless, much of the evidence indicates that 17β-estradiol may mediate the stress-induced disruption of pregnancy. DeCatanzaro et al. (1991) demonstrated that injections of estradiol benzoate, at doses much lower than that for androstenedione and dehydroepiandrosterone, can completely disrupt pregnancy. Also, during the implantation period, radioimmunoassay (RIA) revealed elevated levels of 17β-estradiol in pregnant rats exposed to chronic restraint stress compared to pregnant rats left undisturbed (MacNiven, deCatanzaro & Younglai, 1992). Subsequently, administration of monoclonal antibodies against 17β-estradiol significantly reversed the pregnancy disrupting effects due to restraint stress and exposure to strange males (deCatanzaro, MacNiven, Goodison &

Richardson, 1994; deCatanzaro, Muir, O'Brien & Williams, 1995a). The results of these experiments suggest that estrogen levels play a significant role in early pregnancy disruptions. It is not surprising, therefore, that adrenal androgens have pregnancy-disrupting effects since they metabolize to estrogens in the body (Harper, 1967a,1967b, 1969).

The role of progesterone in implantation failure is not as well established. Progesterone levels have been observed to both increase and decrease in response to stress. For example, MacNiven et al. (1992) showed that progesterone increased in restraint stressed pregnant female mice as revealed by RIA. In another experiment, decreased levels of progesterone were observed following restraint stress in early pregnancy (Wiebold et al., 1986). Furthermore, only a weak effect of sustained pregnancy was observed when progesterone was administered to females exposed to restraint stress or predators (MacNiven & deCatanzaro, 1990). Thus, studies examining the influence of sex steroids indicate that 17β-estradiol appears to have the most significant effects on early pregnancy.

Evidence suggests that early pregnancy is also dependent on behavioural interactions between the strange male and inseminated female. When males and females have direct contact, sexual activity correlates with the Bruce effect, with intromissions in particular being positively correlated with pregnancy disruption (deCatanzaro & Storey, 1989). Furthermore, castrated strange males do not disrupt pregnancy, however testosterone administration restores their ability to disrupt pregnancy (deCatanzaro &

Storey, 1989; deCatanzaro, Wyngaarden, Griffiths, Ham, Hancox & Brain, 1995c). Behaviourally, castrated testosterone-treated novel males, as well as intact novel males, are very assertive with inseminated females, whereas untreated castrated males exhibit passive behaviour (deCatanzaro et al., 1995c). Therefore, sexually-motivated behaviour displayed by novel males may be eliciting an endocrine response in inseminated females that can prevent implantation (deCatanzaro & MacNiven, 1992).

In order to reduce behavioural interactions, an indirect exposure paradigm has been developed in which two strange males are housed above a pregnant female, separated from it by a wire grid (deCatanzaro, Zacharias & Muir, 1996). A substantial Bruce effect is observed. Yet, this paradigm does not totally eliminate behavioural interactions between males and females as oral-genital contact still occurs. Another study has demonstrated that behavioural inactivation of novel males by a major tranquillizer, chlorpromazine, does not diminish the Bruce effect (deCatanzaro, Muir, Spironello, Binger & Thomas, 2000). In this study, females could still contact the male through the wire-grid but the males were totally inactive and showed motor retardation. In this case, males, even though apparently unaroused behaviourally, could still disrupt pregnancy. Nevertheless, the females exhibited exploratory behaviour toward the males, approaching, sniffing, and touching them through the wire-grid.

Since a significant number of females abort in the indirect exposure paradigm, it is proposed that some form of chemical transmission from the male may be involved. Extensive research has been done in the area of individual recognition of pheromones

released in novel male urine. Exposure to these pheromones leads to activation of a neuroendocrine response in females, resulting in the decreased luteotrophic support of the corpus lutea.

Pheromonal Hypothesis

Investigators in many previous studies have attributed the Bruce effect to the female's sensitivity to pheromones released from the strange male. Convincing evidence suggests that this secreted chemical is androgen-dependent. As mentioned previously, castrated males do not disrupt pregnancy. However, if injected with testosterone, castrated males can prevent implantation (deCatanzaro & Storey, 1989; deCatanzaro et al., 1995c). Furthermore, unlike normal females, androgenized females will disrupt pregnancy (deCatanzaro et al., 1995c; Dominic, 1965).

The pheromone involved in the Bruce effect also appears to be non-volatile and acts on the female through contact. In the indirect exposure paradigm, housing the female above novel males does not disrupt pregnancy; females need to be housed below (deCatanzaro et al., 1996). Also, increasing the number of males housed above increases the probability of disruption (deCatanzaro et al., 1996). These findings suggest that a substance secreted from novel males contacts inseminated females below, and the greater the amount of excretions, the greater the Bruce effect.

Studies have shown that the pregnancy-disrupting substance is found in novel male urine. Exposure to the soiled bedding of unfamiliar males prevents implantation of the fertilized ova into the uterine walls (Parkes & Bruce, 1962; Dominic, 1966a). As well,

topical application to the nasal area of urine or salted out urinary proteins of strange males disrupts pregnancy (Dominic, 1965; Marchlewska-Koj, 1977, 1981). These experiments may not always have involved full control for the stressful effects of human handling, which in itself may induce early pregnancy loss (Runner, 1959). In some cases, females exposed to the soiled bedding of novel males were housed in containers with restricted air ventilation (Bruce 1960b, Parkes & Bruce, 1962). This may have resulted in poor air quality. Also, pregnancy was indirectly assessed by measuring cell cornification taken from vaginal smears approximately 4-7 days following insemination. It is conceivable that vulval stimulation involved in vaginal smears could disrupt pregnancy. A recent study was designed to address these issues (deCatanzaro et al., 1995c). Human handling and vulval stimulation were minimized by measuring litter sizes rather than cell cornifications. Also, females were housed in cages with proper air ventilation and novel males' urine was painted on the nasal area of females. In this experiment, urine from strange males did not substantially disrupt pregnancy compared to direct exposure to strange males (deCatanzaro et al., 1995c). Recently, however, deCatanzaro, Muir, Sullivan, and Boissy (1999) found that a reliable pregnancy disruption was obtained from male urine collected when males were in the presence of females, compared to urine of males lacking such stimulation. This indicates that males release the pregnancy-disrupting substance in the presence of females more so than when they are isolated from contact with females. Furthermore, these studies confirm that females come into contact with a non-volatile chemical that is present in the urine of strange males.

The source of the pheromone is still unknown. Males' androgen-dependent accessory glands have been implicated in the pheromonal regulation of estrus in females as well as the regulation of intermale aggression (Jones & Nowell, 1973; Marchlewska-Koj, Pochron & Sliwowska, 1990). However, studies by deCatanzaro et al. (1996) and Zacharias, deCatanzaro, and Muir (2000) have demonstrated that males' preputial and vesicular-coagulating glands are not the source of the pheromone, since removing them did not diminish the capacity of novel males to induce the Bruce effect. Other potential sources include the kidneys and the liver, which produce androgen-dependent metabolites and urinary proteins respectively (Clissold, Hainey & Bishop, 1984; Hoppe, 1975). Thus, further studies need to be conducted to determine the physiological origin of the pregnancy-disrupting substance in the males' system.

Olfactory Memory

Once the pheromone is excreted, it is thought to act on the female's olfactory system, specifically the vomeronasal organ projecting to the accessory olfactory bulb (AOB) as opposed to the main olfactory bulb (MOB). Removal of the vomeronasal organ in the AOB prevents pregnancy disruption, whereas lesions to the MOB did not affect the ability of strange males to disrupt pregnancy (Lloyd-Thomas & Keverne, 1982; Rajendren & Dominic, 1984, 1986). In the AOB, formation of an olfactory memory of the sire during mating is thought to occur (Brennan & Keverne, 1997). Re-exposure to the stud male does not disrupt implantation as the female is thought to have formed an olfactory memory of the stud male (Bruce, 1960b; Thomas & Dominic, 1987b). An imprint of the

sire's pheromones is believed to occur in the AOB since infusion of the anaesthetic, lignocaine, into the AOB following mating results in the sire disrupting his own pregnancy (Kaba, Rosser & Keverne, 1989). This suggests that the female did not recognize the sire's pheromones since her AOB was anaesthetized.

According to Brennan, Kaba, and Keverne (1990), the formation of a memory to male pheromones requires the association of two conditions. First, females need to be exposed to male pheromones for about four hours immediately after mating. Secondly, a coitally induced increase in NE in the AOB is required immediately after mating. During mating, NE in the AOB is increased resulting from vaginocervical stimulation. Increased NE activity lasts for at least four hours after mating, which correlates with the exposure time to pheromones required to form an imprint. Blockade of α-adrenergic receptors, by local infusions of phentolamine into the AOB during the critical period after mating, prevented memory formation (Kaba & Keverne, 1988). The duration of the memory trace for a stud male fades within 30-50 days of the original mating since re-exposure to the original male's pheromones after a 30-50 day interval did disrupt pregnancy (Kaba et al., 1989).

The AOB consists of only three classes of neurons (Brennan et al., 1990). Mitral neurons, found in the AOB, receive the input from the pheromone receptors or vomeronasal nerve, and transmit the pregnancy disrupting signal centrally. Glutamate and aspartate are thought to be the main excitatory transmitters of mitral cells. A second class of neurons is called granule cells. These are the main class of interneurons in the AOB.

They form reciprocal dendrodendritic synapses with mitral cells and provide a gamma-aminobutyric acid (GABA)ergic inhibitory feedback. In other words, vomeronasal neurons activate mitral cells in the AOB which then depolarize granule cells by means of glutamate released at dendrodendritic synapses. This depolarization, in turn, releases GABA from granule cells and hyperpolarizes mitral cells. Activity at these reciprocal mitral-granule cell synapses potentially regulates mitral cell output. The third class of neurons, known as periglomerular cells, have not been implicated in olfactory memory formation.

According to Brennan et al. (1990), the sequence of events concerning the formation of an olfactory memory and subsequent pregnancy outcome are as follows. During mating, NE projections, from the locus ceruleus, reduce the GABAergic feedback inhibition exerted by granule cells on a subset of mitral cells responding to a sire's pheromones. This results in the sustained excitation of a subset of mitral cells activated from pheromonal stimulation. Over the 4-hour period of NE release and pheromone exposure during mating, activated mitral cells will produce sustained excitation of a subset of granule cells. After mating, when NE returns to premating levels, this subpopulation of mitral cells will be subject to increased inhibition from granule cells. Eventually, increased NE levels, during mating, results in long-lasting increases in mitral-granule cell feedback inhibition in a subpopulation of cells responding to the sire's pheromone. During subsequent exposure, the subset of mitral cells responding to these familiar pheromones would be subject to greater reciprocal inhibition. This selectively increased inhibition need

not completely block the signal from familiar pheromones. Merely its disruption might be sufficient to prevent the activation of the neuroendocrine mechanisms leading to pregnancy disruption. Conversely, exposure to pheromones from novel males would excite a subset of mitral cells without increased feedback inhibition and result in pregnancy failure. Therefore, unfamiliar male pheromones would act on a different population of mitral cells, without enhanced feedback inhibition. In this case, the pregnancy-disrupting signal would be successfully transmitted, activating the neuroendocrine mechanisms causing pregnancy disruption.

During exposure to strange male pheromones, the pregnancy-disrupting signal is hypothesized to travel from the AOB to the corticomedial amygdala then to the hypothalamus, which in turn causes the release of dopamine from the tuberinfundibular arcuate nucleus (Li, Kaba, Saito & Seto, 1989). Dopamine binds to D_2 lactotroph receptors and inhibits prolactin secretion from the pituitary gland, thereby removing luteotrophic support and terminating pregnancy (Dominic, 1966b; Rajendren & Dominic, 1988b). Hypophyseal prolactin is necessary for the maintenance of the corpus lutea during the pre-implantation period (Bartke, 1971). Prolactin stimulates the corpus luteum to increase the binding capacity for LH and to produce progesterone. Administration of bromocriptine, a dopamine agonist that blocks hypophyseal prolactin secretion, reduces ovarian LH-binding and progesterone secretion in the rat and prevents implantation of blastocysts (Muller, Bauknecht & Siebers, 1980). Bellringer, Pratt, and Keverne (1980) have also shown that alpha-bromocriptine can cause pregnancy disruption in mice.

Furthermore, a study by Rajendren and Dutta (1988) demonstrated inhibition of the Bruce effect if newly inseminated female mice were injected with haloperidol, a catecholamine antagonist, that not only blocks activity of dopamine but also NE. Therefore, the inhibition of the Bruce effect appears to be due to dopaminergic stimulation of prolactin release which prevents luteal failure in strange-male exposed females.

Pregnancy disruption is significantly reduced if the pregnant female is exposed to the novel male in the presence of the sire (Parkes & Bruce, 1961). The sire appears to have a protective effect on implantation in females exposed to strange males. The nature of the stimulus that is involved in this protective effect is not yet known. Thomas and Dominic (1987a) proposed that both bodily contact and pheromonal exposure may be important in inhibiting the Bruce effect. Although the sire will not disrupt implantation for about 30 days, the sire can only elicit a protective effect for about 5-6 days after insemination (Acharya & Dominic, 1997). The brevity of the effective duration might ensure that sires can only prevent an immediately subsequent novel male from disrupting pregnancy. Familiar males exposed to females during the pericopulatory period can also elicit a protective effect (Kumar & Dominic, 1993). However, males must be exposed to inseminated females immediately following mating in order to elicit a protective effect and not interfere with implantation (Thomas & Dominic, 1989a, 1989b). According to these studies, exposure during mating is necessary to form an olfactory memory. Although another experiment has demonstrated that the protection effect may not rely on olfactory memory. The presence of novel females can decrease pregnancy disruption to a degree

proportional to the size of the group (Bruce, 1963). Since grouped females inhibit estrus (Van der Lee & Boot, 1955), they may be able to counteract the estrus-inducing effects of novel males. Furthermore, the sire has recently mated which may alter its sexually motivated behaviour and the amount of androgen-dependent excretions directed at the inseminated female.

Pheromonal Identification

Relatively little is known about the chemical nature of the pheromone or pheromones involved in the Bruce effect. According to the olfactory memory theory, pheromones involved in the Bruce effect must be of different variants that can code for individuals, be non-volatile, and carry a message of maleness. Several volatile ligands present in mouse urine have been reported to possess primer pheromonal activity in other cases of chemical communication. The adrenal-mediated urinary metabolite, 2,5-dimethylpyrazine, appears to be important in suppressing estrus among grouped females in the absence of males (the Lee-Boot effect) (Ma, Miao & Novotny, 1998). Also, the induction of estrus (the Whitten effect) and acceleration of puberty (the Vandenbergh effect) by males may be mediated by 6-hydroxy-6-methyl-3-heptanone, 2-(*sec*-butyl)-4,5-dihydrothiazole (SBT), dehydro-*exo*-brevicomin (DHB), as well as two molecules derived from the preputial gland, E,E-alpha-farnesene and E-beta-farnesene (Andreolini, Jemiolo & Novotny, 1987; Ma, Miao & Novotny, 1999; Novotny et al., 1999a; Novotny, Ma, Wiesler & Zidek, 1999b). SBT and DHB have also been implicated in having releaser effects of promoting aggression in males and acting as attractants to females (Jemiolo,

Alberts, Sochiniski-Wiggins, Harvey and Novotny, 1985; Novotny, Harvey, Jemiolo & Alberts, 1985). Some of these ligands seem to activate a unique, non-overlapping subset of neurons in the vomeronasal organ (Leinders-Zufall et al., 2000). However, it is unlikely that any of these chemicals, on their own, have the polymorphic capacity to code for individual recognition important in the Bruce effect. Also, the vomeronasal organ is more suited for sensing non-volatile pheromonal components (Brennan & Keverne, 1997). One study has demonstrated that a combination of SBT and DHB applied to the oronasal groove of recently inseminated females was ineffective in disrupting pregnancy (Brennan, Schellinck & Keverne, 1999).

Nevertheless, there are non-volatile proteins secreted in mouse urine that bind volatile molecules. These major urinary proteins (MUPs) are members of the lipocalin family and are derived from about 30 different genes and pseudogenes with many allelic variants (Robertson, Hurst, Bolgar, Gaskell & Beynon, 1997). Expression of MUPs varies across inbred strains and is sexually dimorphic, with males expressing more proteins than females (Hastie, Held & Toole, 1979; Robertson, Cox, Gaskell, Evershed & Beyon, 1996). Thus, the polymorphic nature and strain specificity of MUPs makes them ideal candidates for involvement if individual discrimination is involved in the Bruce effect. MUPs have also been shown to be bound with a number of pheromones such as DHB and SBT (Bacchini, Gaetani & Cavaggioni, 1992). Therefore, one possible mechanism for pregnancy disruption may involve the MUP acting as a carrier to deliver a small, volatile molecule to the vomeronasal organ. In this instance, the MUP would be involved in the

transduction process and code for the strain. Another possibility could include a mixture of pheromones binding to MUPs in a variety of ways, thereby exposing selected parts of the pheromone to vomeronasal receptors.

MUPs act not only as pheromone carriers, but can also have pheromonal properties. MUPs from adult male urine have been shown to accelerate puberty in female mice (Mucignat-Caretta, Caretta & Cavaggioni, 1995). Although, in this study, the MUPs were added to pre-pubertal male urine which might on its own contain chemicals that are a component of the pheromonal signal. Yet, there is further evidence to suggest that MUPs are critical in relaying a pheromonal signal in the AOB. The expression of c-fos mRNA, indicating neuronal activity, is markedly increased when DHB and SBT bound to MUPs were applied to the oronasal groove in female mice (Guo, Zhou & Moss, 1997). This effect was not observed when the MUPs were removed (Guo et al., 1997). Expression of another immediate early gene, egr-1, has also been shown to slightly increase in mitral neurons in the vomeronasal organ when exposed to ligand-stripped MUPs but not to a combination of DHB and SBT (Brennan et al., 1999). However, the authors mention the possibility that very tightly bound ligands may have still remained after extraction. Furthermore, the role of immediate early gene activity in pheromonal transmission in the AOB is not entirely clear. The results from this study also demonstrate that unless MUPs from novel males are combined with urine from other males, MUPs will not disrupt pregnancy when nasally applied to recently inseminated females.

Although MUPs and some of their ligands have been implicated in affecting

endocrine and reproductive function in conspecifics, the chemical nature of the pregnancy-disrupting substance released by novel males has yet to be established. The role of sex steroids and their possible involvement in the pheromonal transmission of the Bruce effect has not been sufficiently investigated. Some of the compounds that have been isolated, such as brevicomin and the thiazolines, are androgen-dependent (Schwende, Wiesler, Jorgenson, Carmack & Novotny, 1986). They decrease following castration, but are restored with testosterone treatment (Schwende et al., 1986). Furthermore, the production and excretion of MUPs demonstrate a similar dependence on androgens (Rajendren & Dominic, 1984). Thus, steroids may have an important role in regulating pheromonal secretion. However, the question of whether steroids themselves may be acting as pheromones in strange-male-induced pregnancy disruption has not been examined.

Importance of Sex Steroids

It is well known that mammalian implantation is dependent on the actions of steroids on the uterus. Specifically, after copulation the progesterone-primed uterus requires estrogen to induce implantation of blastocytes. Treatment with progesterone for 48 hours followed by a single injection of a small amount of estrogen can induce uterine receptivity for 24 hours in a mouse (Psychoyos & Casimiri, 1980). It appears that estrogen, in particular, is required to induce implantation. Administration of an estrogen polyclonal antibody prevents implantation in monkeys (Ravindranath & Moudgal, 1990). However, slight deviations from the critical level of estrogen required to induce

implantation can have detrimental effects on pregnancy. In inseminated female mice, small elevations in 17β-estradiol are observed during chronic restraint stress (MacNiven et al, 1992). Injections of a 17β-estradiol monoclonal antibody will reverse pregnancy disruption due to restraint stress and exposure to novel males (deCatanzaro et al., 1994, 1995a). Also, small amounts of estradiol benzoate administered during the implantation period will disrupt pregnancy completely (deCatanzaro et al., 1991). The mechanisms underlying hormonal regulation of implantation remain elusive. Increased levels of estrogen may increase uterine contractility, delay transport of ova through the oviduct ("tube-locking"), decrease the cleavage rate, and/or inhibit the development of the blastocyte (Burdick & Whitney, 1937; deZiegler, Fanchin, deMoustier & Bulletti, 1998; Pauerstein, Sabry & Hodgson, 1976; Roblero & Garavagno, 1979). Thus, a potent pregnancy-disrupting pheromone would lead to an elevation in 17β-estradiol in inseminated females.

Steroids in novel males also appear to be important in inducing the Bruce effect. Removing the primary source of testosterone via castration will reduce novel males' ability to disrupt pregnancy (deCatanzaro & Storey, 1989). This effect is reversed when castrated males are given testosterone or 17β-estradiol (deCatanzaro & Storey, 1989; deCatanzaro, Smith & Muir, 1995b). This suggests that the pheromone secreted by novel males is androgen dependent. Yet, it also suggests that behavioural interactions between novel males and females may be important. Testosterone treatment potentiates sexual

activity in castrated males (deCatanzaro & Storey, 1989). Given direct access to inseminated females, testosterone-treated castrated males will mate vigorously and completely disrupt pregnancy. Castrated males without hormonal treatment attempt few mounts and intromissions with females. The number of intromissions has been correlated with pregnancy outcome. Thus, the sexual motivation of novel males plays an important role in the Bruce effect. One objective of this thesis is to examine the effect of recent sexual experience on novel males' ability to disrupt implantation.

There is evidence to suggest that the pheromone involved in the Bruce effect is a product of testosterone metabolism. Cyproterone acetate, which blocks androgen receptors, does not diminish the Bruce effect (Rajendren & Dominic, 1988a). This suggests that the pregnancy-disrupting substance is not a product of an androgen-dependent tissue since the receptors are blocked, but may be a product of androgen metabolism. As well, Marchlewska-Koj (1977, 1981) has demonstrated that a male mouse urinary fraction containing low molecular weight peptides has pregnancy-disrupting effects. Castration leads to a decrease in the levels of protein in urine and a loss of pheromonal activity when these proteins were applied to the nasal region of inseminated females (Marchlewska-Koj, 1977). Testosterone-treatment of castrated males and females results in the appearance of pheromonal activity in the protein fraction following nasal application on inseminated females (Marchlewska-Koj, 1977). When radio-labelled testosterone was injected into males, most of the radioactivity was observed in the protein fraction of urine (Marchlewska-Koj, 1984). These results suggest that the pregnancy-

blocking substance may be a product of testosterone catabolism bound to a peptide and excreted in male urine.

Therefore, androgens and estrogens are involved in the capacity of novel males to disrupt implantation and can also be elevated in inseminated females experiencing some forms of early pregnancy disruption. Testosterone easily aromatizes to 17β-estradiol and both lipid-soluble molecules can reach all parts of the body and have long-term actions on reproduction (Burdick & Whitney, 1937, deCatanzaro et al., 1991). Thus, I hypothesize that androgens and/or estrogens excreted by novel males in their urine may have detrimental effects on ovoimplantation, either through absorption, ingestion, or actions on the olfactory system in the female. Estrogen and androgen receptors have been isolated in several areas of the vomeronasal projection pathway including the mitral and granule cells in the AOB, the medial nucleus of the amygdala, the bed nuclei of the stria terminalis, the medial preoptic area and the arcuate nucleus of the hypothalamus (Pfaff & Keiner, 1973; Simerly, Chang, Muramatsu & Swanson, 1990). Furthermore, estrogen has been shown to have modulating effects in the vomeronasal system. This sex steroid enhances accessory olfactory information relayed to tuberoinfundibular arcuate neurons by acting on the amygdala (Li et al., 1989; Li, Kaba, Saito & Seto, 1992). Also, more directly within the AOB, estrogen can regulate the GABAergic system by increasing the response of glutamic acid decarboxylase to male odours (Navarro Becerra, Grigorjev & Munaro, 1996). As well, implants of 17β-estradiol appear to decrease the duration of olfactory memory, possibly by enhancing neurogenesis in the vomeronasal organ (Kaba, Rosser &

Keverne, 1988). Therefore, it is possible that estrogen and/or testosterone present in male urine can have direct effects within the system implicated in olfactory memory.

Since reduction of steroid levels in males can affect their ability to disrupt pregnancy and possibly their androgen and estrogen urinary output, the present studies were designed to examine the effect of novel males' excretory steroids on their ability to disrupt pregnancy. In order to measure the concentration of urinary and fecal testosterone and 17β-estradiol from novel males, a novel enzyme-linked immunosorbent assay (ELISA) has been developed. There are several benefits to measuring sex steroids metabolites in urine and feces using an ELISA. This technique provides an inexpensive, non-toxic alternative to RIAs. Also, unlike collection of blood, urine and fecal samples can be collected for prolonged periods of time without manipulating or stressing the animal. This is essential since the Bruce effect takes place over a five day exposure period to novel males. Daily human handling can cause stress and may alter the production of hormones being monitored. For example, Kamel and Frankel (1978) have shown that simply transferring males to an empty mating arena can increase testosterone levels.

Determining the concentrations of steroids from excretions reduces the number of animals needed since a large number of animals would be sacrificed if blood were to be monitored. Also, individual samples can be collected over several days. Thus, within-subject designs can be performed which can decrease between-subject variability. Furthermore, the concentration of testosterone within an individual mouse is extremely variable and results in episodic peaks of plasma testosterone (Bartke & Dalterio, 1975).

Assessment of the overall or average levels of testosterone, requires repeated sampling from individuals.

Another advantage in monitoring steroids in urine and feces is that steroid metabolite concentrations are two to four orders of magnitude higher than that of the parent steroid in blood (Peter, Critser & Kapustin, 1996). This is ideal since smaller volumes of sample are collected from small animals such as mice. An important consideration is that urinary steroid conjugates reflect plasma steroid levels. Several studies suggest that urinary steroid levels accurately reflect steroid levels in blood (Kirkpatrick, Lasley & Shideler, 1990; Lasley, Shideler & Munro, 1991; Munro, Stabenfeldt, Cragun, Addiego, Overstreet & Lasley, 1991). Also, urinary hormone concentrations can be indexed by urinary creatinine concentrations in small samples (Peter et al., 1996). Since the daily production and excretion of creatinine remains relatively constant, the ratio of hormones to creatinine in urine should not change unless there are changes in hormone production.

Previously, ELISA procedures have been validated for measuring concentrations of urinary and fecal steroids in a variety of other species (Carroll, Abbott, George, Hindle & Martin, 1990; Graham, Goodrowe, Raeside & Liptrap, 1995; Lee, Whaling, Lasley & Marler, 1995; Munro et al., 1991). Part of the goal of this thesis was to adapt this technique for use with the common laboratory mouse. Novel male urine may contain biologically active testosterone or 17β-estradiol that may come into contact with inseminated females. Since the pregnancy-disrupting chemical is transmitted via urine,

direct measurement of steroids in males' excretions rather than blood is preferred.

Focus and Outline of Experimentation

The general objective in this thesis is to gain a better understanding of the roles of sex steroids and behaviour in strange-male-induced pregnancy disruption. I have investigated two qualities of novel males that can substantially affect their capacity to disrupt pregnancy, sexual satiety and castration. I have also measured excreted steroids from novel males under various conditions. Furthermore, the effects of steroids nasally applied to inseminated females were examined.

Chapter 2

The chemosignal involved in the Bruce effect relies on androgens in males. Castrated males do not disrupt implantation (deCatanzaro & Storey, 1989). Administration of testosterone can restore this ability (deCatanzaro et al., 1995c). A reduction in male testosterone levels after successful copulation may diminish a novel male's ability to disrupt pregnancy. It has been demonstrated that copulation diminishes testosterone levels in rats (Batty, 1978; Bliss, Frishat & Samuels, 1972). I hypothesized that pregnancy disruption might be reduced by allowing males to copulate before exposing them to novel inseminated females.

Experiment 1

The purpose of this experiment was to measure the effect of novel male sexual satiety on their capacity to disrupt pregnancy. Each inseminated female was indirectly

exposed in the double decker cage system to either two novel unsated males or two novel sated males from two different strains, CF-1 and heterogeneous (HS). In the control condition, females were housed alone and not exposed to males. This experiment has demonstrated a slight, but non-significant, reduction in implantation failure among females exposed to sated males.

Experiment 2

This study was designed to enhance the degree of sexual satiety in HS novel males by introducing them to more than one hormonally-primed ovariectomized female prior to exposure to an inseminated female in the indirect exposure paradigm. The results indicated that recent sexual experience reduces the capacity for novel males to disrupt pregnancy. A higher proportion of females remained pregnant when exposed to sated males as compared to unsated males.

Chapter 3

Although castration diminishes novel males' ability to disrupt pregnancy, Bruce (1960a, 1965) demonstrated that the capacity to prevent implantation was not immediately lost following castration. The following experiments were performed to assess the time course after castration of the loss of the ability of novel males to disrupt early pregnancy in inseminated females. Studies using RIAs have shown a variable rate of loss of serum or plasma testosterone in castrated male rats (Amatayakul, Ryan, Uozumi & Albert, 1971; Ando et al., 1988; Coyotupa, Parlow & Kovacic, 1973; Gittes, Altwein, Yen & Lee, 1972; Gupta, Zarzycki & Rager, 1975; Keating & Tcholakian, 1983). It is possible that a certain

level of circulating androgens remains, which might be excreted as testosterone or 17β-estradiol. These secreted steroids from recently castrated males were also measured using an ELISA procedure.

Experiment 3

Males were castrated, and at various intervals of time following surgery inseminated females were exposed to these males in the indirect exposure paradigm. After exposure, pooled samples of urine were collected from paired castrated males and assessed for concentrations of testosterone and 17β-estradiol. Parturition was recorded among females exposed to castrated males. The results from this study demonstrated that as the interval of time increases after surgery, there was also an increase in the proportion of parturient females exposed to castrated males. Furthermore, the concentration of testosterone and 17β-estradiol being excreted by castrated males decreased as a function of time after castration. Data from this experiment indicate that the excretion of steroids from the body depletes slowly following surgery. This parallels the rate of decline of castrated males' ability to disrupt pregnancy and may be a contributing factor. Males' excreted steroids may cause a hormonal imbalance in females that might produce suboptimal conditions for implantation. As the length of time following testicular removal increases, inseminated females are exposed to fewer steroids from castrated males.

Chapter 4

These experiments were designed to examine steroid concentrations in urine and feces of intact novel males exposed to recently inseminated females, using the ELISA

technique. Since 17β-estradiol and testosterone are both present in measurable quantities in the excretions of males, the levels of these steroids may be altered when males are introduced to pregnant females. Serum testosterone levels in males are known to increase in response to direct and indirect access to females (Macrides, Bartke & Dalterio, 1975). It might be the case that endogenous androgens are excreted as testosterone or after metabolism to 17β-estradiol. Inseminated females would come into contact with these excretory steroids, which might affect their reproductive physiology.

Experiment 4

This experiment was intended to measure testosterone and 17β-estradiol in urinary and fecal samples taken from novel males during the Bruce effect. Excretions from males were collected before exposure (baseline) and on days 2, 3, 4, and 5 of pregnancy of the corresponding female. The results showed correlations of baseline concentrations of 17β-estradiol and testosterone in males' excretions with pregnancy disruption among inseminated females.

Experiment 5

In the previous experiment, males were temporarily separated from females during the process of collection of urine and feces. This may have complicated the results since exposure to males' excretions is a necessary component of the Bruce effect (deCatanzaro et al., 1999; Dominic, 1966a; Parkes & Bruce, 1962). A modified collection apparatus was used in this next study to allow for the collection of urine and feces from males while

in the presence of a female on day 3 of pregnancy. Each male was adjacent to one pregnant female, separated by a wire partition, while samples were collected. Data from this experiment indicated that males release a greater amount of 17β-estradiol when in the vicinity of recently inseminated females, as compared to being housed individually without contact with females. However, males' testosterone excretion was not similarly affected by exposure to females.

Chapter 5

Since the concentration of steroids from males is altered when they are exposed to pregnant females and correlates with parturition, these steroids may be contributing to strange-male-induced pregnancy disruption. Administration of 17β-estradiol or adrenal androgens subcutaneously will disrupt pregnancy (deCatanzaro et al., 1991). Yet, in order to consider steroids as potential pregnancy-disrupting substances, the mode of transfer must occur on the skin or at the nasal area and not through injection. Urine from novel males painted on the nasal area of pregnant females will interrupt implantation (Dominic, 1965; Marchlewska-Koj, 1977, 1981). This next experiment was designed to examine whether testosterone or estrogen applied to the nasal region of inseminated females would affect pregnancy.

Experiment 6

During the first five days of pregnancy, females were given eight applications of either various concentrations of 17β-estradiol or 17β-estradiol benzoate or testosterone propionate or vehicle (peanut oil) on the nasal area. Consistent with subcutaneous

injections of steroids (deCatanzaro et al., 1991), 17β-estradiol disrupted pregnancy at the lowest concentrations. This was closely followed by 17β-estradiol benzoate. However, testosterone propionate had no effect on implantation; all females remained pregnant. These data suggest that 17β-estradiol is very potent in disrupting pregnancy when nasally applied.

Chapter 2

Sexual Satiety Diminishes the Capacity of Novel Males to Disrupt Early Pregnancy in Inseminated Female Mice (*Mus musculus*)

Spironello, E. and deCatanzaro, D. (1999).

Journal of Comparative Psychology, 113(2), 218-222.

Early pregnancy in mammals is vulnerable to a variety of adverse environmental stimuli, particularly prior to intrauterine implantation of fertilized ova (deCatanzaro & MacNiven, 1992). One of the most potent stimuli for disrupting early pregnancy is exposure to males other than the sire. In several rodent species, exposure to novel males during the period between insemination and implantation can terminate the pregnancy (*e.g.* Bruce, 1959; 1960a; deCatanzaro et al., 1996; Heske & Nelson, 1984; Milligan, 1976; Storey, 1986), a phenomenon known as the Bruce effect.

When given direct access to recently inseminated females, novel males are activated, attempting to mount and reinseminate such females (deCatanzaro & Storey, 1989). This frequently disrupts and replaces the existing pregnancy, with pups born with gestation length and phenotypes indicative of reinsemination by the novel male. When direct sexual contact between the novel male and the previously inseminated female is prevented by separation via a wire-mesh grid, reinsemination cannot occur, but many

pregnancies are simply terminated and not replaced (deCatanzaro et al., 1996). Placement of males directly above females, such that their excretions fall into the bedding of the female, tends to disrupt pregnancy, whereas placement of the males below the females does not. While in proximity to females, males generally become agitated and difficult for humans to handle, excrete milky-white and odorous substances, and become exceptionally aggressive if other males are in their proximity (deCatanzaro et al., 1996).

Accordingly, behavioural arousal in novel males may contribute to their capacity to disrupt pregnancy in inseminated females, even when direct sexual activity between males and females is prevented. Nevertheless, much evidence implicates pheromonal factors in the Bruce effect. Novel males disrupt the hormonal balance in females not only through their behaviour but also by releasing pregnancy-disrupting substances in their excreta (deCatanzaro et al., 1996; Dominic, 1965; 1966a; Marchlewska-Koj, 1977; 1981; Parkes & Bruce, 1962). Whatever the mechanism, these actions rely on the presence of androgens in novel males. Castrated males do not block pregnancy, but testosterone administration restores their ability to disrupt pregnancy (deCatanzaro & Storey, 1989; deCatanzaro et al., 1995c; Dominic, 1965). When given access, castrated testosterone-treated males demonstrate sexual behaviour with inseminated females, whereas untreated castrated males show few mounts and no intromissions (deCatanzaro & Storey, 1989).

If housed with its own mate during the period of implantation, the sire does not show substantial mounting behaviour and does not disrupt the pregnancy (deCatanzaro & Storey, 1989). Unlike novel males, the sire has ejaculated and may be sexually satiated for

some days after insemination (McGill & Blight, 1963). It is thus conceivable that recent sexual experience, and sexual satiety insofar as it exists, might diminish the Bruce effect. The current study was designed to examine this possibility.

Proximity to novel females alters the qualities of males' excretions. Some (Dominic, 1965; 1966a) but not all (deCatanzaro et al., 1995c) reports indicate that pregnancy can be disrupted merely by painting urine of novel males on the nasal region of inseminated females. A recent study (deCatanzaro et al., 1999) found such an effect using urine taken from males housed in proximity to females without sexual access, but not employing urine taken from males not housed near females. This suggests that females stimulate males' release of the pregnancy disrupting substance. Other studies have found that the introduction of novel females, with or without physical contact, results in increased levels of testosterone and a potentiation of sexual behaviour (Bliss et al., 1972; Macrides et al., 1975; Batty, 1978; Pfeiffer & Johnston, 1992; Mosig & Dewsbury, 1976). Following ejaculation, testosterone levels decrease, and mice show a refractory period during which sexual responses cannot be elicited for hours or even days (Bliss et al., 1972; Batty, 1978; McGill & Blight, 1963). Copulation diminishes testosterone levels in rats, whereas sexually excited yet "frustrated" males, separated from females by a wire partition, show increased testosterone levels (Bliss et al., 1972).

Therefore, we reasoned that allowing novel males to copulate prior to housing them in proximity to previously inseminated females, and thus inducing a degree of sexual satiety, might diminish their capacity to disrupt early pregnancy, through behavioural or

pheromonal means or both. To investigate this possibility, we used two methods of inducing sexual satiety, involving either insemination of an intact female or exposure to multiple ovariectomized females made sexually receptive by estrogen and progesterone, just prior to exposure to the inseminated female subjects. Previously satiated or nonsatiated novel males were housed above inseminated female subjects, separated by a wire-mesh grid, in a double-decker apparatus. Two males were housed above each female subject, because this produces a more reliable effect than does just one male (deCatanzaro et al., 1996), in order to maximize sensitivity to potential effects.

Experiment 1

The first experiment involved exposing pregnant females to novel males from days 1 to 6 of pregnancy. The novel males had either just been mated with other females or had no recent sexual experience, and were either of the same strain as females and the inseminating males (CF-1) or of a distinct strain (HS). Parkes and Bruce (1961) reported that males of a distinct strain disrupt pregnancy more than do males of the same strain as the inseminated females. Previous work in this laboratory (deCatanzaro et al., 1996) shows a robust effect of outbred HS males on early pregnancy of CF-1 females, so these conditions were replicated here. We also examined males of the same strain, following reasoning that CF-1 sexually sated males could closely resemble the sire.

Method

CF-1 strain mice were obtained from Charles River Breeding Farms of Canada (La

Prairie, Quebec) or bred in this laboratory from such stock. HS mice were bred in this laboratory from stock originally obtained from the Department of Zoology (University of Toronto) and subsequently genetically enriched by interbreeding with C57, DBA, CF-1, and Swiss-Webster strains. All animals were maintained under a reversed 14:10 light/dark cycle at 21°C. Housing involved standard polypropylene mouse cages measuring 28x16x11 cm with wire grid tops permitting continuous access to food and water. Before insemination, females were housed in groups of 4 or 5. Males were all housed individually in such cages. Males of both strains had previous sexual experience prior to the commencement of the current study, but were deprived of any access to females for at least two weeks before experimental procedures began.

When aged 70-100 days, females were each housed alone with one male of CF-1 strain. The hindquarters of females were checked on three occasions per day for the presence of sperm plugs. When a plug was detected, the female was designated as a subject, with the day of detection of a sperm plug designated as day 0 of pregnancy. On day 1, 1-3 hours after the start of the dark phase of the light cycle, females were removed from the male and randomly assigned to an experimental condition, with the date of insemination counterbalanced across conditions.

Each inseminated female was indirectly exposed to either two novel males with no recent sexual experience (unmated condition) or to two novel males that had just mated with another female (mated condition). The novel mated males had each been caged with an intact female and had deposited a sperm plug in its vagina on the same day that the

female subject, with which it was matched, had received a sperm plug from the inseminating male. Exposure to novel males in both conditions was achieved via a double decker cage system as described by deCatanzaro et al. (1996). Briefly, this involved two males in an upper compartment, separated from each other by a double opaque Plexiglas shield to prevent aggressive interactions between the males. The female subject was placed in a lower compartment below both of the two males' compartments, separated from the males by a stainless steel wire mesh that allowed males' excretions to drop into the female's compartment. In a third (control) condition, females were housed alone in the lower compartment of the apparatus, with no males above. Initially, the experiment was conducted with novel males being of the same (CF-1) genetic strain as were the female subjects and the inseminating males. Subsequently, it was replicated with novel males taken from HS strain.

On day 6 of pregnancy, after approximately 120 h in the double-decker apparatus, each female was transferred to a clean normal cage with fresh bedding, then left undisturbed for the duration of gestation. Commencing on day 18 and until day 25 after detection of a sperm plug, females were checked three times daily for parturition. Pregnancy outcome was measured simply by counting the number of pups born.

Results

Data from this experiment are summarized in Table 1. For females exposed to CF-1 males, 20 of 23 were parturient in the control condition, 17 of 23 exposed to mated novel males were parturient, and 11 of 21 exposed to unmated novel males were

Table 1

Mean (±SE) Number of Pups Born and Percent Parturient in Inseminated CF-1 Females Indirectly Exposed to No Male (Control) or to Recently Mated or Unmated Novel Males of the Same (CF-1) or a Different (HS) Strain in Experiment 1

Outcome	Control	Mated males	Unmated males
CF-1 Novel Males			
Number of pups (all females)	11.04±0.99	9.39±1.24	6.62±1.45
Number of pups (pregnant only)	12.70±0.44	12.70±0.49	12.63±0.66
Percent parturient	87	74	52
HS Novel Males			
Number of pups (all females)	10.44±0.78	3.76±1.47	2.44±1.34
Number of pups (pregnant only)	11.83±0.47	12.80±0.66	14.67±1.20
Percent parturient	88	29	17

Note. HS = heterogeneous strain.

parturient. A test of association, relating occurrence of parturition to conditions, showed significant differences among the conditions, $\chi^2(2) = 6.55$, $p < 0.05$. Also, a test of association reached significance when comparing control and unmated groups, $\chi^2(1) = 6.30$, $p < 0.05$, but tests comparing each of the other pairs of conditions did not reach the conventional level of significance. Where inseminated females were exposed to males of HS strain, 30 of 34 control females, 5 of 17 females exposed to mated males, and 3 of 18 females exposed to unmated males were parturient. A test of association on these data indicated a significant overall effect, $\chi^2(2) = 33.36$, $p < 0.001$. Similar tests for all possible pairs of conditions showed significance comparing the control and unmated male conditions, $\chi^2(1) = 26.00$, $p < 0.001$, and the control and mated male conditions, $\chi^2(1) = 18.21$, $p < 0.001$.

Analysis of variance on the number of pups born (with nonparturient females assigned values of zero) yielded significant effects of strain, $F(1,116) = 13.10$, $p = 0.0007$, and of sexual experience, $F(2,116) = 10.77$, $p = 0.0002$, but no significant interaction. Multiple comparisons (Newman-Keuls' test, $p < 0.05$) showed that the control groups differed significantly from the mated and unmated male-exposed groups.

We have also run a small comparison group of CF-1 female subjects exposed to adult nonpregnant HS females, in order to assess whether animals of HS strain disrupt pregnancy due to some characteristic of the strain rather than male characteristics; 14 of 14 females were parturient in this group, with 10.93±0.62 pups per female.

Experiment 2

The results of the previous experiment show a small albeit nonsignificant reduction in the proportion of females losing pregnancy when novel males have recently mated. Since male mice frequently show more than one copulatory sequence in succession, it is possible that sexual "satiety" induced by recent mating was insufficient to produce a more substantial reduction in the capacity to disrupt pregnancy. Moreover, it is well known that male mammals can experience a "Coolidge effect" (Dewsbury, 1981) when exposed to novel females after mating; that is that sexual interest is reinvigorated by novel females subsequent to initial mating experience. The present experiment was designed to enhance the degree of sexual satiety of novel HS males by mating with more than one female prior to exposure to previously inseminated female CF-1 subjects. Females used for sexual satiation were made maximally sexually receptive via estrogen and progesterone treatment after ovariectomy. The period of exposure of female subjects to novel males was also reduced by one day, in order to diminish the probability that novel males would recover sexual motivation.

Method

CF-1 female subjects were prepared as in the previous experiment. These subjects were exposed to novel mated or unmated HS males following the same procedures as in that experiment, except for procedures used to pre-treat the mated males sexually.

For this pretreatment, adult CF-1 females were bilaterally ovariectomized and

made sexually receptive through a procedure described in detail by deCatanzaro and Gorzalka (1979). About two weeks following surgery, these females each received an s.c. injection of 10 μg of estradiol benzoate. Approximately 48 h later, each female received an injection of 500 μg of progesterone, then 6-8 hours later, each was paired with an HS male. This procedure of hormonal injections and exposure to males was repeated after one week, then again after another week, as sexual receptivity increases with such repeated hormone treatment and interactions with males. Subsequently, the hormone injections were again repeated as required, and these ovariectomized females were used for satiation of males as described below.

Preliminary data were collected on 5 HS males to observe the course of sexual satiety. Males were paired with receptive females for 2 hours per day for 5 days, with continuous observation of mounting and ejaculatory behaviour. On day 1, each male ejaculated and produced a sperm plug. On day 2, 4 males mounted and 2 ejaculated. On day 3, 4 males mounted and none ejaculated. On day 4, 3 males mounted and none ejaculated. On day 5, all males mounted and one ejaculated. From this it was estimated that most HS males would show a significant refractory period after mating with two females, although sexual satiety would remain imperfect and variable even with such procedures. We also decided to reduce the time of exposure of experimental females to novel males, relative to Experiment 1, to decrease the probability of sexual interactions during the exposure.

In the experiment proper, HS males in the mated condition were sexually satiated

by housing each with a receptive hormone-treated ovariectomized CF-1 female. When a sperm plug was detected the female was replaced with a new receptive female and the male was left with it until the following day. At that time, HS males for the mated condition were placed in the upper portions of the double decker apparatus. All other procedures were as described for the previous experiments, except that female subjects were removed from the experimental apparatus on day 5 of pregnancy, 96 h after placement in this apparatus.

Results

Data for this experiment are presented in Table 2. Among control females, 29 of 32 were parturient, while 21 of 32 females exposed to mated novel males and 11 of 32 females exposed to unmated novel males were parturient. A test of association comparing pregnancy outcome to conditions showed significance, $\chi^2(2) = 21.94$, $p < 0.001$. When testing between pairs of groups, a significant test of association was found between control and unmated groups, $\chi^2(1) = 21.6$, $p < 0.001$, control and mated groups, $\chi^2(1) = 5.85$, $p < 0.025$, as well as mated and unmated groups, $\chi^2(1) = 6.25$, $p < 0.025$. An analysis of variance on the number of pups born was significant, $F(2,93) = 13.17$, $p < 0.0001$. Multiple comparisons showed that the control condition differed significantly from the unmated condition, and that the mated condition differed significantly from the unmated condition.

Table 2

Mean (±SE) Number of Pups Born and Percent Parturient in Inseminated Females Indirectly Exposed to No Male (Control) or to Mated or Unmated Novel HS Males in Experiment 2

Outcome	Control	Mated	Unmated
Number of pups (all females)	9.91±0.81	7.84±1.08	3.22±0.92
Number of pups (pregnant only)	10.93±0.64	11.95±0.55	9.36±1.38
Percent parturient	90.6	65.5	34.4

Discussion

These data indicate that very recent sexual experience can diminish the capacity of novel males to disrupt pregnancy. This is most clear in the results of Experiment 2, where males mated with two sexually receptive females prior to their use as stimulus animals for previously inseminated female subjects. In that experiment, previously mated males did produce some pregnancy disruption relative to the control condition, but more pregnancies remained among females exposed to mated males than among females exposed to males that had not recently mated.

Sexual "satiety" is clearly an imperfect and variable phenomenon in male mice. This was evident in the preliminary trials conducted for males in preparation for Experiment 2, where it was observed that, after an initial mating, some males would mate again when given access to a second receptive female. The refractory period after ejaculation in mice is a process that is variable across individuals (McGill & Blight, 1963), and some males are capable of two or more copulatory series in succession (deCatanzaro & Griffiths, 1996). Males of a number of mammalian species can be aroused subsequent to mating if presented with a novel female (Dewsbury, 1981). Males are generally aroused by previously inseminated females during the preimplantation period (deCatanzaro & Storey, 1989), even when access is blocked by a wire-mesh grid (deCatanzaro et al., 1996). It is likely therefore that many of the mated males in Experiment 1 were sexually aroused by previously inseminated females when placed in the exposure apparatus.

These data also replicate the established phenomenon that novel males are

generally more effective in disrupting pregnancy when they are of a distinct strain from that of the inseminated females and the sires of the original pregnancy (Parkes & Bruce, 1961). This is clear in data from Experiment 1. One possibility is that males of some strains generally have a greater capacity to disrupt pregnancy than do others. Other possibilities involve distinctiveness of the sire and the novel male. Clearly, it is not novelty *per se* that is responsible for this, as HS females had no impact on pregnancy of CF-1 females in Experiment 2, despite the fact that HS mice are phenotypically very distinct (being multicolored) from uniformly albino CF-1 mice. Nevertheless, some role of recognition, in interaction with male characteristics of novel stimulus animals, could be involved; CF-1 novel males resemble the actual sire more than do HS males. This factor could be important for the female's perception that a male is distinct from the sire, although results of Experiment 2 suggest again that novelty of a male is not sufficient, because mated males are still distinct from the sire but disrupted pregnancy less than did unmated males. It could also be important for the mated males' behaviour toward females; males might be more aroused by females whose phenotypes differ from those which they have just mated. In Experiment 2, where the clearest diminution of pregnancy disrupting capacity was induced by recent sexual experience, we used CF-1 females to satiate novel HS males. The present data are not sufficient to determine which combination of these factors is responsible.

Recently mated males may emit fewer excretions that have an impact upon females' existing pregnancies, due to depletion of the sources of these excretions through mating,

the natural hormonal consequences of mating, reduced behavioural arousal subsequent to sexual satiety, or some combination of these factors. One possibility involves androgen levels in the novel males, since testosterone levels decrease following ejaculation (Bliss et al., 1972; Batty, 1978), and the Bruce effect is known to involve androgen-dependent factors in novel males (deCatanzaro et al., 1995c, 1996; Dominic, 1965).

Chapter 3

Novel Male Mice Continue to Disrupt Early Pregnancy and to Excrete Testosterone and 17β-Estradiol During the Weeks Immediately Following Castration

Pregnancy disruption may result when a recently inseminated female mouse (*Mus musculus*) is introduced to an unfamiliar male during the first five days of pregnancy (the "Bruce effect", e.g. Bruce, 1959, 1960a). Although there may be behavioural components to this effect when novel males are housed directly with females (deCatanzaro & Storey, 1989; Storey, 1996), a substantial amount of evidence suggests that males' chemical emissions or pheromones contribute to this effect (Bruce, 1960a, 1960b; deCatanzaro et al., 1999; Dominic, 1965, 1966a; Marchlewska-Koj, 1981; Parkes & Bruce, 1961, 1962).

Males' capacity to induce the Bruce effect is androgen-dependent. Castrated males do not disrupt pregnancy, however chronic administration of testosterone or 17β-estradiol restores their ability to disrupt pregnancy (deCatanzaro & Storey, 1989; deCatanzaro et al., 1995b; 1995c). Furthermore, unlike normal females, androgenized females can disrupt pregnancy (deCatanzaro et al., 1995c; Dominic, 1965). Although castration immediately eliminates the body's primary source of testosterone, it may take some time for the hormone to be depleted from adipose and other stores in the body and excreted, while the impact of castration upon other tissues like male sex accessory glands may require time. When castrated males were exposed to females 6 and 18 weeks after surgery, Bruce

(1960a) found that 26% of females returned to estrus. In a subsequent study Bruce (1965) demonstrated a 20%, 14%, and 12% implantation failure rate among females exposed to males 3, 6 to 8, and 16 to 20 weeks after castration, respectively. However, the interpretation of these data is not entirely clear, because the novel males were of the same genetic strain (Parkes) as the inseminated females, while other data indicate that novel males of the female's strain are not generally effective in disrupting pregnancy (Spironello & deCatanzaro, 1999). Elsewhere, when castrated males of the wild (feral) type were introduced to pregnant females of the Parkes strain three weeks after castration, 36.4 % of females lost pregnancy (Rajendren & Dominic, 1988a). In the course of previous investigations (*e.g.* deCatanzaro et al., 1995b), recently castrated males have been observed to disrupt some pregnancies in our laboratory.

Castration results in the loss of other androgen-dependent behaviours such as sexual behaviour, but the nature and rate of the decline varies with the species, genotype within species, and degree of sexual experience prior to castration (Manning & Thompson, 1976). In dogs, sexual behaviour may be exhibited for several months after castration, in some cases enduring for years (Beach, 1970). In rhesus monkeys, 30% displayed the complete pattern of sexual behaviour including ejaculation one year after castration (Resko & Phoenix, 1972). In rodents following castration, the ejaculatory response is typically lost first, within a few weeks of the surgery, then later intromissions are lost, and subsequently the male will cease even attempting to mount (Beach & Holz-Tucker, 1949; Davidson, 1966; Whalen, Beach & Kuehn, 1961). Frequency of testing and

sexual experience can also play roles (Beach, 1970; Bloch & Davidson, 1968; Lisk & Heimann, 1980; Rosenblatt & Aronson, 1958).

RIAs have revealed that castration alters androgen levels in male rodents, however the results vary substantially across studies, in part due to assay protocols and sensitivity, strains of rats, handling procedures, and time of day of sampling. In one report (Amatayakul et al., 1971), serum testosterone decreased to half of the control concentration within 21 days and to 20% within 45 days following castration. Other reports (Coyotupa et al., 1973; Keating & Tcholakian, 1983) indicate clearance of testosterone from circulation within less than a day following castration, with the rate of testosterone clearance varying among rats. Other studies indicate significant, albeit incomplete, reductions of plasma testosterone within 4 or 5 days of surgery (Ando et al., 1988; Gupta et al., 1975), and nondetectable levels of serum testosterone two weeks after castration in rats (Gittes et al., 1972).

Our study was designed to assess the time course of castrated males' proficiency in disrupting pregnancy following surgery, in order to shed light on mechanisms subserving the Bruce effect. We exposed castrated males to pregnant females of a different strain using an indirect exposure paradigm that produces reliable disruption of implantation (deCatanzaro et al., 1996; Spironello & deCatanzaro, 1999). Novel males were housed above pregnant females separated by a wire-mesh grid to reduce behavioural interactions and reinsemination, and each female was exposed to two males to increase the size of the effect and produce sensitivity to experimental manipulations.

We also sampled urine from the novel males, and assayed concentrations of testosterone and 17β-estradiol at various intervals after castration using a previously validated (Appendix I) ELISA procedure. This is important given that implantation in females is established to be very sensitive to androgens and estrogens, in particular being disrupted by extremely low exogenous dosages of 17β-estradiol (deCatanzaro et al., 1991; Whitney & Burdick, 1936), and the potential therefore that androgens and estrogens emitted by novel males could contribute to pregnancy loss.

Experiment 3

Method

Castration

During surgical sessions on 5 separate days, 102 mature HS males were castrated through bilateral removal of the testes via a single perineal incision under sodium pentobarbitol anesthetic i. p. and Xylocaine applied at the site of incision. Damage to sex accessory glands was carefully avoided. Once the testes were excised, both the muscle wall and the skin layer were sutured. Animals were kept warm on a heating pad until normal activity resumed. Castrated males were then each housed individually in clean standard polypropylene cages.

Novel male exposure paradigm

The subjects and insemination procedures were the same as those described in Experiment 1. On day 1 of pregnancy, 1-3 hours after the start of the dark phase of the

light cycle, females were removed from the inseminating males. Experimental females were then housed under castrated males in double-decker cages following procedures described in Experiment 1. The time interval since castration of the novel males was varied over a spectrum of times, such that the exposure began at various intervals between 6 and 43 days after surgery. At various points, 70 other inseminated females were randomly assigned to be simply housed alone, without males, in order to provide a control condition and to ensure that a consistent baseline pregnancy rate was maintained throughout the experiment.

In some cases, paired novel males previously used at a shorter interval after castration were reused as stimulus novel males at a longer interval. In order to assess the possibility that repeated exposure to pregnant females may be a factor in diminishing the ability to disrupt implantation, 17 pairs of males without prior experience and 23 pairs of males with previous experience were used as stimulus animals at the same intervals since surgery.

In order to assess whether data from the 6-week interval represent the maximal loss of pregnancy-disrupting capacity, an additional sample of 27 inseminated females was prepared and exposed to castrated males at intervals of 4 to 7 months after the surgery. The exposure procedures were identical to those described above.

Urine collection

At various intervals after castration, two HS males were exposed to a recently inseminated female for the first five days of pregnancy. On day 6 of pregnancy, two hours

after the dark phase of the light cycle, the upper compartment of the double-decker cage was placed on a clean surface until 500 μL of pooled urine was collected from paired castrated males. The urine was aspirated with sterile 1cc syringes with 23 gauge needles and stored without preservatives at 20°C until assayed for steroid content. In addition urine was collected and stored from 20 intact HS males using the same procedure.

Pregnancy outcome measures

After 120 hours of exposure to novel males in the experimental apparatus, each female was removed from the apparatus and placed alone in a clean cage for the duration of gestation. Commencing on day 18 of pregnancy, and daily thereafter until day 25, each female was checked three times each day for parturition. (Gestation length of CF-1 females in this laboratory has never exceeded 23 days.) Pregnancy outcome was measured simply by counting the number of pups born.

Assay procedures

ELISA procedures generally followed those described for other mammals (Munro et al, 1991); validations for this laboratory for adult male mice are reported elsewhere (Appendix 1). For 17β-estradiol, the interplate coefficient of variation (CV) was 8.4% at 30% bound and 4.1% at 70% bound, and the intraplate CV was 8.7%. For testosterone, the interplate CV was 6.7% at 30% bound and 3.4% at 70% bound, and the intraplate CV was 7.1%. Creatinine, 17β-estradiol, and testosterone were obtained from Sigma Chemical. All antibodies (anti-E2 R4972 and anti-T R156/7) and all corresponding horseradish peroxidase conjugates were obtained from the Department of Population

Health and Reproduction at the University of California, Davis. All plates were measured for optical density using a Bio-tek Instruments Inc. EL 312E microplate reader.

Due to variations in fluid intake and output in animals, an index of urinary hormone concentrations is performed on a relatively constant urinary metabolite, creatinine. This procedure is similar to those described by Munro et al. (1991). Standard creatinine values of 100, 50, 25,12.5, 6.25 and 3.12 μg/ml were used, with distilled water as zero. All urine samples are diluted 1:50 urine:phosphate buffer (0.1 mol/L sodium phosphate buffer, pH 7.0 containing 8.7g of NaCl and 1g of BSA per liter). Using Dynatech Immulon flat bottom plates, 50 μl per well of standard were added with 50 μl distilled water, 50 μl 0.75 N NaOH and 50 μl 0.4 N picric acid. The plate was then shaken and incubated at room temperature for 30 minutes. The plate was measured for optical density on a plate reader (Bio-tek Instruments Inc. EL 312E) with a single filter at 490nm. Standard curves were generated, regression lines were fit, and the regression equation was applied to the optical density for each sample to obtain values expressed in ng creatinine/ml urine.

The assay for detection of 17β-estradiol and testosterone was carried out on NUNC Maxisorb plates which were first coated with 50 μl of antibody stock diluted at 1:10,000 in a coating buffer (50 mmol/L bicarbonate buffer pH9.6) and stored for 12-14 h at 4°C. Wash solution (0.15 mol/L NaCl solution containing 0.5 ml of Tween 20 per liter) was added to each well to rinse away any unbound antibody, then 50μl phosphate buffer

per well was added. The plates were incubated at room temperature for 2-5 hours for 17β-estradiol and 30 min for testosterone before adding standards, samples, or controls. For estradiol, urine samples were diluted 1:8 in phosphate buffer before being added to the plate. For testosterone, urine was diluted 1:4 each in phosphate buffer. For each hormone, two quality control urine samples at 30% and 70% binding (the low and high ends of the sensitive range of the standard curve) were prepared. For all assays, 50μl estradiol- or testosterone- horseradish peroxidase was added to each well, with 20μl of standard, sample, or control for estradiol or 50μl of standard, sample, or control for testosterone. The plates were incubated for 2 hours at room temperature. Next, the plates were washed and 100μl of a substrate solution of citrate buffer, H_2O_2 and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) was added to each well and the plates covered and incubated while shaking at room temperature for 30-60 minutes. The plates were then read with a single filter at 405nm on the microplate reader. Blank absorbance was subtracted from each reading to account for non-specific binding. All values were adjusted for creatinine simply by taking the ratio of the obtained value over the measure of creatinine for the particular sample.

Results

Figure 1 represents the percent of females pregnant following exposure to castrated males at several intervals after surgery. Clearly, the ability to disrupt pregnancy diminished with increasing time since surgery. Figure 2 represents the number of pups

Figure 1
Percent of previously inseminated females parturient after exposure to novel castrated males, presented at six day intervals after surgery.

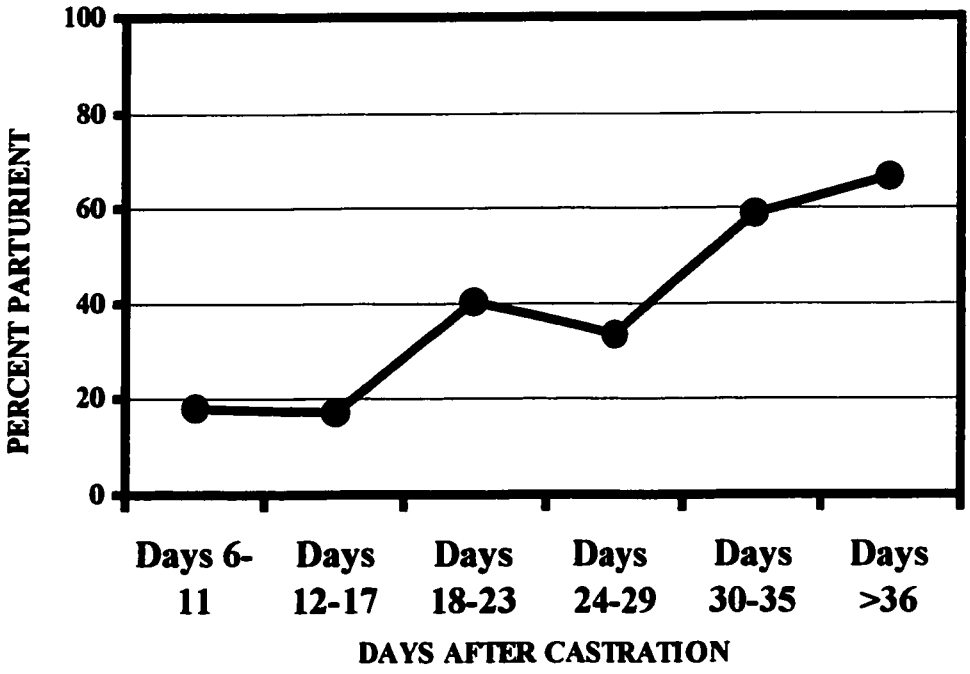
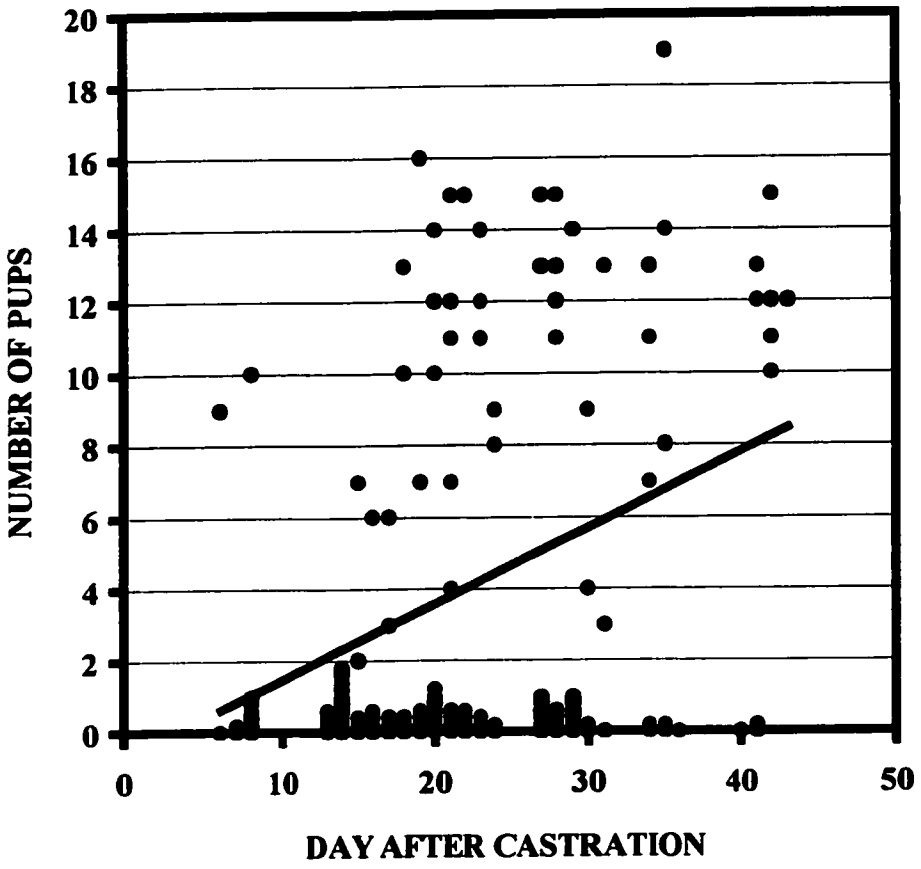


Figure 2
Number of pups born to each previously inseminated female related to the day after castration of the novel males. Each point on the abscissa represents the day of commencement of a five-day indirect exposure to two castrated males. In order to present all data points, overlapping points were slightly displaced. Points equal to zero were stacked between the values zero and one at the bottom of the ordinate, such that all values less than one were actually zero. The least-squares linear regression line is shown.



born from females exposed to males commencing 6 to 43 days after castration. On the abscissa, each point on the graph represents the commencement of a five day exposure to castrated males. The ordinate gives the number of pups observed after parturition was completed. Because some data points overlap, some are slightly displaced so that all data points can be shown. Excluding controls (females housed alone), there was a significant correlation between the time since castration of the stimulus male and the number of pups born, $r = 0.355$, $t(136) = 4.43$, $p < 0.001$. The regression equation gives a predicted number of pups equal to $-1.13 + (0.22 \times \text{day after castration})$. We also assessed whether higher order curves might better depict the relationship (see Pedazur, 1982). The quadratic curvilinear regression yielded an R-value of 0.360, while the cubic trend yielded an R-value of 0.363. *F* tests on the increments in the correlation value did not reach significance, therefore a linear trend best represents the data. We also conducted a point-biserial correlation between the day after castration and the dichotomous variable of pregnancy outcome of the corresponding female (with 0 designated as nonparturient and 1 as parturient); this similarly reached significance, $r = 0.30$, $t(136) = 3.68$, $p < 0.001$.

Table 3 presents pregnancy outcome in females exposed to no males (control) or castrated males at various intervals after surgery. The number of pups born was calculated both considering all females (with zero values for nonparturient females) and considering only those females with litters. Fewer females delivered litters when housed under castrated males soon after surgery compared to weeks or months after surgery. Among control females not exposed to males, 85.7% were parturient. Among females

Table 3

Mean (±SE) Number of Pups Born and Number Parturient in Inseminated CF-1 Females Indirectly Exposed to No Male (Control), Males Commencing 6-8 Days, 40-43 Days, and 4-7 Months After Castration

Outcome	Control	6-8 Days	40-43 Days	4-7 Months
Number of pups (all females)	8.77 ± 0.56	1.73 ± 1.16	9.09 ± 1.82	8.81 ± 1.15
Number of pups (parturient only)	10.23 ± 0.42	9.50 ± 0.50	12.50 ± 0.63	12.53 ± 0.39
n parturient	60	2	8	19
n	70	11	11	27

exposed to males at the shortest interval since castration in the study (6-8 days after castration), 18.2% were parturient. Among females exposed to males at the end of the six-week period (40-43 days after castration), 72.7% were parturient. For females exposed to males 6-8 days after surgery, the number of pups was significantly different from that for controls, $F(1,79) = 22.33$, $p < 0.001$, and from that for females exposed to males 40-43 days after surgery, $F(1,20) = 11.67$, $p < 0.001$. Tests of association were similarly significant, $\chi^2(1) = 24.15$, $p < 0.001$ and $\chi^2(1) = 6.60$, $p < 0.025$, respectively. No differences were found between females exposed to castrated males at the end of the six-week period and control females.

In samples conducted at comparatively long intervals, four to seven months after surgery, 70 percent were parturient (see Table 1), with a mean (±SE) number of pups born being of 8.81 (± 1.15). There were no significant differences between control females and females exposed to males four to seven months after castration.

At intervals since castration of 19 to 31 days, some of the stimulus HS males had previously been used for female subjects at shorter intervals, while others were used for the first time. The dispersion of times since castration in the two groups was similar. These were compared statistically in order to assess the effects of repeated testing on the ability to disrupt pregnancy. Of the 23 females housed below males with previous exposure, 7 (30.4%) delivered litters and had a mean (±SE) number of pups of 3.30 (±1.15). The number of parturient females exposed to castrated males for a single time was 4 out of 17 (23.5%) and had an average of 2.29 (±1.11) pups. A chi-square test of

association and a t-test showed that castrated males that had been repeatedly exposed to females did not differ significantly in their ability to disrupt pregnant compared to castrated males only exposed to females once.

Figure 3 and 4 represent the concentration of testosterone and 17β-estradiol, respectively, in pooled male urine collected from intact males and at various intervals after castration. The regression equation for the predicted levels of urinary testosterone was determined to be equal to 218.0 - (4.43 X day after castration) with a correlation of $r = -0.586$, $t(156) = -9.04$, $p < 0.001$. For urinary 17β-estradiol, regression analysis calculated a predicted concentration of 345.74 - (3.31 X day after castration) with a correlation of $r = -0.326$, $t(156) = -4.31$, $p < 0.001$. Removing the intact male data, the regression equation for the predicted levels of urinary testosterone was determined to be equal to 100.82 (0.64 X day after castration) with a correlation of $r = -0.140$, $t(136) = -1.65$, $p = 0.10$. For urinary 17β-estradiol, regression analysis calculated a predicted concentration of 286.7 (1.39 X day after castration) with a correlation of $r = -0.118$, $t(136) = -1.38$, $p = 0.169$.

Discussion

These data indicate that following castration, males gradually lose their ability to disrupt pregnancy over a 6-week period. Within a week after surgery, males can still disrupt pregnancy in previously inseminated females at rates similar to those induced by intact HS males in the same paradigm (deCatanzaro et al., 1996). The probability that

Figure 3
Concentration of testosterone in the urine of intact males and castrated males, pooled in pairs each exposed to one inseminated female, collected at the end of exposure to the female, at various intervals after castration. The least-squares linear regression line is shown.

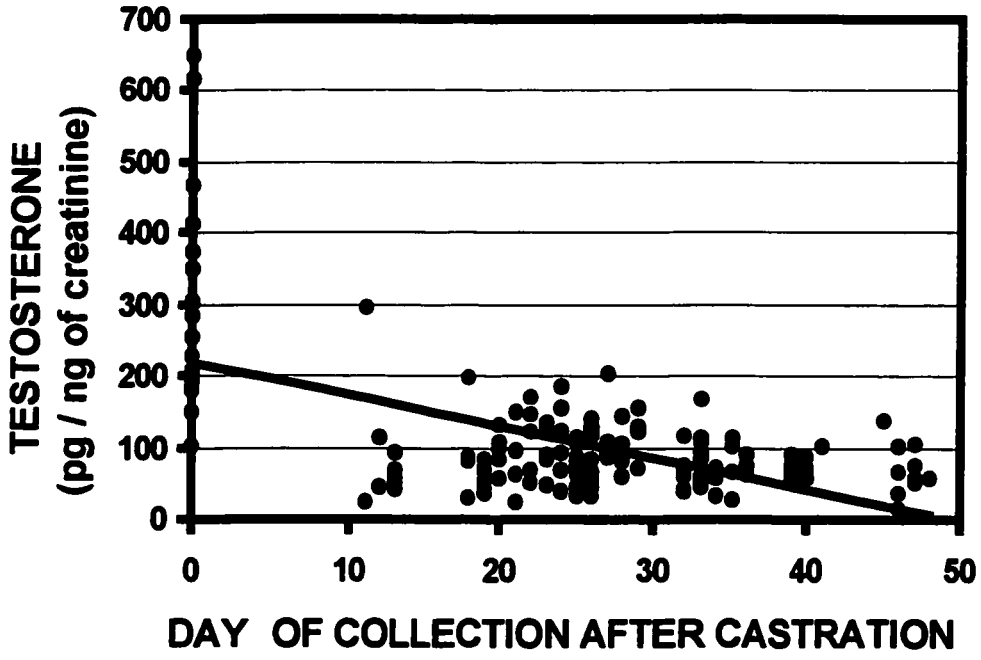
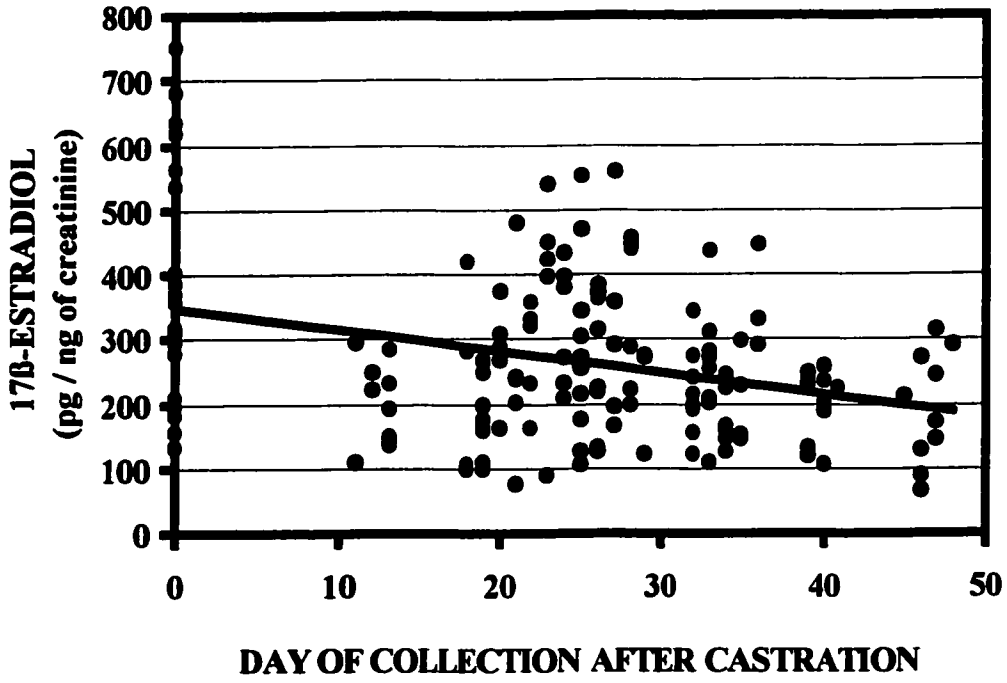


Figure 4
Concentration of 17β-estradiol in the urine of intact males and castrated males, pooled in pairs each exposed to one inseminated female, collected at the end of exposure to the female, at various intervals after castration. The least-squares linear regression line is shown.



males induce pregnancy loss diminished in a roughly linear fashion over time since castration. About 70% of females were parturient after exposure to males that were castrated about 6 weeks previously, and there was no further increase in pregnancy rate when the interval was increased to several months. The effect is probabilistic, as some females remained pregnant after exposure to very recently castrated males while some females were not parturient when exposed to males castrated several weeks previously. The effect is all-or-none, in that litter size among parturient females is similar regardless of condition.

These findings are consistent with the gradual loss of other androgen-dependent functions, such as sexual behaviour, in castrated male rodents (Beach & Holz-Tucker, 1949; Whalen et al., 1961). Bruce (1965) also found a small non-significant decline in the ability to disrupt pregnancy over time following castration, but she did not examine the shorter intervals after castration examined here. She also used the same strain of females and novel males in her study, which could diminish the potential contrast over time compared to the present study.

These data provide the first measures of steroid levels in male mouse urine, in conjunction with validations and preliminary measures provided elsewhere (Appendix I). Testosterone and 17β-estradiol are measurable in male urine, even 50 days after castration, with substantial variation among individuals. It is probable that testosterone and estradiol remain bound to adipose, various receptors, and other tissues following testicular removal, and hence are only gradually lost via various modes of excretion. Also,

the adrenal glands remain as a continuing source of sex steroids after castration, and there is evidence of a small compensatory increase in adrenal androgens in male rats subsequent to castration (Bardin & Peterson, 1967). Without intact male data, the gradual decline in steroid levels is not significant. However, since excretions were not collected immediately following castration, the intact male data provide a measure of normal steroid concentrations in excretions before surgery. The present data (including intact males) are consistent with studies indicating that testosterone levels decline gradually after castration (*e.g.* Amatayakul et al., 1971), and less consistent with those showing rapid decline following the surgery (*e.g.* Coyotupa et al., 1973; Keating & Tcholakian, 1983). The data for 17β-estradiol, which is aromatized from testosterone, are consistent with one study (Hellman & Fishman, 1970) suggesting that circulating estrogen decreases after castration, but not with other studies that show no difference in circulating levels of estradiol following castration (Sinchak, Roselli & Clemens, 1996; Carroll, Weaver & Baum, 1988). However, the current data involve urinary excretions, whereas previous studies have involved blood samples.

With respect to the continued Bruce effect initially following castration, there may be organizational effects of androgens and estrogens upon other processes in the nervous system and other tissues, which would require some time to dissipate. Various male accessory glands, including the preputials, the vesicular-coagulating complexes, and the prostate, are androgen dependent and produce excretions with pheromonal properties. Recent evidence from this laboratory shows that the preputial and vesicular-coagulating

glands are not relevant to the Bruce effect, since removal of such glands does not diminish the capacity of novel HS males to induce this effect (deCatanzaro et al., 1996; Zacharias et al., 2000). MUPs excreted in urine are known to depend on testosterone titer and have been implicated in having a role as a pheromonal carrier in the Bruce effect (Brennan et al., 1999). As yet, the ligand bound to MUPs that would produce pregnancy disruption has not been isolated.

According to several investigators, the pregnancy-disrupting substance involved in the Bruce effect is a product of testosterone metabolism that is released in the males' urine (deCatanzaro et al., 1996; Marchlewska-Koj, 1981; Rajendren & Dominic, 1988a). It is possible that these secreted testosterone metabolites might be absorbed by pregnant females. In particular, excess estrogens can disrupt intrauterine implantation of fertilized ova by altering the timing of transport through the fallopian tubes (Whitney & Burdick, 1936), and they are also known to induce estrus, which may be incompatible with pregnancy, via actions in the hypothalamus (Pfaff, 1980). One possibility is that the males' excreted steroids could themselves induce a hormonal imbalance in females that might produce suboptimal conditions for endometrial receptivity (deCatanzaro et al., 1991).

Chapter 4

Enzyme Immunoassay of 17β-Estradiol and Testosterone in Urinary and Fecal Samples from HS Males Exposed to Inseminated Females

Several reports indicate that 17β-estradiol plays a significant role in early pregnancy disruptions. DeCatanzaro et al. (1991) demonstrated that injections of estradiol benzoate at minute doses will completely disrupt implantation in inseminated females. Also, circulating levels of 17β-estradiol were elevated in females exposed to restraint stress during the implantation period (MacNiven et al., 1992). Pregnancy can be maintained, however, if inseminated females are given 17β-estradiol monoclonal antibodies when exposed to restraint stress or novel males during the first five days of pregnancy (deCatanzaro et al., 1994, 1995a). Thus, a critical level of 17β-estradiol needs to be maintained in order for implantation to occur.

Early pregnancy disruption in the Bruce effect has been attributed to the female's sensitivity to urinary excretions from males other than the sire. Urine from males has been demonstrated to affect other reproductive functions in females (Bronson & Whitten, 1968; Vandenbergh, 1969). Also, females show a preference for the odour of intact males as opposed to that of castrates (Gandelman, 1983). This preference can be abolished by gonadectomy, and restored by administration of testosterone or estrogen (Gandelman, 1983). The pregnancy-disrupting substance is clearly androgen-dependent since castration

reduces novel males' ability to disrupt pregnancy, which can be reversed with testosterone or 17β-estradiol treatment (Bruce, 1965; deCatanzaro et al., 1995b; deCatanzaro et al., 1995c; deCatanzaro & Storey, 1989; Dominic, 1965). Behaviourally, compared to castrated males, intact males are more assertive with females, and with direct access, sexual activity has been shown to correlate with the disruption of pregnancy in proximate inseminated females (deCatanzaro et al., 1995c; deCatanzaro & Storey, 1989). Furthermore, sexual satiety has been shown to diminish novel males' capacity to disrupt pregnancy (Spironello & deCatanzaro, 1999).

The pheromone involved in the Bruce effect also appears to act on the female through contact. Females housed in cages that have previously been soiled by novel males will experience a higher rate of implantation failure than do control females (Dominic, 1966a; Parkes & Bruce, 1962). Topical application of male urine or salted out urinary proteins on the nasal areas of recently inseminated females can lead to pregnancy disruption (Dominic, 1965; Marchlewska-Koj, 1977, 1981). A recent study has shown that urine collected from males housed near females will disrupt pregnancy more effectively than urine collected from isolated males (deCatanzaro et al., 1999). Further evidence suggesting that the pheromone is non-volatile is demonstrated by the observation that females housed above novel males, separated by a wire grid, do not generally lose pregnancy (deCatanzaro et al., 1996). In this indirect exposure paradigm, females need to be housed below males and therefore come into direct contact with the excretions of novel males above (deCatanzaro et al., 1996). These experiments show that inseminated

females are directly exposed to the chemical involved in the Bruce effect through the excretions of novel males.

Several studies have suggested a number of factors that may be mediating the Bruce effect, including an individual memory trace of the sire formed within the vomeronasal organ during mating, and a dopaminergic inhibition of prolactin leading to failure of the luteotrophic support of pregnancy (Brennan et al., 1990; Li et al., 1989; Rajendren & Dutta, 1988). The nature of the pheromone has not been determined, but there are experiments that point to MUPs as potential candidates, since MUPs have enough genetic diversity to possibly code for strain or individual differences (Robertson et al., 1996, 1997). These MUPs are androgen dependent and are found in higher quantities in males than females (Hastie et al., 1979; Rajendren & Dominic, 1984). They generally are bound to ligands that may have pheromonal properties, but have not been identified to have pregnancy-disrupting capabilities (Bacchini et al., 1992; Brennan et al., 1999).

Another pheromonal candidate might be sex steroids excreted by novel males that may be absorbed or ingested by inseminated females. This would cause a hormonal imbalance leading to unfavourable conditions for endometrial receptivity. Castrated males gradually lose their ability to disrupt pregnancy and show a decline in the urinary concentrations of testosterone and 17β-estradiol being excreted (Chapter 3). In order to determine what may be contributing to the transmission of the Bruce effect, the present studies were designed to assay the excretions of males for testosterone and 17β-estradiol using an ELISA technique adapted for mice. The validations and characterization of this

assay for male house mice are outlined in Appendix I. Similar procedures have been employed to measure urinary and fecal steroids in other species such as cats, birds, humans, and non-primates (Carroll et al., 1990; Graham et al., 1995; Lee et al., 1995; Munro et al., 1991). In the present experiments, urine and fecal samples were collected from novel males during exposure to recently inseminated females and analyzed for steroid content. It was hypothesized that either testosterone and/or 17β-estradiol will be secreted in higher quantities when males are introduced to pregnant females as compared to when they are isolated.

Experiment 4

Since circulating levels of testosterone are important for the ability of males to prevent implantation, novel males may be excreting testosterone or 17β-estradiol in their urine or feces. These excreted steroids may have an impact on the inseminated female's hormonal status. In this experiment, recently inseminated females were exposed to males via the double decker cage system for the first five days of pregnancy. Samples of urine and feces were collected beginning on the morning of each day, for days 2-5 of pregnancy of the corresponding female. These samples were then analysed for concentrations of testosterone and 17β-estradiol.

Method

The subjects and insemination procedures were the same as that described in

Experiment 1. On day 1, 1-3 hours after the start of the dark phase of the light cycle, females were removed from the inseminating male and were exposed to no males (control condition) or to two novel HS males, with the date of insemination counterbalanced across conditions.

Inseminated females were exposed to two novel males in a double-decker apparatus similar to that described in Experiment 1. In this paradigm, however, pregnant females, in the lower compartment, were housed on a wire grid floor that was situated on fine wood-chip bedding in a stainless steel tray. This design allowed for the collection of both male and female samples. When urine was collected, each section of the cage was placed on a clean surface until 500 µl of urine and 0.5-1gram of feces from males were collected. Collection of urine and feces generally required 8-12 hours. The urine was aspirated with sterile syringes with 23 gauge needles and the feces were collected with sterile forceps. Urine and fecal samples were collected for each HS male 3-4 days before exposure to inseminated females and also from days 2 to 5 of pregnancy of the corresponding female. An effort was made to collect samples on day 3 of pregnancy, but insufficient urine was produced by males to complete the measure. All samples were stored without preservatives at -20^{0}C until assayed for steroid content.

After 120 hours in the double-decker cage system, females were placed in a standard clean cage and left undisturbed for the duration of gestation. Litter checks began 18 days after insemination and continued until day 30, or until all of the pregnant females had delivered. Pregnancy outcome was measured by counting of the number of pups

born.

Fecal Extraction and Urinary Creatinine Adjustment

Each 0.5 g of fecal sample was treated with 2.5 ml of extraction buffer (27.8 g NaH_2PO_4 in 1L 20% methanol, 28.4 g Na_2HPO_4 in 1L 20% methanol, 8.7 g NaCl, 1 g BSA, 500 ml 20% methanol, pH 7.0), broken up and tumbled 360^0 in test tubes at 20 rpm overnight. The following morning, they were centrifuged at 2500 rpm for 15 min and the supernatant was poured off and stored at -20^0C until assayed for steroid content. For urine, creatinine was measured using the same procedures as that described in Experiment 3.

ELISA Procedures

The ELISA procedures are similar to those described in Experiment 3. For 17β-estradiol, fecal extracts were diluted 1:4 and the urine samples 1:8 in phosphate buffer before being added to the plates. For testosterone, fecal extracts were diluted 1:16 and urine samples were diluted 1:4 in phosphate buffer. For each hormone, two quality control urine and fecal samples at 30% and 70% binding (the low and high ends of the sensitive range of the standard curve) were prepared. After the plates were read with the microplate reader, standard curves were generated, regression lines were fit and sample optical density values were interpolated into the regression equation to calculate sample concentrations.

Results and Discussion

Of females exposed to two novel males, 6 of 10 were parturient, while 9 of 10 of

control females delivered litters. The mean number of pups born, with zero assigned to non-parturient females, was 7.9 ± 2.2 for females exposed to males and 10.5 ± 1.5 for control females. A chi-square test of association for the number of females parturient and an analysis of variance for the number of pups born did not reach significance.

Levels of urinary and fecal testosterone and 17β-estradiol for exposure males are shown in Table 4. Figures 5 and 6 give a breakdown of the urinary testosterone and 17β-estradiol data in relationship to parturition of associated females. Significant correlations were found between the measure taken before exposure and the presence or absence of parturition for testosterone, $r(17) = 0.52$, $p = 0.024$, and for 17β-estradiol, $r(15) = 0.52$, $p = 0.034$ (sample sizes differed as a few samples were of insufficient quantity for measurement), but correlations at other times were not significant.

The high proportion of pregnant females exposed to novel males in this study might be due to the prolonged removal of males from females during the collection of samples. Since the pregnancy-disrupting substance is non-volatile (deCatanzaro et al., 1996), decreased exposure to novel males, and their androgen-dependent emissions, would diminish the disruption of implantation in inseminated females. Also, separating males from females during collection and pooling samples from two males may have made collection of urine more difficult. First of all, males may have been previously excreting most of their urine before collection in an effort to scent mark their own territory, as well as release emissions on the pregnant female. Secondly, during collection, paired males tended to urinate along the division line of the double-occupancy cage. When this

Table 4

Mean (±SE) levels of testosterone (T) and 17β-estradiol (E_2) in urine (creat.=creatinine) and feces from exposure males. Measures were taken 2 days before contact with females, days 2, 4, and 5 after insemination during exposure

Day	T-urine (pg/ng creat.)	T-fecal (ng/g feces)	E_2-urine (pg/ng creat.)	E_2-fecal (ng/g feces)
Before	275.2 ±30.2	5.0 ±.05	377.6 ±47.7	8.1 ±0.7
2	235.7 ±20.0	4.6 ±0.4	326.9 ±28.4	9.4 ±0.9
4	310.0 ±55.8	4.5 ±0.4	284.1 ±31.8	7.6 ±0.5
5	243.5 ±28.0	3.2 ±0.4	340.8 ±39.2	6.4 ±0.6

Figure 5
Mean (±SE) levels of urinary testosterone in males exposed to females during days 1-5 after insemination. Males associated with non-parturient females are presented separately from those associated with parturient females.

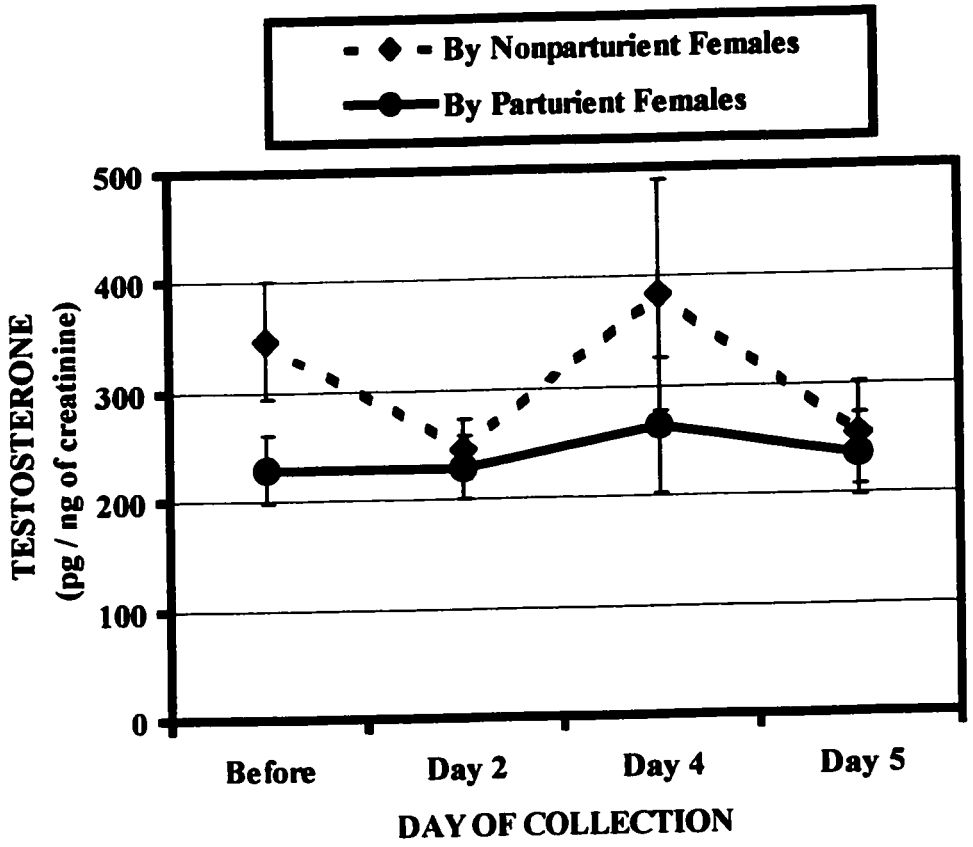
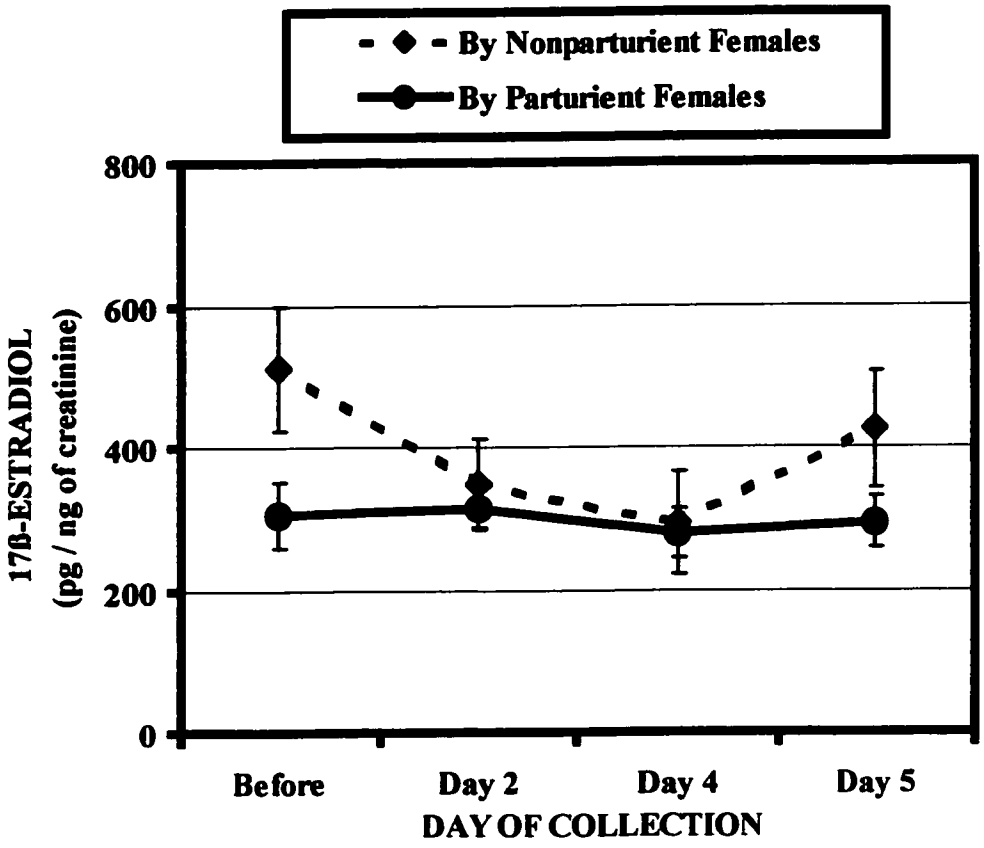


Figure 6
Mean (±SE) levels of urinary 17β-estradiol in males exposed to females during days 1-5 after insemination. Males associated with non-parturient females are presented separately from those associated with parturient females.



occurred, samples could not be collected due to the ambiguity in identifying which male urinated in that area. As such, Experiment 5 was designed to improve upon these difficulties.

Experiment 5

This experiment used a modified caging apparatus that allowed for the collection of individual male's excretions while in the presence of one pregnant female. Therefore, males were separated from females during the collection procedure. Also, obtaining urine from each male as opposed to pooling urine from paired males might shorten the collection time since there would be no ambiguities as to the identity of the urine. Since collection of male urine is generally a more tedious process than that of females, excretions were collected before exposure (baseline) and only on one day of pregnancy (day 3) of the corresponding female. In this study, males were housed beside females, separated by a wire partition. Since males will only disrupt pregnancy when they are housed above females and not beside females (deCatanzaro et al., 1996), parturition will not be measured. Also, since the steroid content of feces did not correlate with pregnancy outcome in Experiment 4, only urine from males was analysed.

Method

Animal and insemination procedures were as in the previous experiment. All 46 males were housed in the collection cages for two days before samples of urine were

retrieved in isolation (baseline) 1-3 hours after the start of the dark phase of the light cycle. After this 2-day period alone in the apparatus and completion of urine collection, 29 males (experimental group) were exposed to inseminated females. Stimulus females for this group were placed into one of the compartments of the collection cage, on the morning of day 1 of pregnancy, with a male beside them in the other compartment. On Day 3 of pregnancy of the stimulus females, urine was collected from the males. The other group of 17 males (control group) was not exposed to inseminated females and a second baseline sample was collected 1-3 hours after the dark phase of the light cycle.

Collection Apparatus

During the collection procedure, subjects were housed in clear Plexiglas cages (30x21x27 cm), divided into two compartments measuring 30x21x13 cm each. Males and females were separated by a wire-mesh grid with square openings measuring 0.71x0.71 cm. The wire-mesh grid permitted some male-female interaction, without risk of insemination. Both the male and female had access to an independent supply of food and water. The food pellets were placed in a small circular Tupperware container, closed with a second wire-mesh grid with openings measuring 1 cm^2. These collection cages were laid on steel trays measuring 85x31 cm. The bottom of each collection cage had a similar wire-mesh grid floor with square openings measuring 0.71 cm^2. This allowed the excretions to fall onto the tray where they could easily be collected.

Urine Collection

For each collection cage, male and female excretions were sectioned off using a

strip of silicone sealant on the steel tray. This sectioning ensured that the excretions of each mouse remained separated, as it fell onto the tray where it was collected. Collection of all samples began 1-3 hours after the start of the dark phase of the light cycle. During collection, the cages were placed on a lower shelf surface momentarily until 500 μl of urine was retrieved with sterile syringes with 23 gauge needles. Collection of urine required approximately 8 hours. Following the morning of day 6 of pregnancy, males and females were removed from the collection cages and housed separately.

Results and Discussion

Table 5 shows the mean testosterone and 17β-estradiol concentrations in male urine under the various conditions. For males not exposed to females, a paired t-test showed no significant differences between two baseline concentrations of urine for either hormone. For males exposed to females, a paired t-test revealed a significant difference between urinary levels of 17β-estradiol excreted during baseline collection compared to that excreted during exposure to a recently inseminated female on day 3 of pregnancy, $t(28) = -2.12$, $p < 0.05$. Furthermore, the second baseline urinary 17β-estradiol measure collected from males not exposed to females was significantly different from males exposed to inseminated females, $t(44) = 2.02$, $p < 0.05$. For urinary testosterone levels, a paired t-test showed no significant change when males were exposed to females on day 3

Table 5

Mean (±SE) levels of testosterone and 17β-estradiol in urine (creat.=creatinine) from males. Measures were taken during two isolated (baseline) conditions as well as during a baseline condition followed by exposure to an inseminated female on day 3 of pregnancy

Control Group	Baseline (pg/ng creat.)	Baseline (pg/ng creat.)
Testosterone	126.57 ± 8.44	156.27 ± 17.19
17β-Estradiol	252.49 ± 17.95	208.92 ± 15.78
Experimental Group	**Baseline (pg/ng creat.)**	**Exposed to Inseminated Females (pg/ng creat.)**
Testosterone	134.17 ± 9.41	131.8 ± 10.40
17β-Estradiol	260.01 ± 15.99	298.29 ± 15.84

of pregnancy compared to baseline levels. These results demonstrate that samples collected when males were housed individually do not differ from one another. However, introduction of a female can increase the level of 17β-estradiol but not testosterone being excreted by males.

Discussion

These results suggest that males' excretions of steroid hormones, especially 17β-estradiol, are affected by exposure to pregnant females and are correlated with pregnancy outcome in proximate inseminated females. Higher concentrations of 17β-estradiol are found in males exposed to females on day 3 of pregnancy compared to isolated males. Also, males excreting higher baseline levels of 17β-estradiol or testosterone were associated with an increase in pregnancy failure in the corresponding females.

Samples collected in Experiment 4 demonstrated that HS males with higher baseline urinary testosterone or 17β-estradiol levels prior to the commencement of the experiment, disrupted pregnancy more effectively than did males that exhibited lower concentrations of testosterone or 17β-estradiol at that time. This finding is consistent with the literature that shows the dependence of pregnancy disruption on testosterone (Bruce, 1965; deCatanzaro et al., 1995c; deCatanzaro & Storey, 1989; Dominic, 1965). However, significant increases in urinary or fecal testosterone or 17β-estradiol during exposure to pregnant females were not observed. Yet, studies have demonstrated

increases in circulating testosterone in males upon introduction to a female (Batty, 1978; Bliss et al., 1972; Macrides et al., 1975; Mosig & Dewsbury, 1976; Pfeiffer & Johnston, 1992). Another study has shown decreased testicular function in group housed males, especially the subordinate males (McKinney & Desjardins, 1973). Therefore, pairing males and collecting samples prior to the beginning of the experiment might have confounded the results by changing their testosterone levels, regardless of subsequent exposure to females.

Collection of male urine is difficult since males tend to urinate episodically and spray in small quantities. Males use their urine as a social resource and aim their urine toward females, thereby affecting females' reproductive status (deCatanzaro et al., 1996; Drickamer, 1995; Reynolds, 1971). The collection process was tedious and would take a number of hours before a sufficient quantity of urine was retrieved. In Experiment 4, males and females were separated during collection which may have complicated the results. Experiment 5 was performed to measure steroids in male excretions while males were exposed to females across a wire grid.

The fifth experiment showed that the quality of male urine changed with exposure to a pregnant female. This was also observed in a study in which chronic housing in proximity to females increased the potency of novel male urine in disrupting pregnancy (deCatanzaro et al., 1999). Therefore, social context or stimulation influences the chemical composition of male urine. Novel males whose sexual motivation has been reduced through castration also produce qualitatively different excretions and their ability

to disrupt pregnancy is diminished (Chapter 3).

The results of Experiment 5 indicate that the level of 17β-estradiol but not testosterone changes in males' urine with exposure to a pregnant female. A prior study has demonstrated that serum testosterone levels increase with exposure to a female (Macrides et al., 1975). Testosterone might then be excreted, at least in part, in the form of 17β-estradiol. Estradiol is important for implantation of ova and is elevated in females experiencing early pregnancy disruption (MacNiven et al., 1992). Administration of antibodies against 17β-estradiol can reverse the effects of restraint stress and strange-male-induced pregnancy disruption (deCatanzaro et al., 1994, 1995a). Since males tend to target their urine at females (Drickamer, 1995; Reynolds, 1971), 17β-estradiol found in male urine might be contributing to the Bruce effect. Novel males may be exposing females to an excess amount of 17β-estradiol that may produce suboptimal conditions for implantation.

Chapter 5

Topical Application of 17β-Estradiol on the Nasal Region Terminates Implantation in Inseminated Female Mice

The previous chapters have demonstrated that the concentrations of testosterone and/or 17β-estradiol in males' urine are affected by castration and exposure to inseminated females. Males gradually excrete less of both steroids following testicular removal. Males also excrete more urinary 17β-estradiol but not testosterone in the presence of a female than when they are isolated.

It is known that 17β-estradiol in the female is highly regulated during early pregnancy (Psychoyos & Casimiri, 1980). Subcutaneous injections of minute levels of 17β-estradiol can cause a complete disruption of pregnancy (deCatanzaro et al., 1991). Also, RIA has demonstrated slight increases in circulating 17β-estradiol levels in female mice exposed to restraint stress during implantation (MacNiven et al., 1992). Additionally, an ELISA has shown that urinary and fecal testosterone and 17β-estradiol are elevelated during the implantation period in females that are not parturient (Muir, deCatanzaro, Spironello & Pisani, submitted). Administration of antibodies against 17β-estradiol can prevent the loss of pregnancy due to restraint stress experienced during the implantation period (deCatanzaro et al., 1994). Similarly, the Bruce effect can be prevented with administration of 17β-estradiol antibodies (deCatanzaro et al., 1995a).

Elevated levels of 17β-estradiol during the implantation period have been shown to be involved with lysis of the corpus luteum, affecting the transportation of the ova into the uterus, and producing adverse conditions for endometrial receptivity (deZiegler et al., 1998; Pauerstein et al., 1976; Roblero & Garavagno, 1979). Furthermore, 17β-estradiol can induce estrus at the ventromedial hypothalamus (Pfaff, 1980).

Since estradiol has the potential to alter females' reproductive status, the presence of estradiol in males' excretions may be involved in early pregnancy disruption. The Bruce effect is mediated by a pregnancy-disrupting substance in urine that comes into direct contact with the female. Application of novel male urine on the nasal area of recently inseminated females can disrupt implantation, especially if urine was obtained from males in the vicinity of females (deCatanzaro et al., 1999; Dominic, 1965). As well, salted out urinary proteins applied to the nasal region can result in failure of the ova to implant (Marchlewska-Koj 1977, 1981). It also appears that the vomeronasal organ in the olfactory system is the site of action of the pheromone. Removal of the vomeronasal organ prevents implantation failure (Lloyd-Thomas & Keverne, 1982; Rajendren & Dominic, 1984, 1986). Therefore, in order to consider estradiol as a potential pheromone, it should be able to have pregnancy-disrupting capabilities when nasally applied to inseminated females.

The chemical in urine that disrupts pregnancy relies on androgens. The ability to disrupt implantation is diminished with castration and can be restored with injections of testosterone or 17β-estradiol (Bruce, 1965; deCatanzaro et al., 1995b, 1995c;

deCatanzaro & Storey, 1989; Dominic, 1965). Males in the presence of females have higher serum testosterone levels and with exposure to pregnant females excrete more 17β-estradiol in their urine (Chapter 4; Macrides et al., 1975). Circulating testosterone readily metabolizes to 17β-estradiol which when present in male urine can directly contact inseminated females and may have estrus-inducing effects.

There have been other suggestions for possible pheromones involved in the Bruce effect. MUPs may act as carriers that transport molecules with pheromonal properties (Bacchini et al., 1992). Although molecules such as brevicomins and dihydrothiazoles have been found to affect female reproductive behaviour, a recent study has shown that these ligands cannot disrupt pregnancy when applied to the nasal area of females (Brennan et al., 1999; Novotny et al., 1999b; Schwende et al., 1986). Since novel males naturally excrete a pregnancy-disrupting substance, 17β-estradiol, in their urine, this study examined whether steroids could prevent implantation when nasally applied to inseminated females.

Experiment 6

Method

CF-1 females were inseminated as described in the previous experiments. Following insemination, each female was removed from the male, housed individually in a clean cage with fresh bedding, and assigned to one of the experimental conditions, counterbalanced across age and date of insemination.

Nasal administration of hormones was achieved by giving each female eight applications of the same dose. For each application a No. 6 artist's paintbrush (Curry's Series 2600, Hamilton, Ontario) was dipped into the solution. The base of the female's tail was held to restrain its movement, and 5 strokes were made with the brush aiming for the hairless tip of the snout proximate to the nostrils. Attempts were made to minimize human handling of the animal during this process. Each application took approximately 10-15 sec. On day 1 of pregnancy, 8 h after commencement of the dark phase of the lighting cycle, each female received the first administration. On each of days 2, 3, and 4 of pregnancy, each female received two additional administrations at 1 and 8 h after commencement of the dark phase. On day 5, at 1 h after commencement of the dark phase, each female received a final administration. The administrator was blind with respect to the dosage of the solutions being applied to particular animals.

Steroids were obtained from Sigma Chemical Co., St. Louis. Concentrations for 17β-estradiol were 18, 6, 2, 0.67, 0.22, and 0 μg of 17β-estradiol in 0.05 cc of peanut oil. Concentrations for 17β-estradiol benzoate were 54, 18, 6, 2, 0.67, and 0 μg of 17β-estradiol benzoate in 0.05 cc of peanut oil. Concentrations for testosterone propionate were 202, 67, 22, 7.5, and 0 μg of testosterone propionate in 0.05 cc of peanut oil. In order to convert these to approximate doses per animal per application, the paintbrush was subsequently weighed before and after the application process.

Following the experimental manipulations, females were left undisturbed for the duration of gestation. Approximately 20 days after insemination, parturition was

measured by recording the number of pups born.

Results

Figures 7 and 8 present the percent of females parturient after nasal administration of 17β-estradiol and 17β-estradiol benzoate, respectively. Tables 6, 7, and 8 provide measures of the number of pups born including nonparturient females, the number born excluding these females, and the sample sizes for each of the experimental groups. All females receiving testosterone propionate were parturient. However, females nasally given 17β-estradiol or 17β-estradiol benzoate were generally not parturient, except at the very lowest doses for each hormone.

For nasal administration of 17β-estradiol, a chi-square test of association showed a significant reduction of pregnancy, $\chi^2(5) = 38.34$, $p < 0.001$. An ANOVA on the number of pups born, including nonparturient females, was significant, $F(5,43) = 25.25$, $p < 0.00001$. Multiple comparisons (Newman-Keuls) showed that the control (0.0) dosage differed significantly from all other conditions, and that the 0.22 concentration group differed from all higher dosages. For nasal administration of 17β-estradiol benzoate, a chi-square test of association revealed a significant reduction in pregnancy, $\chi^2(5) = 42.05$, $p < 0.001$. An ANOVA reached significance on the number of pups born, $F(5,44) = 30.81$, $p < 0.00001$. Newman-Keuls multiple comparisons ($p < 0.01$) showed that the control (0.0) and 0.67 concentration groups differed significantly from all higher dosages,

Table 6

Mean (±SE) Number of Pups Born and Proportion Parturient in Inseminated CF-1 Females Nasally Treated with 17β-Estradiol During Days 1 to 4 of Pregnancy

µg/0.05cc oil	Pups/female	Pups/litter	Proportion parturient
0	11.6 ±1.3	12.6 ±0.8	12/12
0.22	7.1 ±2.2	10.0 ±1.7	5/7
0.67	0.0 ±0.0	0.0 ±0.0	0/7
2	0.0 ±0.0	0.0 ±0.0	0/7
6	0.0 ±0.0	0.0 ±0.0	0/8
18	0.0 ±0.0	0.0 ±0.0	0/8

Table 7
Mean (±SE) Number of Pups Born and Proportion Parturient in Inseminated CF-1 Females Nasally Treated with 17β-Estradiol Benzoate During Days 1 to 4 of Pregnancy

µg/0.05cc oil	Pups/female	Pups/litter	Proportion parturient
0	10.4 ±1.3	11.4 ±0.9	11/11
0.67	9.7 ±1.7	11.1 ±1.1	7/8
2	0.0 ±0.0	0.0 ±0.0	0/8
6	0.0 ±0.0	0.0 ±0.0	0/7
18	0.0 ±0.0	0.0 ±0.0	0/8
54	0.0 ±0.0	0.0 ±0.0	0/8

Table 8

Mean (±SE) Number of Pups Born and Proportion Parturient in Inseminated CF-1 Females Nasally Treated with Testosterone Propionate During Days 1 to 4 of Pregnancy

µg/0.05cc oil	Pups/female	Proportion parturient
0	12.9 ±0.7	7/7
7.5	11.3 ±1.2	7/7
22	12.9 ±1.3	7/7
67	12.7 ±1.8	7/7
202	11.3 ±0.6	7/7

Figure 7
Percent of inseminated females parturient after nasal application of varied concentrations of 17β-estradiol on days 1 to 4 of pregnancy.

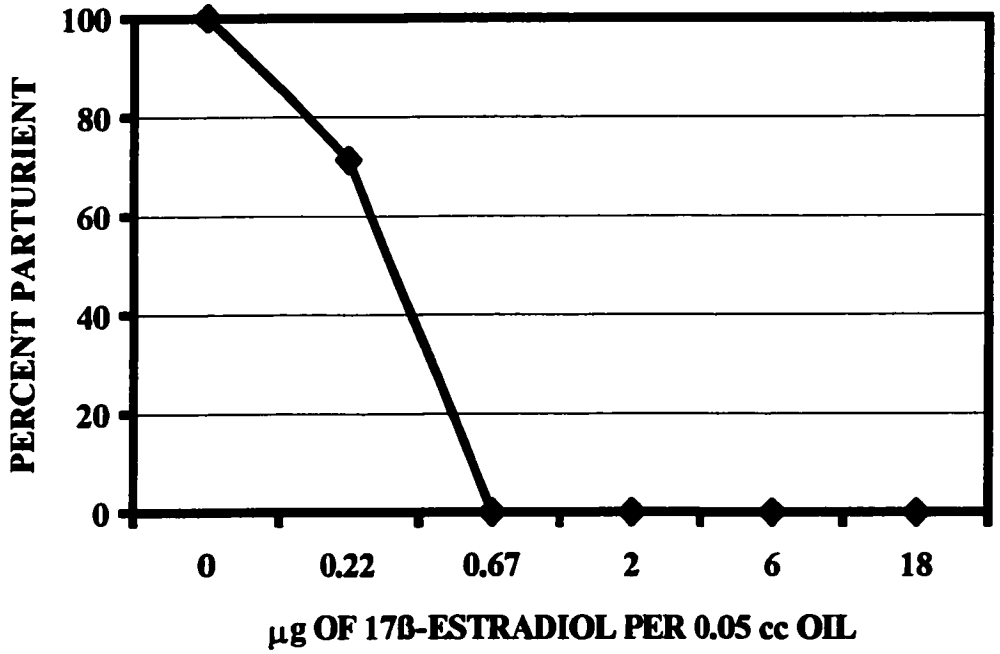
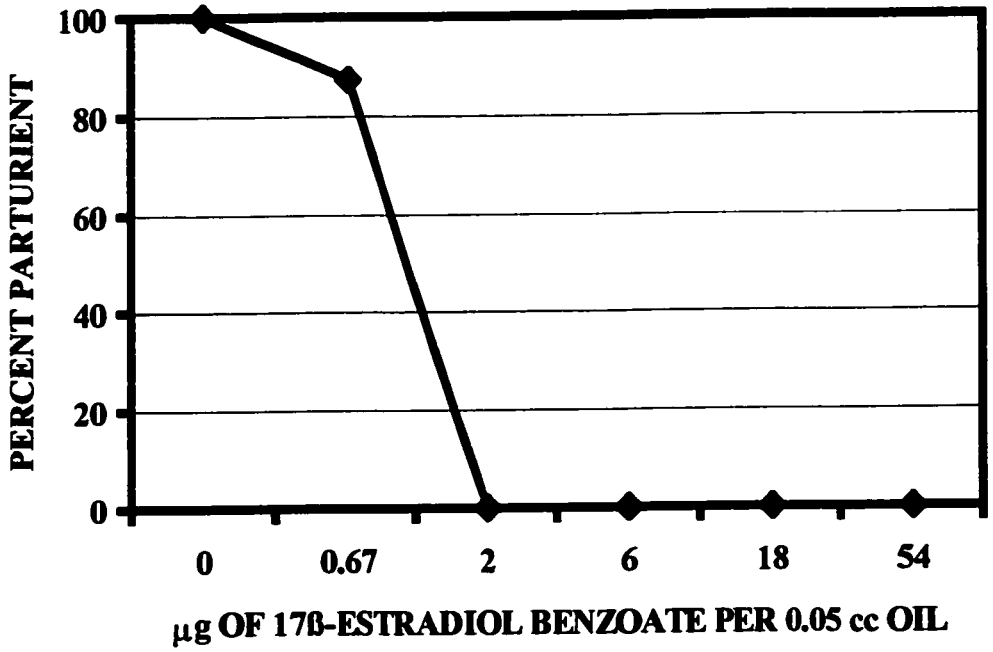


Figure 8
Percent of inseminated females parturient after nasal application of varied concentrations of 17β-estradiol benzoate on days 1 to 4 of pregnancy.



but not from each other. For nasal administration of testosterone propionate, since all females were parturient, statistics are not necessary.

In order to quantify the dosage per animal in nasal application, the paintbrush was weighed before and after application of 0.67 μg 17β-estradiol/0.05 cc oil, the lowest concentration that completely disrupted pregnancy. Ten samples of the first application of this dose yielded a mean (±SE) of 0.0494±0.0034 g on each female. Following reasoning that the quantity deposited could be reduced in subsequent applications because some oil conspicuously remained on the nasal area during repeated administrations, measures were repeated for a second application, yielding values in 9 samples of 0.0379±0.0030 g on each female. Weighing samples of peanut oil, its specific gravity was determined to be 88% of that of water. Therefore, the estimate for the first application was calculated to be approximately 0.75 μg/animal and for the second application, 0.58 μg/animal. For the 0.22 μg 17β-estradiol/0.05 cc oil, which partially disrupted pregnancy, the estimates are 0.25 μg/animal for the first application and 0.19 μg/animal for the second application.

Discussion

These results demonstrate that very low doses of 17β-estradiol or 17β-estradiol benzoate applied to the nasal area of recently inseminated females can completely disrupt pregnancy. However, testosterone applied even at high doses does not prevent implantation. This is similar to subcutaneous administration of steroids where the lowest

concentration to disrupt pregnancy occurs with 17β-estradiol (deCatanzaro et al., 1991). Therefore, 17β-estradiol appears to be the most potent steroidal agent to prevent implantation. This is consistent with the literature that suggests that estrogen levels in the female are an important factor in ovoimplantation. Administration of 17β-estradiol antibodies will prevent pregnancy disruption in the face of restraint stress or exposure to strange males (deCatanzaro et al., 1994, 1995a).

The actual amount of 17β-estradiol reaching the circulatory system may be lower than predicted since some of the oil was displaced to other areas through grooming and females may have ingested the oil. There are several possible sites of action for 17β-estradiol to affect pregnancy. Estradiol may be directly acting on the uterus which can have several consequences leading to implantation failure (deZiegler et al., 1998; Pauerstein et al., 1976; Roblero & Garavagno, 1979). There are also areas in the brain that contain 17β-estradiol receptors. Very small quantities of 17β-estradiol can induce estrus when delivered directly to the ventromedial hypothalamus (Pfaff, 1980). Also, 17β-estradiol may be acting on the vomeronasal organ or the amygdala implicated in olfactory memory (Kaba et al., 1988; Li et al., 1989, 1992). In this case, binding of 17β-estradiol to receptors in the AOB or the amygdala would activate a set of neuroendocrine events leading to decreased luteotrophic support of the corpus lutea. There may also be other unknown actions of 17β-estradiol that could prevent implantation.

Other pure chemicals have not been shown to have a capacity to disrupt

pregnancy. Urine or urinary proteins from males will prevent implantation when administered directly on the nasal region of inseminated females (deCatanzaro et al., 1999; Dominic, 1965; Marchlewska-Koj 1977, 1981), but the precise active agent in urine that will result in the same effect has not been identified. MUPs that are bound to ligands may have pregnancy disrupting effects, however, the appended putative pheromones have not been isolated (Bacchini et al., 1992; Brennan et al., 1999).

Novel males actively direct urine towards previously inseminated females and their sexual behaviour has been correlated with pregnancy outcome (deCatanzaro et al., 1996; deCatanzaro & Storey, 1989; Drickamer, 1995; Reynolds, 1971). Altering males' sexual motivation or hormonal status through sexual satiety or castration affects their ability to disrupt pregnancy (Chapter 2; deCatanzaro et al., 1995c; deCatanzaro & Storey, 1989). Since male urine contains 17β-estradiol and levels of this hormone are elevated in the presence of a pregnant female (Chapter 4), sexually motivated and intact males may be targeting this steroid at inseminated females. To date, 17β-estradiol is the most potent external chemical to disrupt implantation. Males that direct their urine towards females will be exposing them to 17β-estradiol, which may contribute to the disruption of implantation of fertilized ova.

Chapter 6

General Discussion

General Summary of Thesis Findings

The experiments in this thesis have demonstrated two ways in which the capacity for novel males to disrupt pregnancy can be reduced, through sexual satiety and castration. The influences of sexual satiety upon novel males' capacity to disrupt pregnancy have never been previously examined. Using a novel immunoassay, I have also measured for the first time 17β-estradiol and testosterone in males' excretions under various conditions in mice. Furthermore, I have also demonstrated that 17β-estradiol can disrupt pregnancy when applied to the nasal region of inseminated females during the implantation period.

Experiments in Chapter 2 were designed to investigate the role of recent sexual experience in novel males' capacity to disrupt pregnancy. Sexual behaviour, in particular the number of intromissions attempted by intact novel males, has been shown to correlate negatively with measures of pregnancy outcome (deCatanzaro & Storey, 1989). After copulation, males' sexual activity is temporarily suspended (McGill & Blight, 1963), which may affect their ability to disrupt pregnancy. For Experiments 1 and 2, males were allowed to mate with females prior to indirect exposure to previously inseminated novel female subjects. Mated males were less effective at disrupting pregnancy than were

unmated males. Thus, recent copulation can diminish novel males' ability to prevent implantation. This suggests, among other possibilities, that sexually-motivated behaviour might be important in inducing pregnancy disruption.

Another method of decreasing sexually-motivated behaviour in males is via castration (deCatanzaro & Storey, 1989). One purpose of the research reported in Chapter 3 was to examine the time course of the ability of novel males to disrupt implantation after castration. During days 1-5 of pregnancy, previously inseminated females were housed underneath castrated males at various intervals after surgery. Castrated males continued to disrupt implantation a few weeks after surgery. Progressively, the probability of retention of pregnancy increased as a linear function of time since castration. There was an apparent asymptote, where the majority of females remained pregnant, beginning at about 6 weeks following surgery. Therefore, the ability to disrupt implantation is gradually lost following castration.

Although castration might diminish novel males' ability to disrupt pregnancy through behavioural means, a substantial amount of evidence suggests that pheromonal factors contribute to this effect (Bruce, 1960a, 1960b; deCatanzaro et al., 1999; Dominic, 1965, 1966a). Novel males disrupt pregnancy in females by releasing pregnancy-disrupting substances in their excreta. The pheromone is thought to be non-volatile and androgen-dependent. Castration eliminates the primary source of testosterone in males. Yet, some level of circulating androgens remains, which might be excreted as testosterone or 17β-estradiol. The studies in Chapter 3 also examined the concentrations of urinary

testosterone and 17β-estradiol excreted by castrated males at various points after surgery using an ELISA procedure. Both steroids in male urine decreased as a linear function of time after testicular removal.

In Chapter 4, the concentrations of urinary steroids from novel males were further examined during exposure to inseminated females. In Experiment 4, novel males were indirectly exposed to inseminated females on days 1-5 of pregnancy. In this paradigm, the levels of testosterone and 17β-estradiol in novel HS males' urine prior to exposure to females were positively correlated with females' implantation failure. In Experiment 5, novel males were adjacent to females, separated by a wire grid, during the first 5 days of pregnancy. The results from this study show that novel males excreted more urinary 17β-estradiol in the presence of females on the third day of pregnancy compared to isolated males.

Since the concentration of steroids excreted by novel males is altered when they are exposed to inseminated females and correlates with parturition, these steroids may be contributing to the Bruce effect. Injections of 17β-estradiol in females can disrupt implantation (deCatanzaro et al., 1991). However, the pheromone involved in the Bruce effect acts on the female through external contact. Novel male urine can disrupt pregnancy when nasally applied to females (Dominic, 1965; Marchlewska-Koj, 1977, 1981). The research reported in Chapter 5 was designed to investigate whether steroids could affect pregnancy when topically applied to the nasal area of inseminated females.

On days 1-5 of pregnancy, 8 nasal applications of 17β-estradiol, 17β-estradiol benzoate or testosterone were given to females. The results demonstrated that 17β-estradiol and 17β-estradiol benzoate terminate pregnancy at very low doses. For 17β-estradiol, a complete disruption of implantation was observed at all doses greater than or equal to approximately 1 μg per day. All females were parturient when testosterone was nasally applied to inseminated females. The results, taken with those reported in other chapters, suggest that 17β-estradiol excreted in the urine of novel males has the potential to prevent implantation when directly contacting inseminated females.

Implications for the Steroid Hypothesis

The proposed steroid hypothesis suggests that 17β-estradiol, excreted and possibly targeted at females by novel males in their urine, may have adverse effects on implantation, either through absorption, ingestion, or actions on the olfactory system in the female.

Results from the satiety and castration studies suggest two possible means to reduce novel males' capacity to disrupt pregnancy. The reasons why this occurs have never been thoroughly explored. The presence of novel males' excretions is required for the Bruce effect since novel male urine alone can disrupt implantation when applied to the nasal area of inseminated females (Dominic, 1965; Marchlewska-Koj, 1977, 1981). One explanation is that testicular removal and recent sexual experience are altering the quantity of these excretions that can induce implantation failure. Perhaps these two procedures,

mating and castration, are depleting the source of these excretions that can affect females' reproductive physiology. One consideration is that the testes are the source of the pheromone since their removal reduces novel males' capacity to disrupt pregnancy. Yet, the capacity to interfere with pregnancy is not immediately lost after castration and requires some time to dissipate. Thus, the quantity of pheromone released from males may decrease slowly over time following surgery. This has been demonstrated with the concentration of testosterone and 17β-estradiol being excreted from males in urine after castration. Both steroids gradually decrease in quantity after gonadectomy in males.

Behavioural interactions between males and females may also be contributing to the reduction in the ability to disrupt implantation after castration and mating. Recently mated males and castrated males do not disrupt pregnancy as effectively as do intact, unmated males. Copulation and castration both affect males' subsequent sexual behaviour. Castrated or mated male mice show diminished sexual responses and arousal toward females and castrated males become less aggressive toward other males (Batty, 1978; deCatanzaro et al., 1995c; McGill & Blight, 1963). Intact unmated males, however, are sexually activated in the presence of females and generally demonstrate intermale aggression (deCatanzaro et al., 1996). Sexual behaviour has been shown to correlate with the Bruce effect, with the number of intromissions being negatively related to pregnancy outcome (deCatanzaro & Storey, 1989). Furthermore, studies investigating urine marking have found that males will deposit urine on the body of novel females (Taylor, Haller, Bartko & Weiss, 1984). This behaviour is diminished with castration and restored with

testosterone treatment (Taylor, Bartko & Farr, 1987). Therefore, in addition to castration altering the quantity of steroids being excreted, the amount of urine targeted at the female is diminished as well.

Another hypothesis to explain the reduction in the ability of castrated and mated males to disrupt implantation might involve the natural hormonal consequences of mating and castration. Circulating testosterone levels decrease following ejaculation and castration (Bliss et al., 1972; Batty, 1978; Amatayakul et al., 1971), and testosterone replacement can restore the ability of castrated males to disrupt pregnancy (deCatanzaro et al., 1995c). Androgens within the male may be excreted in the form of testosterone or 17β-estradiol and directly contact inseminated females, which might affect their reproductive physiology. The steroid, 17β-estradiol, can have direct effects on females' physiology. Estradiol may be acting on areas important in reproduction, such as the ventromedial hypothalamus or the uterus. In the hypothalamus, 17β-estradiol is known to have estrus-inducing effects (Pfaff, 1980). At the uterus, 17β-estradiol can have detrimental consequences such as transportation or developmental difficulties of the blastocyte that can prevent implantation (deZiegler et al., 1998; Pauerstein et al., 1976; Roblero & Garavagno, 1979).

The concentration of 17β-estradiol in male urine is altered by exposure to females. Novel males excrete more urinary 17β-estradiol in the presence of inseminated females compared to when males are housed alone. A previous study has shown that urine

collected from males housed in proximity to females can disrupt pregnancy when applied to the nasal region of inseminated females (deCatanzaro et al., 1999). This effect is not observed with urine taken from isolated males. Therefore, males release the pregnancy-disrupting substance more so in the presence of females than when they are isolated. The same effect is found with 17β-estradiol.

Since males target their urine at females (Drickamer, 1995; Reynolds, 1971), inseminated females may be exposed to 17β-estradiol in male urine. Estradiol is important for implantation and can disrupt pregnancy through injection or nasal application on inseminated females (Chapter 5; deCatanzaro et al., 1991). There is an elevation of 17β-estradiol in females experiencing early pregnancy disruption which can be prevented with administration of 17β-estradiol antibodies (deCatanzaro et al., 1994, 1995a; MacNiven et al., 1992). Therefore, upon introduction to an inseminated female, novel males excrete more 17β-estradiol in their urine, and when directly contacting females can produce detrimental conditions for endometrial receptivity.

The only substances that have been found to disrupt pregnancy via topical application are urine or salted out urinary proteins from novel males (deCatanzaro et al., 1999; Dominic, 1965; Marchlewska-Koj, 1977, 1981), and 17β-estradiol (Chapter 5). Therefore, it is possible that steroids, in particular 17β-estradiol, contribute to the pheromonal mediation of the Bruce effect. Other chemicals that possess pheromonal activity for other reproductive behaviours, such as DHB, SBT, and MUPs, have not

affected pregnancy when nasally applied to inseminated females (Brennan et al., 1999). Steroids have previously not been implicated as pheromonal agents in early pregnancy disruption. This does not exclude the possibility that a combination of substances may be involved, but it does suggest that 17β-estradiol found in male urine can have pregnancy-disrupting capabilities.

Implications for the Olfactory Memory Hypothesis

The olfactory memory hypothesis (Brennan et al., 1990) suggests that inseminated females recognize individual mice as distinct from each other. In this theory, females are thought to form an olfactory memory of males' pheromones after the mating process. Exposure of inseminated females to novel males' pheromones, for which they have no memory, will lead to a cascade of events in the vomeronasal organ followed by the amygdala and then the hypothalamus where dopamine release causes the suppression of prolactin secretion from the pituitary. This is thought to remove luteotrophic support and result in implantation failure (Rajendren & Dutta, 1988). If females form olfactory memories of males, it is quite possible that 17β-estradiol is involved. Estradiol has been shown to have actions on the AOB and the amygdala involved in this pathway (Navarro Becerra et al., 1996; Kaba et al., 1988; Li et al., 1989, 1992). Furthermore, estrogens have been shown to affect the dopaminergic inhibition of prolactin (Ferland, Labrie, Euvrard & Raynaud, 1979). However, the olfactory memory hypothesis as it has previously been proposed does not account for the behaviour of novel males towards inseminated females and the detrimental actions of 17β-estradiol on implantation.

The sire and familiar males, upon simultaneous exposure with novel males, can reduce the percentage of females aborting (Kumar & Dominic, 1993; Parkes & Bruce, 1961). An explanation of this sire or familiar male protection effect presents challenges for both the olfactory memory and steroid hypotheses. According to the olfactory memory model (Thomas & Dominic, 1989a, 1989b), only males that have been exposed to females during mating can inhibit the Bruce effect. It is thought that females form olfactory imprints of the sire and any proximate male odours during mating. However, this model may not always be appropriate. Grouped females, without prior exposure, also demonstrate a protective effect (Bruce, 1963). Grouped females can inhibit estrus in other females (Van der Lee & Boot, 1955), which might counteract the estrus-inducing effects of 17β-estradiol released by males. Also, unmated males are more effective at disrupting pregnancy than mated males. Since the sire has also recently mated, its ability to disrupt pregnancy may be diminished either through pheromonal or behavioural means or both.

Novel males of a different strain from the inseminated female disrupt pregnancy more effectively than do males of the same strain as the inseminated female (Parkes & Bruce, 1961). According to some investigators that adhere to the olfactory memory model (*eg.* Brennan et al. 1999), the pheromone codes for strain variability. Perhaps MUPs, which have the polymorphic capacity to vary across strains (Hastie et al., 1979; Robertson et al., 1996), are involved in combination with the potent pregnancy-disrupting steroid, 17β-estradiol. Furthermore, it is possible that males of one strain have a greater capacity to disrupt pregnancy than do others. Also, males may be more aroused by

females that are phenotypically different, which may alter their ability to disrupt implantation.

Future Investigations

The sites of action of 17β-estradiol that lead to pregnancy disruption are not fully understood. Urinary 17β-estradiol from novel males might be absorbed through the skin or nasal region of inseminated females. This steroid could then trigger the pathway involved in olfactory memory or could affect other areas important in reproduction, such as the ventromedial hypothalamus or the uterus. It is also conceivable that 17β-estradiol may be secreted by the adrenal glands in females in response to stress elicited by novel males. The origin of 17β-estradiol and its effects could be investigated in a number of ways. Radiolabelled 17β-estradiol could be painted on the nose of inseminated females and its sites of action could be visualized. Furthermore, the levels of an adrenal androgen, dehydroepiandrosterone, that is a precursor for 17β-estradiol, could be measured in inseminated females' urine and feces during exposure to either no males (control) or novel males. Elevated levels of dehydroepiandrosterone during exposure to males compared to control conditions would indicate that the adrenal glands are the source of heightened 17β-estradiol observed in females in the Bruce effect. This would suggest that the females' adrenal glands were activated as a result of a stress response.

Using the ELISA procedure, studies could be designed to investigate whether excreted steroids from the sire would be altered with exposure to a female carrying its

own offspring. Other studies could examine the concentrations of steroids in excretions from mated and unmated males during isolated conditions and during exposure to an inseminated female. Preliminary data from this laboratory show a trend that unmated males secrete more 17β-estradiol than mated males during exposure to pregnant females.

Most physiological systems important for survival and reproduction use multiple redundant mechanisms to function in an efficient and effective manner. It is not surprising, therefore, that novel males would excrete a naturally occurring substance such as 17β-estradiol that can disrupt pregnancy during the implantation period. In order to maximize their inclusive fitness, novel males could simply implement an existing physiological system by targeting urine containing estradiol at inseminated females. This would be adaptive since 17β-estradiol can prevent implantation and allow novel males to reinseminate females, increasing their own reproductive success.

Appendix I

The validation of an Enzyme-Linked-Immunosorbent Assay for the Detection of Estrone Conjugates, 17β-Estradiol, and Testosterone in Male Mouse Urine and Feces

In order to assess the concentration of steroids (testosterone, 17β-estradiol and estrone conjugates) that novel males are excreting, our laboratory has adapted a fecal and urinary ELISA (Munro et al., 1991) for the house mouse (*Mus musculus*). Validations have previously been reported for such steroid measures for female urine and feces (Muir et al., submitted); the present work will describe validations for male excretions.

For each hormone (estrone conjugates, 17β-estradiol, and testosterone), a series of validation tests must be performed in order to demonstrate the efficacy and validity of the assay. These tests include: generating a standard curve, performing parallelisms, and determining the sensitivity, precision, recovery and extraction efficiencies of a given hormone. These validations were essential before analysis of samples could occur.

The standard curve represents a range of optical densities for known concentrations of a two-fold serially diluted hormone (Kemeny, 1991). This curve serves as a reference for interpolating the concentration of unknown samples based on their optical densities (Kemeny, 1991). To account for possible inter-plate differences, standard curves were generated for each plate. Consistent standard curves provide a strong

measure of internal experimental reliability. During sample analysis for each hormone, standard curves were obtained from the same serially-diluted standard solutions.

A test for parallelism determines whether the samples behave immunologically in a similar manner as the standards (Kemeny, 1991). If the samples dilute out in the same way as the standard curve, then the sample and standard curves should run parallel to one another. This allows for interpolation of the concentration of an unknown sample based on the standard curve (Kemeny, 1991). Furthermore, this test indicates if the antigens (hormones) of interest are present in measurable quantities. Samples were run at 50% binding where the slope of the standard curve is steep and a change in colour reflects a change in concentration (sensitive range). Thus, sample curves that fell below 50% binding, which reflects a high concentration of hormone in the sample, were considered to have measurable quantities of the antigen (Kemeny, 1991).

In order for an assay to be valid, the sensitivity and precision of the procedure needs to be determined (Kemeny, 1991). Sensitivity can be defined as the lowest concentration of hormone that can be detected from the zero concentration of standard (only buffer) (Munro et al., 1991). The precision of the assay can be assessed by determining the coefficient of variation (CV) both between plates (interplate) and within a plate (intraplate) (Munro et. al., 1991). For inter-assay variations, control (pooled) samples binding at approximately the upper and lower limits of the standard curve (30% and 70%) were performed for each plate. Variations within a plate can be determined by running 40 identical aliquots of the sample on one plate (Munro et al., 1991). The formula

for calculating CV is as follows:

$$CV = (\text{standard deviation} / \text{mean}) \times 100$$

where the standard deviation and mean are the optical densities of the control samples for the interplate CV, and are for the 40 aliquots for the intraplate CV. The results should fall within the acceptable range of the assay (10-20%), otherwise there has been a problem with the binding properties of the antibodies or standards (Kemeny, 1991).

Recovery studies and extraction efficiencies were performed to account for procedural losses of hormone (Kemeny, 1991). For recovery studies, pooled samples of urine or fecal extracts were spiked with steroid standards in increasing amounts and the percentage of steroid recovered was determined. In order to evaluate the efficiencies of fecal extractions, feces were also spiked with different concentrations of steroid standards and extracted for measurement. The percentage recovered should reflect what was added plus the amount in the original sample.

The following work represents the validations for fecal and urinary enzyme immunoassays as required for publication purposes. For each validation, pooled samples of isolated HS male urine and feces were used.

Method

The subjects were sexually experienced HS male mice weaned at 30 days of age and bred in our laboratory by interbreeding C57, DBA, CF-1, and Swiss-Webster strains. All males were housed individually in standard polypropylene cages measuring 28x16x11

(height) cm with wire grid tops allowing continuous access to food and water. When urine and feces were collected, the males were placed into cages measuring 30x21x27 cm with wire mesh bottoms with 0.71x0.71 cm openings. These cages were placed on a clean surface until 500µl of urine and 0.5-1gram of feces from males were collected. The urine was aspirated with sterile syringes with 23 gauge needles and the feces were collected with sterile forceps. Urine and feces were separately pooled and stored without preservatives at -20^{0}C until assayed for steroid concentration.

Assay Procedure

These ELISA procedures, fecal extractions and creatinine assay are similar to those described in Experiments 3 and 4. All hormones (estrone conjugates, testosterone, and 17β-estradiol) and creatinine were purchased from Sigma Chemical. All antibodies (anti-E2 R4972, anti-E1C R522, and anti-T R156/7) and all corresponding horseradish peroxidase conjugates were purchased from the Department of Population Health and Reproduction at the University of California, Davis. The cross reactivities for the E1C R522 antibody are: estrone-3-glucuronide, 100.0%, estrone-3-sulfate 66.6%, estrone 238.0%, 17β-estradiol 7.8%, estradiol-3-glucuronide 3.8%, estradiol-3-sulfate, 3.3%, and all other structurally similar steroids <0.1%. For anti-E2 R4972 the cross reactivities are: 17β-estradiol 100%, estrone 3.3%, progesterone 0.8%, testosterone 1.0%, and androstenedione 1.0%, while all other steroids were less than 0.1%. The cross reactivities for anti-T R156/7 are as follows: testosterone 100%, dihydrotestosterone 57.4%,

androstenedione 0.27%, and androsterone, DHEA, cholesterol, 17β-estradiol, progesterone and pregnenolone <0.05%. All plates were measured for optical density using a Bio-tek instruments inc. EL 312E microplate reader.

Fecal and Urinary ELISA for Estrone Conjugates, 17β-estradiol and Testosterone

The levels of plasma estrogens are reflected by the concentration of estrone conjugates and include estrone sulfate and estrone glucoronide. For all hormones, each assay was carried out on NUNC Maxisorb plates which were first coated with 50 µl of antibody stock diluted at 1:50,000 for estrone conjugates and 1:10,000 for 17β-estradiol and testosterone in a coating buffer (50 mmol/L bicarbonate buffer pH9.6) and stored overnight at 4°C. Wash solution (0.15 mol/L NaCl solution containing 0.5 ml of Tween 20 per liter) was added to each well to rinse away any unbound antibody, then 50µl phosphate buffer per well was added. The plates were incubated at room temperature for 2-5 hours for estrone conjugates and 17β-estradiol and 30 min for testosterone before adding standards, samples, or controls.

For each hormone, standards and samples were serially diluted two-fold and two quality control urine and fecal samples at 30% and 70% binding (the low and high ends of the sensitive range of the standard curve) were prepared. Then, 50 µl of estrone conjugates- or 17β-estradiol- or testosterone-horseradish peroxidase were added to each well, with 50 µl of standard, sample or control for testosterone or 20 µl of standard, sample or control for estrone conjugates and 17β-estradiol. The plates were incubated for

2 hours at room temperature. Next, the plates were washed with wash solution and 100 µl of a substrate solution of citrate buffer (pH 4.0), H_2O_2 and 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid) was added to each well. The plates were then covered and incubated while shaking at room temperature. After approximately 30-60 minutes, the plates were read with a single filter at 405 nm on the microplate reader. Blank absorbance was subtracted from each reading to account for non-specific binding. Zero wells were considered to have 100% enzyme conjugate binding to the antiserum. Standard and sample curves were generated by dividing all values by the mean zero reading thereby giving a percent binding value.

Assay Validation

In addition to the parallelism and interplate controls performed above, the intraplate CV was calculated by running 40 aliquots of the same urinary sample at 50% binding on the same plate for each hormone with its corresponding antibody. The sensitivity of the assay was determined by measuring the least amount of hormone in urine that was consistently significantly different from the zero concentration on the standard curve using a t-test ($p<0.05$). Furthermore, recovery and extraction efficiencies were generated by adding a known amount of steroid to pooled samples of urine or feces and assaying for hormone content. The final value reported represents the percentage of added steroid plus the amount in the original sample recovered.

Assay Characterization

Figures 9-11 show standard curves and parallelisms for each hormone validated.

All samples measured, except for estrone conjugates in feces, could be serially diluted in parallel to their corresponding standard dilutions. In Figure 9, estrone conjugates found in urine did not fall below 50% binding. Also, fecal estrone conjugates did not run parallel to the standard curve. Therefore, it was concluded that there were inadequate amounts of estrone conjugates in urine and feces for detection. For 17β-estradiol and testosterone, Figures 10 and 11 demonstrate that both hormones are detectable in measurable concentrations in urine and feces and behave immunologically in a similar manner as standard hormones. Therefore, further validations were only performed on 17β-estradiol and testosterone.

For 17β-estradiol, the interplate CV for urine samples was 8.4% at 30% bound and 4.1% at 70% bound, and for fecal samples was 5.4% and 4.2% for the 30% and 70% bounds, respectively. For testosterone, the interplate CV for urine samples was 6.7% at 30% bound and 3.4% at 70% bound, and for fecal samples was 7.9% (30% bound) and 5.9% (70% bound). For urine samples, the intraplate CV for 17β-estradiol was 8.7% and for testosterone was 7.1%. The testosterone assay had a range of 312.5-2.4 pg/well with a sensitivity of 48 pg/ml for urine samples. The 17β-estradiol assay had a range of 250-0.49 pg/well with a sensitivity of 39 pg/ml for urine samples. For both hormones, 100% of the added steroid in urine was recovered. Fecal extraction efficiency for the testosterone assay was 27.70 ± 0.49%. For the 17β-estradiol assay, the extraction efficiency was calculated to be 60.82 ± 2.98%.

Figure 9
Serially diluted male urine and fecal samples binding to antibody in parallel with estrone conjugate standards.

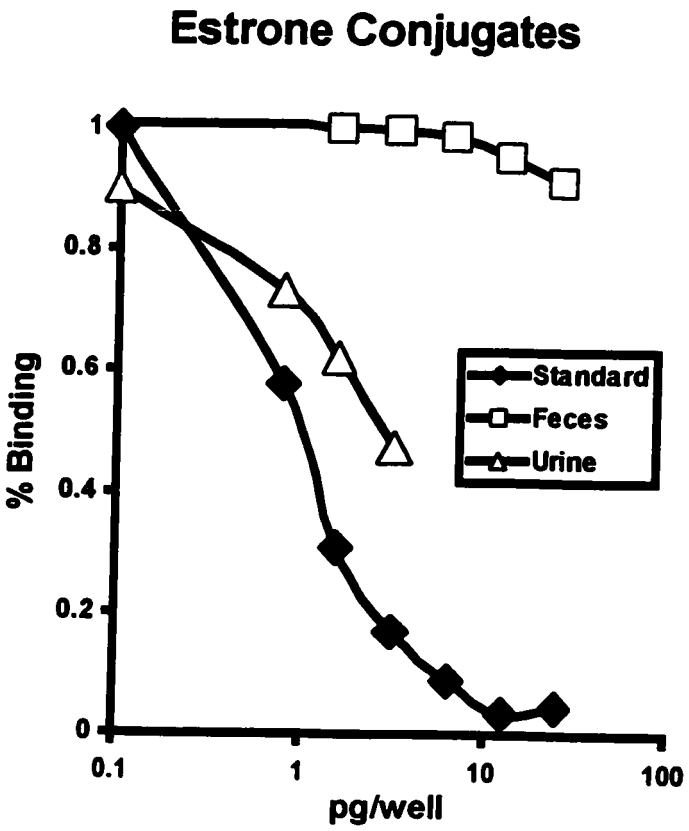


Figure 10
Serially diluted male urine and fecal samples binding to antibody in parallel with 17β-estradiol standards.

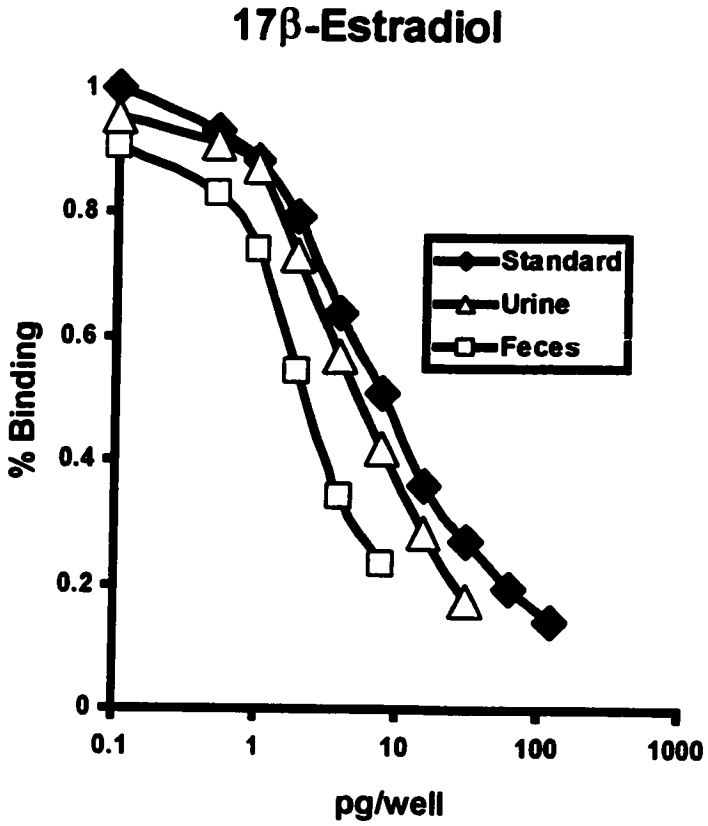
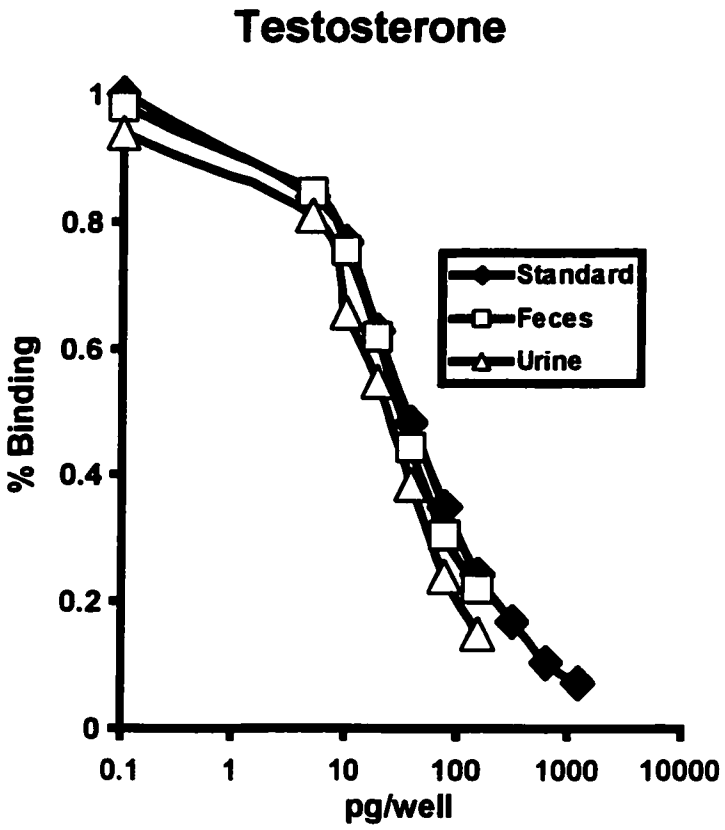


Figure 11
Serially diluted male urine and fecal samples binding to antibody in parallel with testosterone standards.



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