THE ROLE OF STEROIDS IN NOVEL-MALE INDUCED PREGNANCY DISRUPTIONS IN MICE (THE BRUCE EFFECT)

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ABSTRACT

Mammalian reproduction is vulnerable to psychological and physiological stress. This research focuses on the psychophysiological mechanisms that lead to the disruption of pregnancy by the failure of implantation of fertilized ova into the uterine walls. The underlying hormonal mechanisms of implantation failure are not well understood. It is known that many environmental events have a substantial impact on hormonal dynamics in mammals. These environmentally induced hormonal changes can disrupt implantation. This thesis focuses on the hormonal dynamics of female mice that lose their pregnancy when exposed to a novel male during the implantation period (the Bruce effect).

In Study 1, a repeatable and reliable Bruce effect was established by indirectly exposing inseminated females during the implantation period to novel males housed above them separated by a wire grid floor. Separating the animals allowed for the independent study of the chemical transmission from male to female, and the physiological transduction within the female. The findings from this study suggest that females must come in direct contact with the excretions of the stimulus novel males. The more excretions the females encounter, the greater the chance is of pregnancy disruption.

The Bruce effect is known to be dependent on androgens in the stimulus males, since castration eliminates their capacity to disrupt pregnancy. Study 2 showed that surgically removing the androgen-dependent preputial glands from the stimulus males does not diminish their capacity to disrupt pregnancy. Study 3 showed that administering

17β-estradiol to castrated males can restore their capacity to disrupt pregnancy. This suggests that 17β-estradiol as well as testosterone is involved in the chemical transmission of the Bruce effect. It has been hypothesized that 17β-estradiol is elevated in females that fail to implant in the presence of a novel male. Administering an antibody specific to 17β-estradiol to females during their implantation period can lower the hypothesized increase in 17β-estradiol and implantation takes place despite the exposure to novel males.

Finally, in Study 4 testosterone, 17β-estradiol and its major metabolites the estrone conjugates were quantified in females' urine and feces while exposed to novel males during implantation. It was found that testosterone and 17β-estradiol were significantly elevated in females that failed to implant while exposed to novel males.

In conclusion, this line of research reveals a potential role of steroids in novel male induced pregnancy disruptions in mice. Elevated testosterone and 17β-estradiol are shown to be related to the prevention of implantation in mice. These hormonal dynamics may be partially responsible for the physiological transduction of the Bruce effect.

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LIST OF ABBREVIATIONS

ACTH	Adrenocorticotropic hormone
CRF/CRH	Corticotropin releasing factor/hormone
DHEA	Dehydroepiandrosterone
ELISA	Enzyme-linked immunosorbent assay
GABA	Gamma-aminobutyric acid
Glu	Glutamate
GnRH	Gonadotropin releasing hormone
HPA	Hypothalamic-pituitary adrenal
LTP	Long-term potentiation
MUP	Major urinary protein
PGF2α	Prostaglandin F2α
PPX	Preputialectomized

Chapter 1

INTRODUCTION

Reproduction is vulnerable to psychological and physiological stress. Many environmental events have a substantial impact on hormonal dynamics in mammals. Some hormonal variations can be detrimental to reproduction, especially during early pregnancy. This research focuses on the psychophysiological mechanisms that lead to the disruption of pregnancy by the failure of implantation of fertilized ova into the uterine walls. The careful synchronization of specific endocrine events is important to the implantation process in mammals.

Investment in reproduction in females is much greater than males, which makes female reproductive physiology more vulnerable to the effects of stress than is male physiology (Liptrap 1993). A stressor, in this context, has extensive meanings and can be defined in many ways. Stress can be defined as the psychophysiological consequence of any event challenging an organism's capacity to cope (Selye 1956). Similarly, it has been defined as any influence on an organism's steady state, which requires adjustment or adaptation (Hinkle 1977). In mammals, this could include excessive heat, cold, overcrowding or physical restraint.

Generally, there is an inverse relationship between stress and reproductive behavior especially in female mammals. This is mediated in large measure by steroid hormones. Steroids are conserved in molecular structure across species in their functions

in both reproduction and stress, and these systems tend to work antagonistically. There are only two sources of steroids in mammals and those are the adrenal glands and the gonads. The adrenal glands are responsible for adaptation to stressors and maintaining homeostasis in many physiological systems. The gonads are responsible for maintaining sexual physiology and behavior. Typically, the size and activity levels of these glands are inversely proportional to each other (Genuth 1988).

Stress Responses

Environmental stressors can elicit a stereotyped physiological response in mammals. In response to stressors, the central nervous system of most mammalian species evokes physiological responses that ultimately result in activation of the hypothalamo-pituitary-adrenocortical (HPA) axis and the sympatho-adrenal axis. The responses of these major systems generally have adaptive and homeostatic value during periods of stress (Minton 1994).

Short-term stress

The typical short-term stress response, or the "fight or flight" response, can be characterized by an increase in activity in the sympathetic-adrenal system. Stimulation of the sympathetic division of the autonomic nervous system ordinarily produces generalized physiological responses rather than discrete localized effects. This results in part from the sympathetic fibers innervating all major organs (Guyton 1976). Norepinephrine is the major neurotransmitter involved in the synapse between the sympathetic fibers and the target organ. The sympathetic response involves the rapid

activation of the adrenal medulla. This releases epinephrine and norepinephrine into the blood, which can augment the sympathetic response. Exposure to cold, pain or strong emotions such as fear and rage evoke sympathetic activity (Genuth 1988). The functions of the gastrointestinal tract are suspended and blood is shunted away from the abdominal viscera. Heart rate and blood pressure increase, the coronary arteries dilate, and the bronchioles of the lungs widen. These physiological responses occur rapidly and enable an organism to cope temporarily with extreme physical or psychological conditions.

Long-term stress – The HPA

Under chronically stressful conditions, the actions of the hypothalamo-pituitary-adrenal axis regulate and adapt many physiological functions. The hypothalamus receives input from almost all possible sources in the nervous system and from various hormonal substances acting on central locations. Much of this input is used in regulating the secretions of the pituitary gland. The pituitary gland controls the release of many steroid hormones from the adrenal glands that target and regulate various physiological mechanisms including stress and reproduction. Numerous factors such as corticotropin releasing factor (CRF), vasopressin, oxytocin, angiotensin II (and conceivably other hormones) can stimulate the release of the stress hormone adrenocorticotropic hormone (ACTH) (Aguilera 1994). Typically, when a chronically stressful stimulus is encountered, the hypothalamus responds by releasing CRF, which targets the anterior pituitary. The anterior pituitary then releases ACTH, which regulates the growth and secretions of the adrenal cortex. ACTH stimulates the growth of those specific zones of the adrenal cortex concerned with secretion of glucocorticoids, sex steroids and their

precursors (Genuth 1988). Glucocorticoids serve many functions including suppression of inflammation and immunity, regulation of carbohydrate production from proteins and lipid metabolism. However, glucocorticoids can inhibit the secretion of ACTH by acting at the hypothalamic and/or pituitary level.

The multiple factors responsible for the control of the pituitary-adrenal axis may be due to the great variety of stress stimuli. Each factor could have specific roles in various stress situations. A multiple-factor control can provide a highly sensitive mechanism regulating, very finely, the stress hormones in response to a variety of endogenous and exogenous stimuli (Gaillard & Al-Damluji, 1987). Depending on the type of stressor, they may singly or in combination affect the amount and duration of ACTH and steroid secretion. The released glucocorticoids may then produce their numerous effects on inflammatory and immunological processes, carbohydrate metabolism and water balance. These multiple regulatory effects may be important in order to prevent host responses from over-reacting to stress and threatening homeostasis (Gaillard & Al-Damluji, 1987).

The Hypothalamo-Pituitary Gonadal axis

During normal function, the hypothalamic-pituitary complex also regulates the production of sex steroids from the gonads as well as the adrenal glands. In the female, this pattern is cyclical and involves a feedback mechanism. The release of gonadotropin-releasing hormone (GnRH) from the hypothalamus causes the anterior pituitary to release the gonadotropins, luteinizing hormone and follicle-stimulating hormone. These target the ovaries and stimulate the secretion of estrogens and progesterone that are responsible

for follicular maturity and release, menstruation, and the maintenance of sexual behaviors. During implantation and pregnancy, they are the major chemical communicants between the fertilized ova, placenta and endometrium. During normal cycling, they feedback to the hypothalamus and pituitary and regulate their own synthesis. The production of adrenal androgens and estrogens is low relative to such production by the ovaries or testis. The concentrations of sex steroids released by the adrenal glands are high enough to interfere with female gonadotropin cycling and can interfere with female sexual responses and early pregnancy (Guyton 1976).

Stress and Suppression of Reproduction

There exists a complex interaction between stress and reproductive mechanisms. Stress has many qualities, and it can modulate reproduction at many levels. Chronically housing male mice in groups can inhibit their sexual behavior in the presence of sexually receptive females (deCatanzaro, Douglas, Griffiths & Muir 1996, deCatanzaro & Gorzalka 1979, 1980, deCatanzaro & Ngan 1983). Suppression of the estrous cycle and delay of ovulation have been seen in sheep and cows that are stressed by transportation and exposure to a novel environment (Averill 1964, Moberg 1975). A number of stressful stimuli can disturb implantation of fertilized ova in the uterine walls in early pregnancy (deCatanzaro & MacNiven 1992) and some stimuli can disrupt pregnancy once implantation has taken place (Storey & Snow 1990).

A sex difference exists in the response of the HPA axis to stress, with females reacting more robustly than do males. It has been demonstrated that in females, products of the HPA axis inhibit reproductive function (deCatanzaro & Graham 1992, Harper

1992, Magiakou, Mastorakos, Webster & Chrousos 1997, Viau & Meaney 1991). Furthermore, the sex differences in HPA function are in part due to differences in the circulating gonadal steroid hormone milieu (Handa, Burgess, Kerr & O'Keefe 1994). However, corticosteroids, ACTH, and stress can exert both inhibitory and facilitory effects on reproduction (Brann & Mahesh 1991). It has been suggested that gonadal steroid hormones modulate HPA activity in order to prevent the deleterious effects of excessive HPA activation on reproductive function (Handa et al. 1994). Effects of the HPA on the gonadal axis are consistent with conservation and redirection of valuable resources towards homeostasis during times of stress (Torpy & Chrousos 1996).

The HPA axis exerts profound, multilevel inhibitory effects on the female reproductive system. Corticotropin-releasing hormone (CRH) and CRH-induced proopiomelanocortin peptides inhibit hypothalamic GnRH secretion. Glucocorticoids from the adrenals suppress pituitary luteinizing hormone and ovarian estrogen and progesterone secretion and they render target tissues less sensitive to estradiol (Chrousos, Torpy & Gold 1998). Several components of the HPA axis and their receptors are present in reproductive tissues as regulators of various physiological active endogenous substances. The ovaries and endometrium contain CRF receptors that are thought to participate in the inflammatory processes of the ovary, such as ovulation and luteolysis, and of the endometrium, such as blastocyst implantation and menstruation. The placenta also contains CRF receptors that may participate in the physiology of pregnancy and the timing of labor and delivery (Chrousos et al. 1998, Magiakou et al. 1997).

The communication between the HPA and the HPG works reciprocally. Some researchers have found that estradiol can enhance chronic stress responses through the

mediation of ACTH and glucocorticoids. Ovarian estrogens have the capacity to enhance various basal and stress-induced activities of the body (Carey, Deterd, Koning, Helmerhorst & de Kloet 1995). In female rats with normal estrous cycles, higher peak ACTH and corticosterone responses were found in reaction to stress during proestrus, when estradiol levels are highest, compared to during the estrous and diestrous phases (Viau &Meany 1991). In addition, estradiol can maintain enhanced glucocorticoid release after the termination of stress (Viau 1991). Furthermore, in response to footshock stress, ACTH and corticosterone responses are prolonged in the presence of estradiol (Burgess & Handa 1992). It has also been found that estradiol can impair glucocorticoid receptor-mediated negative feedback (Burgess & Handa 1992). These results suggest an ovarian influence on both activational and inhibitory components of HPA activity.

Chronic stress response plays an important role in suppressing reproductive function in females. Under stressful conditions, investment in pregnancy or rearing offspring could be maladaptive and many mammals have evolved to avoid reproducing under stressful conditions. Typically in females, sexual behaviors are suppressed by short-term acute stressors (deCatanzaro & Graham 1992). Although one study reports that sexual behavior can be facilitated by chronic psychosocial stress (Gorzalka, Hanson & Brotto 1998) other data indicated that chronic adrenal activation antagonizes female sexual behavior in the rat and mouse (deCatanzaro 1987, deCatanzaro, Knipping & Gorzalka 1981). It has also been shown that during chronically stressful situations implantation during early pregnancy is disrupted (deCatanzaro & MacNiven 1992).

Implantation Physiology

Mammalian implantation can be defined as the state of physiological receptivity of the uterus to fertilized blastocyst and the adhesion or fixation of the blastocyst to the endometrial surface of the uterus (Davies & Hessedahl 1971). This implantation 'window' can be generalized to include an embryonic 'developmental window', an endometrial 'receptive window' and a coordinated 'ovum transport window' (Harper 1992). The tight synchrony of these events will lead to the successful establishment of early pregnancy with the displacement of the uterine luminal epithelium by blastocyst-derived trophectoderm (Hunt & Roby 1994).

As the fertilized ovum travels down the fallopian tube, it matures and sends out chemical signals, such as prostaglandins and growth factors that reach the endometrium and prepare it for implantation. Similarly, the endometrium communicates with the ovum and supplies it with supporting nutrients for maturity. The major endocrine factors which induce the biological and morphological changes in the endometrium are ovarian steroids, particularly 17β-estradiol and progesterone that can be produced in the ovaries, endometrium or blastocyst. These can act in an endocrine, paracrine and/or autocrine manner (Findlay & Salamonsen 1991). Estrogens released from the trophoblast and the ovaries stimulate the production of prostaglandin F2α (PGF2α) in the endometrial epithelium that stimulates an inflammation reaction preparing the endometrium for implantation. Simultaneously, the corpus luteum secretes progesterone that stimulates the endometrium to produce glycoproteins for nutritional support for the trophoblast (Smith 1991). If the cycle is infertile and there is not a viable ovum, then the corpus

luteum will release PGF2α that acts in an autocrine fashion as a luteolysin and the corpus luteum will 'self destruct'. If the ovum is viable at this time then it will release chorionic gonadotropin to inhibit the PGF2α in the endometrium and stop the inflammatory response. This chorionic gonadotropin is involved in a 'corpus luteal rescue' by inhibiting the PGF2α released at the corpus luteum. This stops deterioration of the corpus luteum, and allows vital progesterone to be produced by the corpus luteum that maintains the nutritional support for the ovum in the uterus. The physical attachment of the blastocyst can then take place (Ahmed & Smith 1992, Ford & Christenson 1991).

Implantation and Stress

Various studies have focused on the involvement of ACTH and adrenal corticosteroids in the suppression of implantation during stress. Earlier reports indicated that exogenous ACTH during the implantation period reduces the capacity for implantation (Velardo 1957), and that ACTH can disrupt estrous cycling in rats (Hagino, Watanabe & Goldzieher 1969) and mice (Christian 1971). More recent studies showed that glucocorticoids (Bitman & Cecil 1967, deCatanzaro MacNiven & Ricciuti 1991, Schlough 1971) or epinephrine (deCatanzaro & Graham 1992, Trend & Bruce 1989) alone do not have the capacity to disrupt implantation in rats and mice. Trend and Bruce (1989) studied the effects of the adrenal medullary catecholamine, epinephrine, in rats. This study showed no significant effect on pregnancy even at concentrations well above natural levels found in chronically stressed rats. DeCatanzaro and Graham (1992) found similar evidence of this when they injected epinephrine into mice during early pregnancy. In this investigation, epinephrine was injected at extremely high concentrations and many

of the mice in the high dose group died while the others in the group remained pregnant. These studies show that catecholamines released from the adrenal medulla during short-term stress do not necessarily play a role in the disruption of pregnancy. However, ACTH controls not only the release of glucocorticoids but also that of adrenal androgens and estrogens, which may play a role in the disruption of early pregnancy.

Hyperprolactinemia, which accompanies chronically elevated ACTH levels in pregnant mice, has also been implicated in early pregnancy disruptions (Kittinger, Gutierrez-Cernosek & Pasley 1980). It is known that the anterior pituitary hormone prolactin is necessary for the development and maintenance of a corpus luteum. Prolactin normally acts to stimulate progesterone secretion from the corpus luteum for the initiation of implantation and pregnancy. The corpus luteum releases progesterone that activates the endometrium to provide the incoming ova with nutritional support. Prolactin may also act to stimulate sex steroid production by the adrenal glands and thereby to suppress pituitary gonadotropin secretion and subsequent steroid output (Bohnet, Dahlen & Wuttke 1976, Boyar et al. 1974).

It is thought that sex steroids are released from the adrenal cortex in times of chronic stress and that they can help regulate early pregnancy and implantation. Administration of dehydroepiandrosterone (DHEA) and androstenedione during the implantation period can disrupt pregnancy (deCatanzaro et al. 1991, Harper 1967,1969). These steroids are produced under stressful situations in the cortex of the adrenal glands and are precursors for other androgens and estrogens. These steroids may metabolize to estrogens and mediate early implantation failure. Some recent findings have supported this hypothesis. It has been found that exogenous 17β-estradiol can disrupt pregnancy

with the same efficacy with a dose thousands of times lower than DHEA or androstenedione, when administered daily during the implantation period (deCatanzaro et al. 1991). Radioimmunoassay of plasma estrogens in pregnant restraint stressed rats revealed that during their implantation period there was elevated 17β-estradiol compared to unstressed controls (MacNiven, deCatanzaro & Younglai 1992). In addition, injections of 17β-estradiol antibodies given to restraint stressed pregnant female mice significantly increased the capacity for them to bear litters compared to controls (deCatanzaro, MacNiven, Goodison & Richardson 1994). These experiments emphasize the importance of 17β-estradiol in the disruption of early pregnancy.

These hormonal dynamics have been studied in several paradigms that elicit similar neuroendocrine responses. Various types and durations of physical restraint (Kvetnansky & Mikulaj 1970, Kvetnansky et al. 1978, MacNiven, deCatanzaro & Younglai 1992, Wiebold, Stanfield, Becker & Hillers 1986), extreme temperatures (Castro-Vazquez, Esquivel, Fernandez-Cano1958, MacFarlane, Pennycuik & Thrift 1957, Martin & Rosner 1975, Wettemann & Bazer 1985), nutritional stress (Archunan & Dominic 1989), predator exposure (deCatanzaro 1988) overcrowding (Barrett & Stockham 1963, Christian & LeMunyan 1958, 1971) and swimming stress (Guo 1993) have been used to induce early pregnancy failure. Increased hypothalamo-pituitary-adrenal axis activity has also been shown to disrupt the female estrous cycle and delay ovulation in sheep and cows that are stressed by transportation and exposure to a novel environment (Averill 1964, Moberg 1975).

The Bruce Effect

In 1959, Bruce discovered that if a newly inseminated female mouse is removed from the stud male and brought into the vicinity of another male, implantation fails and the female returns to estrus as if mating had never taken place (Bruce 1960a, 1960b). The female will typically not lose the pregnancy if the stud male is of the same strain of mouse (Bruce 1968, Marsden & Bronson 1965). This effect is thought to act through olfactory stimulation of the female by pheromones in the urine of the males (Bellringer, Pratt & Keverne 1980, Rajendren & Dominic 1984). The effect also acts through the females' contact with the males' excretions or pheromones which are thought to be non-volatile (Rajendren & Dominic 1985).

Pheromones

Various pheromonal effects have been observed in mice with respect to reproductive maturation. The presence of sexually mature males or their urine odor can accelerate sexual maturation in prepubertal female mice and induce estrus in sexually mature female mice (Vandenbergh 1967, 1969, Whitten, Bronson, & Greenstein 1968). Furthermore, the presence of pregnant, lactating or large groups of females or their urine alone can delay the onset of puberty in juvenile female mice (Drickamer 1977, 1984). Both the duration and timing of pheromone stimulation appear to be critical factors affecting pheromone-induced influences of physiological events such as puberty delay and puberty acceleration in female mice.

It is typically thought that inseminated females do not experience intrauterine implantation of fertilized ova due to physiological effects occurring as a result of the exposure to novel male pheromones. Originally, Parkes and Bruce (1962) reported that housing inseminated females in cages soiled by novel males would disrupt pregnancy. It has also been reported that if females had their olfactory bulbs surgically removed or their olfactory tracts transected, the effect would not take place (Bruce & Parrott 1960, Rajendren & Dominic1985). On the other hand, it has also been reported that the stud male can disrupt pregnancy if the female's vomeronasal system is destroyed (Keverne & de la Riva 1982). Male mice will increase urine output dependent on their sexual status, and females can discriminate among differing males' urine (Drickamer 1992, 1995, Hurst 1990a, 1990b, 1993). Olfactory preference studies have shown that pregnant female mice are attracted to the soiled bedding of the stud male over clean bedding or the bedding of a novel male (Drickamer 1989). Novel male urine has been reported to be sufficient to disrupt pregnancy when applied to the snout of the inseminated female (Dominic 1966a, 1966b). These studies suggest that either urine or a pheromone within the urine is responsible for the disruption of pregnancy.

Researchers have attempted to isolate and determine the parameters of such a pheromone. Fractionation and thin-layer electrophoresis techniques on male urine isolated a substance with pregnancy blocking pheromonal activity in the peptide fraction (Marchlewska-Koj 1981). The pheromones that induce estrus and early puberty in mice have been tentatively identified as the brevicomins and dihydrothiazoles (Keverne 1998, Liebich Zlatkis, Bertsch, Van Dahm & Whitten 1977, Nishimura, Utsumi, Yuhara, Fujitani, & Iritani 1989). These volatile constituents of male mouse urine have been

synthesized and tested for their ability to induce estrous cycle in female mice (the Whitten effect) (Jemiolo, Harvey & Novotny 1986). The synthetic compounds, when added together in appropriate concentrations to previously inactive urine of castrated males, or even to water, were found as effective as normal male urine in inducing estrous cycling. However, these chemicals have been found to have no such effects when tested for inducing the Bruce effect (Brennan, Schellinck & Keverne 1999).

These relatively simple chemicals lack the structure to differentiate individuals or even differing mouse strains when tested in an olfactory paradigm (Brennan et al. 1999). It has been found that these substances are capable of binding to the less volatile urinary constituents, such as proteins or peptides (Novotny et al. 1999). Although these chemicals by themselves do not induce physiological changes in mice, they do when they are bound ligands on major urinary proteins (MUPs). MUPs are involved in olfactory communication processes in mice and come from a multi-allelic family of proteins (Robertson, Hurst, Hubbard, Gaskell, & Beynon 1998). They are capable of binding small hydrophobic volatile molecules in a central cavity. These molecules have been implicated in the signaling responsible for dominance relationships, onset of puberty and individual recognition in mice (Robertson et al. 1998). It has been suggested that MUPs may serve as transporters for the dihydrothiazoles and brevicomins. Since these MUPs differ across strains and individuals, these proteins may participate in pheromone recognition when combined with brevicomins and dihydrothiazoles (Brennan et al. 1999). It has been hypothesized that the same vomeronasal, neural and neuroendocrine pathways are also activated during pregnancy disruption which makes these compounds possible pregnancy blocking pheromones. However, this has not been studied extensively and

recent results suggest that these compounds are not involved with the Bruce effect (Brennan et al. 1999). A major problem with this line of research is that it has not been well established that mice can differentiate among individual mice of the same strain.

Various biological activities have been tested with purified urinary protein or "pheromone". Differential responses have been found with respect to age, gender and hormonal/physiological status of the mice exposed to the "pheromone". Molecules that co-purify with male MUPs, possibly the brevicomins and dihydrothiazoles have been found to attract adult females and repel adult males (Mucignat-Caretta, Caretta & Baldini 1998). In prepubertal animals, females are repelled and males are attracted by these same chemicals. These purified proteins and bound ligands have been found to have puberty-accelerating properties (Novotny et al. 1999). Whole male urine as well as preparations of MUPs that had been stripped of their ligands, have been investigated in female mice for their pheromonal properties. Expression of the immediate early gene egr-1 was used as a marker for the induction of activity in the accessory olfactory bulb (Brennan, Hancock & Keverne 1992, Brennan et al. 1999). It was found that the anterior and posterior halves of the accessory olfactory bulb processed different aspects of the male pheromone signal (Brennan et al.1999). It has been suggested that the differential responses to the MUPs in the main and accessory olfactory bulbs reflect a mechanism for mouse strain recognition. It has also been suggested that this may be a potential mechanism for olfactory memory in the Bruce effect.

The roles of pheromonal and sex steroid interactions have not been studied sufficiently in the Bruce effect. It has been found that any pheromonal activity is dependent on androgens (Hoppe 1975, Mucignat-Caretta 1998, Novotny, Schwende,

Wiesler, Jorgensona, & Carmack 1984). It has also been found that male urine can change the concentration of gonadotropin-releasing hormone and norepinephrine in the olfactory bulb tissue and serum of female voles (Dluzen, Ramirez, Carter, & Getz 1981). Increases in GnRH in the female and the possibility of biologically active steroid hormones in male urine can have detrimental consequences to pregnancy.

Stud Male Protection and Prolactin

Several studies indicate that if a recently inseminated female is left housed with the stud male, then a novel male cannot induce implantation failure (Acharya & Dominic 1997, Kumar & Dominic 1993, Thomas & Dominic1987, Thomas & Dominic 1989). This phenomenon has been referred to as stud male protection. It is thought that olfactory memories of the stud are induced in the females vomeronasal organ during mating. Subsequent encounters with the stud suppress any olfactory activity that might trigger neuroendocrine events that are detrimental to pregnancy.

The mechanism for the stud male's protection is thought to be the suppression of dopamine release in the hypothalamus by the inhibition of the olfactory neurons that are involved with an olfactory memory. When the female is kept in the vicinity of the stud male, while exposed to a novel male, dopamine is not released from the hypothalamus. Dopamine has the capacity to suppress the release of prolactin from the anterior pituitary. Prolactin is known to maintain the corpus luteum during pregnancy. The stud male has also been shown to also protect pregnancy in paradigms involving pregnancy loss due to nutritional stress (Archunan & Dominic 1989). The stud male can also induce pseudopregnancy in females exposed to novel males up to 4 days post coitum (Archunan

& Dominic 1992, Thomas & Dominic 1987). This suggests that females retain the memory of the stud for several days. However, if pregnancy is disrupted in females by the injection of bromocriptine, a dopamine agonist, the stud male cannot prevent the pregnancy loss.

The Bruce effect can be partially prevented by injecting females with progesterone (Rajendren & Dominic 1987, 1988, 1993). It is suggested that exogenous progesterone given early during novel male exposure prevents luteal failure and maintains pregnancy in females (Rajendren & Dominic1993). Rajendren and Dominic (1988) showed that the corpus luteum of newly inseminated female mice could synthesize progesterone for at least up to 48 hr after the beginning of alien male exposure. Furthermore, administration of prolactin to newly inseminated females, beginning the time of alien male exposure, can prevent implantation failure. However, when prolactin is administered between 24 and 48 hours after novel male exposure, pregnancy is lost. Their results suggest that the corpora lutea of newly inseminated females cease to respond to prolactin within 24 hr of alien male exposure, although they exhibit the capacity to synthesize progesterone for a longer period. The dopamine agonists bromocriptine and cabergoline have also been reported to disrupt pregnancy through the suppression of prolactin thereby inhibiting progesterone biosynthesis by the ovary (Archunan & Dominic 1992, Negishi & Koide 1997).

Olfactory Memory

Although there has been much work on olfactory memories in the Bruce effect, these studies tend to focus only on long-term potentiation (LTP). This study of LTP is

the investigation of neural mechanisms of Hebbian (association-dependent) changes in synaptic efficacy in neural tissue. Investigators of olfactory memory look for these changes in the accessory olfactory bulb (Brennan, Kaba & Keverne 1990, Breer, Raming & Krieger 1994, Kaba, Hayashi, Higuchi & Nakanishi 1994, Shepard 1994). The hormonal dynamics below the level of the pituitary gland are not investigated in these studies and there has been little inquiry into events in the adrenal glands or the ovaries.

A considerable amount of the recent work on the Bruce effect has focused on the idea that female mice form an olfactory memory to the sire's pheromones during mating. This involves the study of the formation of olfactory memories and the LTP in the accessory olfactory bulb (Brennan et al. 1990, Brennan, Kendrick & Keverne 1995, Kaba et al. 1994). This involves an increase in synaptic efficacy between the mitral and granule cells of the accessory olfactory bulb. This olfactory memory is independent of the hippocampus since lesions there do not affect the memory (Selway & Keverne 1990). It is thought that the pheromones of the sire form an olfactory imprint in the olfactory bulb of the female. The increase in efficacy of these synapses allows the neurons involved in the imprint to be inhibited during subsequent encounters with the stud male (Lloyd-Thomas & Keverne 1982). However, a novel male activates a different subset of olfactory neurons, which fire and trigger certain neuroendocrine events in the female leading to implantation failure (Kaba et al. 1994). The induction of the Bruce effect is thought of as an indication of the formation of an olfactory memory. In these paradigms, a successful Bruce effect is used as a measure of a successful imprinted olfactory memory of the stud.

The hypothesized pathway which leads to the disruption of pregnancy begins with the pheromonal activation of olfactory receptor neurons at the vomeronasal epithelium that converge in a given pattern to the glomeruli. Glomerular outputs diverge slightly to the mitral cells of the accessory olfactory bulb (AOB) which project sub-cortically through the amygdala to the hypothalamus (Breer et al. 1994, Shepard 1994). Dopamine is released here which inhibits the production of prolactin (Dominic 1966a, Marieb 1989). Prolactin is known to have leuteotropic effects and to promote the production of progesterone in the corpus luteum (Dominic 1966a). Progesterone targets the endometrial layer of the uterus, which provides support in the form of glycogen for the fertilized ova. Once the production of progesterone is terminated, so is implantation. A recent study (Rajendren & Dominic1988) showed that the drug haloperidol, which is known to induce pituitary prolactin release by the inhibition of hypothalamic dopaminergic neurons, could prevent early pregnancy loss by the Bruce effect. However, haloperidol is known to have tranquilizing effects on the females and this could confound any behavioral component of the effect by relaxing an otherwise stressed female.

It is thought that an olfactory memory occurs in recently inseminated female mice because there is a long lasting increase in the inhibition of a subset of mitral cells that respond to the stud male's pheromones (Kaba et al. 1994). The synaptic changes that occur in the formation of this memory occur in the accessory olfactory bulb. When normal odor activation occurs, mitral cells are depolarized by the vomeronasal inputs. They project to the amygdala but they also release glutamate (Glu) into the dendrodendritic granule cell reciprocal synapse. The granule cells will release γ aminobutyric acid (GABA) reciprocally in a probabilistic fashion (Brennan & Keverne

1989). Activity at the dendrodendritic synapse between the mitral and granule cells normally regulates mitral cell activity by a negative feedback mechanism. If GABA is released then the mitral cell is inhibited and an odor imprint is made by a combination of excited and inhibited mitral cells (Kaba et al. 1994). This GABA release is dependent on the activation of the ionotropic subset of Glu receptors but this exact mechanism is unknown.

For a mating induced pheromonal olfactory memory to be formed, mating and pheromonal exposure must be combined to induce the increase in the efficacy of the mitral and granule cell reciprocal synapse (Brennan et al. 1995). During mating, norepinephrine (NE) is globally increased and it reduces the GABA mediated feedback inhibition of the mitral cells (Kaba et al. 1994). The memory is formed by the sustained excitation of a pheromone specific population of mitral cells. This sustained excitation is made possible by the inhibition of the synapse by NE released from the centrifugal fibers projected from the locus ceruleus. During mating, NE is released globally and when the occurrence of inhibition of the dendrodendritic reciprocal synapse is paired with the mitral cell activation from pheromonal stimulation, this results in an increase in synaptic efficacy (Kaba et al. 1994). As in hippocampal LTP, which also involves a co-active mechanism, this memory formation requires the concomitant action of NE release from mating and the presence of pheromonal stimuli (Brennan et al. 1990).

After the induction of the memory, the novel male and the stud male activate different olfactory pathways. The pathway activated by the stud is easily inhibited due to LTP while the pathway activated by the novel male falls outside of this imprint. Re-exposure of the female to the stud male activates the same neuronal firing pattern as was

activated at memory formation. This triggers the reciprocal synapse to inhibit the mitral cells and synapses and the transmission of the signal is terminated. The inhibition is mediated by the release of glutamate from the mitral cells, which subsequently stimulates the granule cells. GABA is released which hyperpolarizes the mitral cells and inhibits the entire "stud male pheromone " neural burst-firing pattern (Brennan 1994). Exposure to a strange male results in a different pattern of mitral cells firing and there is much less inhibition of the signal to the amygdala and other higher processing centers. There may be some overlap of the signal, since the pheromones are specific and conserved molecules, however the mitral cells which fall outside of the stud male's "imprint" will still fire. If any of the signal gets through the prolactin suppression chain reaction occurs (Brennan 1994).

These olfactory memory models do not account for some recent findings. Some criticisms of this work include the fact that it has not been well established that mice can differentiate among individual mice of the same strain. It is also known that sexual satiety diminishes the capacity for novel males to disrupt pregnancy (Spironello & deCatanzaro 1999). This should not be the case unless sexual satiety changes the composition of male urinary constituents. This olfactory memory research concentrates primarily on the interactions at the level of the vomeronasal organ through to the pituitary gland. In this work, what may be overlooked are the steroid hormonal dynamics that occur at the level of the adrenal glands and the ovaries and their subsequent influences on uterine receptivity and ova transport and viability.

The Role of Steroids in the Bruce Effect

Typical adrenal stress hormones, such as the glucocorticoids, have been dismissed as mediators of the Bruce effect but there is still controversy over the role of the adrenal glands in the effect. Researchers have reported that adrenalectomy failed to prevent the ovoimplantation block by exposure to novel males (Sahu & Dominic 1981) while others report the opposite (Snyder & Taggert 1967). There may be a confounding variable in these studies, the poor health of mice that have had their adrenal glands removed. These conflicting reports and this confound suggest that no conclusions can be drawn about the involvement of the hypothalamo-pituitary-adrenal axis in the Bruce effect.

It has been accepted that elevated androgens and estrogens in pregnant females disrupt pregnancy. A weak but consistent pregnancy disruption in mice can be obtained by administering, during the implantation period, dehydroepiandrosterone (DHEA) and androstenedione (deCatanzaro et al. 1991, Harper 1967,1969). These steroids are produced under stressful situations in the cortex of the adrenal glands and are precursors for other androgens and estrogens. Harper suggested that these steroids metabolize to estrogens and may mediate early implantation failures induced by stressors. Some recent findings have supported this hypothesis. First, it has been found that pregnancy can be disrupted by 17β-estradiol with the same efficacy as DHEA and androstenedione at a fraction of the dose when administered daily in the implantation period (deCatanzaro et al. 1991). Furthermore, radioimmunoassay of plasma estrogens in pregnant restraint stressed rats revealed elevated 17β-estradiol during their implantation period compared to unstressed controls (MacNiven, deCatanzaro & Younglai 1992). Finally, injections of

monoclonal antibodies specific to 17β-estradiol given to restraint stressed pregnant female mice significantly increased the capacity for them to bear litters compared to controls (deCatanzaro, MacNiven, Goodison & Richardson 1994). These studies instigated the idea that estrogens may play a role in the Bruce effect.

Sex steroids in the novel males are also important in the transmission of the Bruce effect. Castration of the stimulus males completely abolishes the capacity for them to disrupt pregnancy when exposed to inseminated females both with direct exposure, where the males fail to mount the females (deCatanzaro & Storey 1989), and when they are exposed indirectly through a wire mesh grid (deCatanzaro et al. 1995). This also occurs if castrated males' urine is used alone (Bruce 1965, Dominic 1965). Testosterone replacement restores the males' capacity to disrupt pregnancy (deCatanzaro et al. 1995, Dominic 1965). Exposure to females is known to alter the hormonal state of males. It has been found that males exposed to females have increased levels of testosterone (Macrides, Bartke & Dalterio 1975). It is also known that sexual satiety diminishes the capacity for novel males to disrupt pregnancy (Spironello & deCatanzaro 1999). However, it is not yet known if sexual satiety in males correlates with decreased testosterone levels. The ability to disrupt pregnancy can be stimulated in females when they are given testosterone (deCatanzaro et al. 1995, Dominic 1965) or castrated males when given depo-testosterone epiandrosterone, androstenedione or androsterone; which all metabolize to testosterone (Hoppe 1975). It is a possibility that biologically active androgens or estrogens are being transmitted to the pregnant female through urine and being absorbed through the skin, lungs or olfactory organs. This could unfavorably elevate estrogen levels in the inseminated females and disrupt pregnancy.

Sex steroids and their mediators can exert a profound effect on activity in the olfactory bulb. The effect of ovarian steroids and pheromones on the activity of glutamic acid decarboxylase, the enzyme that synthesizes GABA, was studied in the rat main and accessory olfactory bulbs (Navarro Becerra, Grigorjev & Munaro 1996). It was found that estrogens could regulate the synthesis of GABA in the main and accessory olfactory bulbs and potentially olfactory memory. However, this estrogen control of GABA synthesis is attenuated by the presence of male pheromones. The results from this study suggest that the hormonal status of pregnant females may play a role in the mediation of olfactory memory. Similarly, female prairie voles exposed to a single drop of male urine on the upper lip show changes in concentrations of GnRH and norepinephrine in olfactory bulb tissue and rapid increases in serum concentrations of luteinizing hormone (Dluzen et al. 1981). This suggests that GnRH and norepinephrine in the olfactory bulb may mediate luteinizing hormone release in response to external chemical cues. These results suggest that further study of estrogens in the Bruce effect is needed.

Estrogens are known to have detrimental effects on implantation. A deleterious effect of high plasma estradiol levels and the resulting elevation in the estradiol/progesterone ratio have been recognized to produce suboptimal endometrial receptivity (Suginami 1995). The estrogenic mediation of various physiological parameters involved in implantation is still poorly understood. Estrogens are known to control muscular contraction, ciliary movement and flow of secretions as well as other mechanisms of progression and retention of ova through the fallopian tubes. Manipulations in the time course of ovum transport in the oviduct may influence implantation by creating asynchrony between the embryo and the endometrium or by

creating an inappropriate tubal environment for the early embryo (Croxatto & Ortiz 1975, Land Evers, Boeckx & Brosens 1987). It has also been suggested that in lower mammals the oviduct secretes factors that are toxic to embryo development after the period of normal tube transport (Pauerstein & Weinberg 1980). High plasma estradiol has been shown to decrease the rate of travel or "lock" fertilized ova in the fallopian tubes (Burdick & Whitney 1937, Hodgson, Nork, Heesch & Johns 1980, Morris & VanWagenen 1973, Overstrom, Bigsby & Black 1980, Pauerstein, Sabry, Hodgson 1976, Pauerstein & Weinberg 1980, Whitney & Burdick 1936) and can also induce lysis of the corpus luteum (Greenwald 1964). Withholding estrogen from target tissues during ovum transport results in accelerated oviductal transport and expulsion of ova from the uterus (Bigsby, Duby & Black 1986).

Assays for Fecal and Urinary Steroids

Part of the goal of this thesis is to determine the role of androgens and estrogens in the Bruce effect. Recent evidence has shown that steroid hormones might be involved either in the transmission from male to female or transduction within the female of the Bruce effect. Novel male urine may contain biologically active androgens or estrogens that are being transmitted to the inseminated females. The transduction of the effect may be mediated by elevated 17β-estradiol or testosterone in the female. An analysis of these sex steroids in the females during implantation is justified. This can be done using an enzyme-immunoassay. If these steroids were elevated in the females losing pregnancy, they would potentially be contributing to the implantation failure.

Assays currently exist for analysis of sex steroids in blood, urine and feces for many mammalian species (Peter, Kapustin & Critser 1996). However, existing assays can not be used for all species or all media. The species-specific nature of both the structure of the reproductive protein hormones and the patterns in which they are secreted are limiting factors for applying existing assays to all mammalian species (Lasley Shideler & Munro 1991). Although not all species metabolize androgens and estrogens similarly, assaying for steroid hormones is possible due to the stability of steroid hormone metabolites in blood and excretia and the conservation of steroid hormone molecules across taxa (Lasley & Kirkpatrick 1991). What makes these assays useful is the fact that excretion profiles of gonadal steroid metabolites reflect gonadal activity and provide an accurate assessment of overall endocrine status (Munro et al. 1991).

For small fragile animals, such as mice, there are many benefits for testing urine and feces, as opposed to blood, by way of immunoassay. They eliminate human handling or restraint, and avoid the use of chemical immobilization in animals, which prevents alteration of the hormone levels to be measured. This is beneficial because protocols can be imposed for long periods without stress to the animal (Lasley & Kirkpatrick 1991). Daily samples of urine and feces can be taken whereas for blood, sufficient quantities can not always be taken from small fragile animals. As with plasma samples, urine and fecal samples can be frozen and stored for long periods without preservatives. However, the advantage of blood is its rapid secretory dynamics and the ability to measure momentary changes in hormones (Peter et al. 1996). Non-specific antibodies can be used for total excreted hormone or a more specific antibody can be used for specific major conjugates. In addition, steroid concentrations are often 2-4 times higher in urine and fecal samples

than in blood (Munro & Lasley 1987, Peter et al. 1996). Typically, a close relationship exists between plasma estradiol and urinary estrone conjugates. Steroids hormones are lipid soluble and when they are processed in the liver, hydroxyl groups are conjugated to make them soluble in urine. In feces, steroid hormone metabolites are unconjugated (Lasley 1991).

Measuring hormone levels in urine and feces also has some disadvantages. Since the entire amount of steroid may be in a combination of both urine and feces one must first learn the excretory route of the steroid of interest. The investigation of the route of excretion can be done by recovering and measuring radiolabeled exogenously administered steroid (Schille, Wing, Lasley & Banks 1984). One must also know all free forms and all metabolites of the steroid of interest as well as different cross-reactivities for the antibodies to be used.

This type of technology can be useful in a variety of ways. In non-domesticated and zoo animals, fecal and urinary assays for sex steroids are useful in characterizing ovarian cycles and reproductive status (Peter et al. 1996). Observed behaviors (estrous or other sexual) can be correlated with hormone levels to predict estrus and estrous cycle lengths. Pregnancy itself can be distinguished from pseudopregnancy since pseudopregnancy only lasts 1/2-2/3 of normal gestation length. Assessments can also be made of sexual maturity, fertility, reproductive status, effectiveness of experimental contraception, as well as diagnosing specific reproductive dysfunction. The applications of noninvasive endocrine assessments to free-ranging wildlife are also numerous. Precise reproductive status of animals can be determined without altering the subjects' behaviors.

A number of researchers have taken advantage of these advancements and applied these techniques on several species. The main objectives in most research has been to develop and validate methods of extracting and measuring fecal estradiol and progestins and to use fecal and urinary analysis as a noninvasive means for monitoring ovarian steroids. This has been done in Goeldi's monkey (Carroll, Abbott, George, Hindle & Martin 1990), various endangered felids (Graham, Goodrowe, Raeside & Liptrap 1995), red buffalo, yak, Grevy's zebra and Nubian ibex (Safar-Hermann, Ismail, Choi, Mostl & Bamberg 1991), domestic pigs (Schwarzenberger, Toole, Christie & Raeside 1993), and humans (Munro et al. 1991) as well as many other exotic animal species (Czekala, Gallusser, Meier & Lasley 1986, Lasley & Kirkpatrick 1991). At present, this technology is most useful in aiding in reproduction and maintaining viable populations of exotic and wild animals and it has many applications in human research (Peter et al.1996).

Although this type of steroid measurement has not yet been adapted successfully for the common laboratory mouse, it has been adapted for many other species. The adaptation of this assay for the mouse would enable the quantitative analysis of androgens and estrogens in novel-male-exposed inseminated mice. Samples of urine and feces could be collected for the first five days of pregnancy for each inseminated female exposed to novel males and steroid content could be compared to that of inseminated control females. The amount of urine and feces required to accurately measure the steroids is about what could be collected from a mouse in a 12-hour period. Although this would not provide information on momentary fluctuations of hormone, it would provide a useful measure for each animal for each day.

Focus and Outline of Experimentation

The general objective of this thesis was to gain a better understanding of the role of androgens and estrogens in the transmission and transduction of the Bruce effect. This is done by examining some parameters of the effect previously unclarified, particularly if elevated steroids in the females are contributing to the disruption of pregnancy.

Chapter 2

Published work (deCatanzaro, Muir, Sullivan, Boissy 1999, deCatanzaro, Zacharias, Muir1996)

Rationale – The first objective of this thesis was to redefine some of the parameters involved in the Bruce effect. Some similar experiments have been criticized for methodological concerns involving confounding male/female mating behavioral interactions, excessive handling by experimenters of inseminated females, poor air ventilation in the females' cages and unsanitary conditions. These factors, as well as the exposure to novel males' excretions may have contributed to the disruption of early pregnancy. Experiments 1, 2, 3 and 4 were run to define a Bruce effect paradigm in our laboratory. The use of a double-decker cage minimized contact between the females and stimulus males while providing proper air exchange. These experiments also define the role of urine and feces in the Bruce effect by varying the quantities of each excretion required to induce the effect.

Experiment 1

The purpose of experiment 1 was to assess the effect of differences in emission strength on the female's pregnancy outcome. It can be hypothesized that the more males the pregnant females are exposed to, the more urine, feces or other emissions they will encounter, which should lower their chance of bearing litters. This experiment was designed to expose pregnant females to different magnitudes of novel male pheromones or emissions by varying the number of males above the female and assessing pregnancy outcome. It was found that the more males and excretions a female is exposed to, the lower the chances were of remaining pregnant.

Experiment 2

Based on the findings from experiment 1, it was hypothesized that females must come in contact with the excretions of males in order for pregnancy to terminate. This experiment was designed to expose pregnant females to three novel males housed below them and to compare their pregnancy outcomes to females who were exposed to three novel males housed above them. Females housed above the males should not come in contact with the emissions and should remain pregnant. The results of this experiment show that males housed beneath females do not disrupt pregnancy as well as males housed above.

Experiment 3

This experiment was designed to determine if male urine alone could disrupt pregnancy when painted directly on the noses of inseminated females. The quality of the male urine was varied. Urine was either taken from novel males housed in proximity to a

female or from novel males housed alone. Female pregnancy outcome from each of these groups was compared to females that had tap water painted on their noses. The results showed that novel males must be in proximity to a female in order for their urine alone to disrupt pregnancy.

Experiment 4

The results from experiment 3 showed a diminished Bruce effect in both urine conditions compared to a Bruce effect where urine and feces can both contaminate the females' environment. Male feces have never been implicated as a vehicle in the Bruce effect. This experiment tested the role of the male feces. The males' feces were prevented from entering the female's cage by a fine wire mesh and pregnancy outcome was compared to that of females that could contact the males' feces. This experiment was originally designed as a procedure to collect female urine and fecal samples without contaminating them with the males' feces. The results suggest a role for the males' feces in the transmission of the Bruce effect.

Chapter 3

Published work (deCatanzaro, Zacharias, Muir1996)

Rationale – A second objective of this thesis was to isolate the source of a potential pheromone. In previous studies of the Bruce effect, urine had always been investigated as the vehicle for a pheromone. Observations from our laboratory suggested that a pheromone might be transmitted in an oily pungent smelling secretion from the stimulus males that had been observed on the ventral surface near the genitals saturating the fur. This secretion, which had not been reported in the literature, also covered subordinate

males when three males were housed above a pregnant female. If the source of the secretion could be localized, then it could be analyzed further. The preputial glands in the males were chosen as the potential source since they are secondary sex organs and have been implicated in the literature in secreting various social chemosignals.

Experiment 5

This experiment was designed to isolate the source of a potential pheromone. Females were exposed to novel males that had their preputial glands removed. Their pregnancy outcome was compared to females that were exposed to intact males. These results showed that the preputial glands are not required in order for males to disrupt pregnancy.

Chapter 4

Published work (deCatanzaro, Smith, Muir 1995, deCatanzaro, Muir, O'Brien, Williams 1995)

Rationale – A third objective of this thesis was to determine the role of androgens and estrogens in the Bruce effect. It is known that castrated males given exogenous testosterone will retain their capacity to disrupt pregnancy. It is suspected that some of this steroid may be excreted in the urine and feces of the male and contaminating the environment of the inseminated female. It is known that extremely low doses of estrogens in the females are detrimental to implantation. If estrogens are given to males and are excreted in biologically active form in the urine, then inseminated females might not implant. There has been no direct evidence that elevated estrogens are involved with implantation failure in the Bruce effect. If the loss of early pregnancy is due to an

increase in 17β-estradiol, then administering a monoclonal antibody specific to 17β-estradiol to pregnant females, should lower the hypothesized increase in 17β-estradiol and reduce the frequency of pregnancy disruptions.

Experiment 6

This experiment was designed to test the effects of indirect exogenous 17β estradiol on pregnancy outcome. Exogenous 17β-estradiol was administered to castrated novel males housed above pregnant females. The females were exposed to the excretions of the males in the form of urine and feces and their pregnancy outcome was measured and compared to females who were exposed to castrated males given a vehicle injection. The results of this experiment showed that castrated males given 17β-estradiol disrupt pregnancy as well as intact males.

Experiment 7

The results from the previous experiment strengthen the hypothesis that 17β-estradiol may be involved in the disruption of pregnancy. This experiment was designed to lower the hypothesized increase in 17β-estradiol in pregnant females exposed to novel males. Females were exposed to novel males and injected with 17β-estradiol antibody for the first five days of pregnancy and their pregnancy outcome was measured and compared to females who did not receive the antibody. These results showed that females receiving the antibody maintained their pregnancy.

Chapter 5

Rationale – Experiment 6 showed that antibodies for 17β-estradiol significantly prevented the Bruce effect. Based on this, the final objective of this thesis was to

measure androgens and estrogens in inseminated females exposed to novel males. It is possible that implantation is failing due to elevated androgens and estrogens in the inseminated females. These steroids have been shown detrimental to implantation. It can be hypothesized that in the Bruce effect females these hormones may be elevated.

Experiment 8

This study was designed to determine whether elevated androgens and estrogens correlated with pregnancy loss. Measurements were taken of testosterone, 17β-estradiol and estrone conjugates in the excretions of inseminated females during days 1 to 5 of pregnancy. The females were either exposed to novel males or housed alone. It was found that these hormones were elevated significantly in the females exposed to novel males that lose their pregnancy.

Chapter 2

Refinement of parameters in the chemical transmission of the Bruce effect: The role of male urine and feces

This chapter addresses certain issues involving the experimental paradigm used in studying the Bruce effect. Current researchers of this effect have built on an original experimental paradigm (Brennan et al. 1999, Kumar & Dominic 1993, Thomas & Dominic 1989) that has been criticized for some of its potential confounds (deCatanzaro & MacNiven 1992). Several reports have implicated pheromones, probably contained in the urine from the novel male, as a mediator of the Bruce effect (Bruce 1960c, Dominic 1965, 1966a, 1966b). However, certain methodological concerns have been raised in some studies regarding the experimental design (deCatanzaro & MacNiven 1992, deCatanzaro et al. 1995). Earlier studies would induce the Bruce effect by placing the inseminated female in bedding soiled by multiple males or funneling the urine of several males into the cages of the females (Bruce 1960b, Parkes & Bruce 1962). It has been noted (deCatanzaro & MacNiven 1992) that this design involved changing the bedding twice daily and keeping the female in a container with restricted air ventilation. Poor air quality and excessive handling were concerns that may have confounded these experiments. Excessive handling and manipulation of inseminated females by itself can induce early pregnancy loss (Runner 1959). Human handling and unsanitary conditions in these experiments may have played a large role in stressing the females and inducing a pregnancy disruption through physiological mechanisms other than those induced by the males' excretions.

Behavioral interactions between the inseminated female and novel male have also been reported to be correlated with the disruption of early pregnancy (deCatanzaro & Storey 1989, Storey & Snow 1990). Close contact of the inseminated female with the novel male often brings about mating behavior. This is known to induce ovarian hormonal changes (Diamond 1970). Many of these experimenters (Dominic 1965, 1966a, 1966b, 1966c, Marchlewska-Koj 1981) have used daily vaginal smears, taken post-coitum, and cell cornification as the measure of pregnancy. Vaginal stimulation can simulate mounting and intromission behavior and induce hormonal changes that could impair implantation or disrupt pregnancy post implantation. Furthermore, it has been noted that in some of these studies (Brennan et al. 1990, Brennan et al. 1999, Keverne & de la Riva 1982) implantation sites were counted as a measure of pregnancy. Counting implantation sites involves sacrificing the animal and such methodologies may not account for delayed implantation. This can lead to lower pregnancy rates for the experimental groups and misinterpretation of some results. These types of measures of implantation have not been validated in terms of actual pregnancy outcome. It is important that these issues are addressed and a current paradigm designed to minimize many of these potential confounds.

In this laboratory, we have studied the Bruce effect by housing females below novel males in a double-decker cage. Mating behavior and possible reinsemination by the male is prevented by a wire mesh grid that separates the top and bottom halves of the cage. This allows the males' excretory products to fall into the female's cage while minimizing human handling and allowing proper air ventilation. Pregnancy outcome is performed by counting the litters and number of pups occurring in each group.

This chapter addresses various parameters and properties of the chemical transmission of the Bruce effect. The quality and quantity of urine and feces required from novel males to induce the effect is tested. First, the quantity of male urine and feces required to induce a significant effect is examined by exposing females to varying numbers of males housed above. This experiment establishes the importance of the urine or feces as a vehicle for the transmission of the effect. It effectively partitions out any behavioral component of the effect by preventing male/female interactions with the use of the mesh grid separating the top and bottom halves of the cage. The next experiment tests the importance of female contact with male excretions in the chemical transmission of the Bruce effect. By comparing the pregnancy outcome of females housed above 3 novel males with females housed below 3 novel males it can be determined if the pheromone is volatile or if females need to physically contact the urine and feces. Next, urine alone is tested to induce a Bruce effect and the social context of the novel male it is collected from is varied. By comparing urine from males housed alone or near novel females the quality of the urine may be changed and its pregnancy disrupting properties can be assessed. Finally, the role of the novel male feces is investigated. The transmission of the Bruce effect is tested in the absence of male feces.

Experiment 1 – Variation in the number of novel males in the female's environment

The purpose of experiment 1 was to assess the effect of differences in emission strength on the female's pregnancy outcome. It can be hypothesized that the more males a pregnant female is exposed to, the more urine, feces or other emissions they will encounter and this should lower their chance of bearing a litter. This experiment was

designed to expose pregnant females to different magnitudes of strange male pheromones and to assess the conditions under which these pheromones are effective. Pregnancies may be disrupted more reliably if the concentration of male chemical cues is increased by housing two or three males above a pregnant female instead of just one.

General Methods

CF-1 strain mice were obtained from Charles River Breeding Farms, La Prairie, Quebec at 55-60 days of age or bred in this laboratory from the same stock. HS (heterogeneous strain) mice were bred in this laboratory from stock originally obtained from the Dept. of Zoology, University of Toronto. Prior to the experiment, females were housed in groups of three or four. All males were housed individually. All housing was in standard polypropylene cages measuring 28 × 16 × 11 (height) cm with straight-wire tops allowing continuous access to food and water. The colony room was maintained under a reversed 14:10 light/dark cycle at 21°C.

When female subjects were between 75-100 days of age, they were each housed alone at the commencement of the dark phase of the light cycle with one male of the same strain in a cage like that described above. The males were sexually experienced and had been deprived of access to a female for at least 7 days. After every 2-1/2 h period following pairing, the hindquarters of each female were inspected for the presence of a sperm plug. At the end of the dark phase, all females with obvious plugs were identified as subjects, and the day of detection was designated as day 0 of pregnancy. Each female remained housed with the inseminating male until the morning after detection of a sperm plug, about the start of the dark phase of the light cycle. The female was then removed

from the male and assigned to one of the experimental conditions. Assignment to conditions was counterbalanced across age and date of insemination.

Females were then housed individually in clean cages with fresh bedding. For each experimental female, this was in an apparatus that resembled normal caging in dimensions but allowed the female to be exposed to a novel male through a wire-mesh partition. This was a double-decker cage, constructed from clear Plexiglas, measuring 30 × 21 × 27 cm, divided into two compartments measuring approximately 30 × 21 × 13 cm. These two compartments were separated by a wire-mesh grid with square openings measuring 0.5 cm^2. The upper compartment was covered with standard straight-wire mouse cage lid providing continuous access to food and water. The lower compartment was filled with approximately 0.5 L of clean bedding and had a waterspout protruding through one wall. Food in the lower compartment was delivered through a cylindrical (4 cm diameter, 5 cm depth) plastic cup, with the open end providing access to food through a wire mesh, oriented so as to prevent urine and feces from the animal above from contacting the food. Each female was housed in the lower compartment of such an apparatus.

Experimental females were continuously in the double-decker cage until 5-6 h after commencement of the dark phase of the light cycle on day 6 of pregnancy. At that point each female was directly transferred to a clean standard mouse cage and left undisturbed until pregnancy outcome measures began. Beginning at day 18 after the detection of the sperm plug, females were checked on two occasions each day for the birth of litters. Such inspections continued until 30 days after detection of the plug.

Pregnancy outcome was measured through the presence or absence of a litter and by counts of the number of pups born.

Experimental Procedure

On the first day after the detection of the sperm plug, approximately 4 hours after the onset of the dark phase of the light cycle, females were randomly assigned to one of four conditions. Control females were left completely undisturbed for the duration of their pregnancy in a clean cage with fresh bedding. Experimental females were placed in the lower compartment of a double-decker cage with one, two or three novel males housed together above.

Pregnancy Outcome Measures

On the sixth day after the detection of the sperm plug, the experimental females were housed singly in separate clean mouse cages with fresh bedding and left undisturbed until day 18. On day 18, the mice were inspected for the birth of litters twice a day until day 30. Pregnancy outcome was measured by the presence of litters and by the total number of pups born.

Results

Figure 1 summarizes the pregnancy outcomes from experiment 1. The control group had the highest pregnancy outcome with 84 percent pregnant, followed by the one male above group with 53 percent pregnant. The two males above group had 27% pregnant and the three males above group had only 13 percent pregnant. A Chi-squared

Experimental females were either housed, in a double-decker cage, directly below three novel sexually experienced HS males or directly above three novel sexually experienced Hs males. Pregnancy outcome was measured by the presence of litters and by the total number of pups born.

Results

Figure 2 is a summary of the proportion of females in each group bearing litters in experiment 2. The control group had the highest proportion of pregnancies (88 %), followed by the three-males-below group (63 %), and the three-males-above group with the lowest at (19 %). A Chi-square test of association relating the conditions to the presence or absence of litters showed significance when all three groups were compared, χ^2 (2) = 12.38, $p < 0.005$. Table 2 illustrates the average number of pups born per group (± SEM). An analysis of variance showed significance, when completed across all conditions, on the number of pups born to each group considering all females, F (2, 45) = 10.5, $p < 0.005$. Multiple comparisons identified differences between the males above condition and the two other conditions. The differences in the number of pups per group were proportional to the number of females bearing litters since litter sizes among all groups were similar when nonparturient females were excluded.

Housing 3 novel males in close proximity to a female they cannot access unexpectedly induced intense intermale aggressive behavior. In several instances, death of a male was a result of these interactions. This result was an incidental finding in this experiment and death of these stimulus males was not intended. The deceased males were often found covered completely from head to tail with an oily pungent smelling

substance that appeared to be secreted by the dominant male. This substance was found on the ventral surface surrounding the genital region saturating the fur. In a few cases, victims were found alive but sluggish and did not appear to have sufficient wounding to account for their demise. When found alive, these males were placed in a clean cage and offered food and water. In all cases these males died within 10-12 hours. Deceased males were removed when detected and replaced with another novel male of the same strain age and sexual experience.

Discussion

The data from experiment 2 show that the female must come in direct contact with the emissions of the males for the Bruce effect to take place. Females that were housed above novel males had a greater number of pregnancies than females housed beneath novel males. If the chemical cue coming from the males is contained in the urine, feces or as an emission on its own, then the females' pregnancy may be disrupted more reliably if positioned beneath the males in direct contact with their excretory products. If the female were living above the males, then the female would only be exposed to the smell of the males and not come in contact with their waste. The data support the idea that the smell is not enough to block pregnancy. From Experiments 1 and 2 we can see that there is a chemical mediation of the Bruce effect. The more the female is in contact with this chemical mediator, the less chance she has of producing a litter.

The multiple male groups were subjectively observed to have aggressive encounters often leading to the death of one of the males. In order to eliminate this

confound, subsequent experiments had multiple males partitioned above the female with a double wire grid partition. Although these males could not physically touch each other, males were still often found dead with absence of any physical wounding. Despite this lack of physical contact, these males were also covered in the foul smelling oily substance. To remedy this, an opaque plastic partition was put in the cages in such a manner that the males could not interact behaviorally. This removed all aggression and mortality

Experiment 3 – The Bruce Effect in the Absence of Male Feces

This experiment was originally designed as a procedure to collect female urine and fecal samples without contaminating them with the males' feces. A fine mesh was to prevent the male feces from falling into the bedding of the female while the female samples were collected. Preliminary studies showed that there was a small decrement to the Bruce effect, so the experiment was repeated. In this experiment, inseminated females were exposed indirectly to novel males either with a regular grid floor that allowed the passage of feces or with a fine mesh screen that captured the feces.

Experimental Procedure

The subjects and insemination procedure were identical to that described for experiment 1. On the first day after the detection of the sperm plug, approximately 4 hours after the onset of the dark phase of the light cycle, the females were put into one of three conditions. Control females were housed individually in a clean cage and left undisturbed for the duration of the experiment. Experimental females were put into the

lower level of a double-decker cage with two novel males above with or without a fine mesh screen that prevented the male feces from falling into the lower cage. After the first five days of pregnancy, the females were individually housed in standard clean cages for the duration of pregnancy.

Litter checks began 18 days after the detection of the sperm plug and were conducted three times a day until day 30 or until all of the pregnant females had delivered. Pregnancy outcome was measured by the presence of litters and by the total number of pups born.

Results

Figure 3 shows the proportion pregnant in each group and the mean number of pups born to females in each group. When the feces were removed from the females' environment with a screen 57 percent had litters compared to only 33 percent when the feces were present and 100 percent of controls when no male was present. A chi-squared test of association between conditions and the presence or absence of litters showed significance when all conditions were compared to each other, χ^2 (2) = 28, $p < 0.001$. Significance was also found when comparing the control and no-screen groups, $\chi^2(1) = 28.49$, $p < 0.05$ and the control and screen groups, χ^2 (1) = 15.74, $p < 0.05$, but not the screen and no-screen groups. Table 3 shows the mean (± SEM) number of pups born for all females. An analysis of variance on the number of pups born to each group showed significance, F (2, 82) = 15.91, $p < .00002$. Newman-Keuls multiple comparisons tests showed significance between the control and each experimental condition ($p < 0.05$) but not between the 2 other experimental conditions. The differences in the number of pups

per group were proportional to the number of females bearing litters since litter sizes among all groups were similar when nonparturient females were excluded.

Discussion

The absence of feces in the female's bedding did not significantly decrease the strength of the Bruce effect although there was a small trend showing some influence on pregnancy. However, fewer females did experienced pregnancy failure when there were no feces present. There have been no studies reported in the literature that implicate male feces as a vehicle in the transmission of the Bruce Effect; all studies have focused on urine. It is common knowledge that mice eat their own feces as well as the feces of other mice. It is possible that the feces from the male contain biologically active androgens or estrogens, which if ingested or physically manipulated, could influence pregnancy to a much lesser extent than urine. It is an objective of this laboratory to chemically analyze the feces of the males in this effect for the presence of estrogens or androgens using an ELISA procedure. These data might reach statistical significance between the groups exposed to novel male feces and those not exposed if the sample size were increased.

Experiment 4 – Topical Application of Novel Male Urine

These experiments demonstrate that the quantity of the males' excretions is important in the transmission of the Bruce effect but there is reason to believe that the quality of the excretions may also be important. Male urine alone has been reported to disrupt pregnancy when painted directly on the noses of the inseminated females during

the implantation period (Dominic 1966c, 1966a). A recent study showed that urine collected from males housed alone was insufficient to induce a Bruce effect when painted on the noses of females (deCatanzaro et al. 1995). This prompted an attempt to modify the quality of the urine placed on the females noses. If the motivational state of the stimulus males is changed, they might produce qualitatively different excretions. This can be done by exposing the novel males to females they cannot access while their urine is being collected. This has been reported to increase plasma testosterone in the males (Macrides et al. 1975).

This experiment is designed to replicate experiments by Dominic (1966c, 1966a) in which he reported a Bruce effect with the use of novel male urine alone. Dominic did not compare the urine of male mice to control urine but instead used tap water as a control. It is possible that the handling of the inseminated females was stressful to the females or that the application of any type of animals' urine on the females' nose could disrupt pregnancy. Recently, a study in our laboratory (deCatanzaro et al. 1995) failed to reproduce this effect with the urine put directly into female bedding or painted directly on the noses of the pregnant females. However, the urine in that study was collected from novel males who were housed alone.

Experimental Procedure

The subjects and insemination procedure were identical to that described for experiment 1. Males of the HS strain were housed in pairs in the upper half of a clear double-decker Plexiglas cage (30×21×27 cm) with a wire grid floor and a double-layer opaque Plexiglas partition separating the males from each other. The males had either an

HS female housed below them that served to sexually stimulate them, or the males were housed above nothing and their urine served as a control. Urine was collected by placing the top half of the double-decker cage on a stainless steel surface until the males urinated.

On the first day after the detection of the sperm plug, approximately 4 hours after the onset of the dark phase of the light cycle pregnant females were put into either experimental or control conditions. Control females were housed in a clean standard cage with fresh bedding and received repeated topical applications of tap water to the snout area for the first five days of pregnancy. Experimental pregnant females were placed in a clean standard cage with fresh bedding and had either unstimulated male urine or stimulated male urine painted on their noses for the first five days of pregnancy. The unstimulated urine was collected from males that were not in the context of a female while the stimulated urine was collected from males that were housed above females that they could not access. These applications were applied at 2.5, 6.5, 9.5 and 13.5h after the start of the dark phase with a No. 6 artist's paintbrush to avoid human handling. On the sixth day after the detection of the sperm plug, the females were housed in standard cages and left undisturbed for the remaining gestation period. Repeated measurement of the quantity of fluid delivered by this method, by weighing the brush before and after application, indicated a mean weight of fluid delivered in a single application of 82.8 mg with a range of 55 to 104 mg. Different brushes were used for each condition. Urine was applied within two minutes of collection. The stainless steel surface and paintbrush were cleaned and dried at the end of each day to prevent bacterial growth.

Litter checks began 18 days after the detection of the sperm plug and were conducted three times a day until day 30 or until all of the pregnant females had delivered

litters. Pregnancy outcome was measured by the presence of litters and by the total number of pups born.

Results

Figure 4 shows the proportion bearing litters in each condition for experiment 3. It appears that there was no difference in efficacy between water and unstimulated urine at disrupting pregnancy. Of the females who received tap water, 87.5 percent produced litters, whereas 78.6 percent of females that received unstimulated urine produced litters while only 58.6 percent of females that received stimulated urine produced litters. Table 4 shows the mean (± SEM) number of pups born per group. A test of association comparing all three conditions with the presence or absence of parturition showed significance, χ^2 (2) = 9.25, $p < 0.05$. When the water exposure group and the stimulated urine exposure group were compared there was also significance, χ^2 (1) = 8.58, $p < 0.05$, but there was no significance with the other pairs. Analysis of variance on the number of pups born was significant, F (2, 107) = 6.99, $p < 0.05$. A Newman-Keuls multiple comparisons test ($p < 0.05$) showed differences between the water and stimulated urine groups and between the unstimulated urine and stimulated groups but not between the water and unstimulated urine groups. The differences in the number of pups per group were proportional to the number of females bearing litters since litter sizes among all groups were similar when nonparturient females were excluded.

Discussion

Urine collected from novel males housed above females was able to induce pregnancy disruption when painted on the noses of inseminated females. Urine collected from males housed alone was unable to do this. These data help settle the discrepancy between previous data in this laboratory that failed to obtain such results (deCatanzaro et al. 1995) and other published studies that support these findings (Dominic 1966a, 1966c). These previous reports did not specify the social context of the males' urine collection.

Evidence suggests that housing males in the proximity of females can alter their endocrine state and raise testosterone levels (Bartke & Dalterio 1975, Bliss, Frischat & Samuels 1972, Macrides, Bartke & Dalters 1975) as well as augment aggressive behavior (deCatanzaro et al.1981). Castration of the male eliminates this effect (deCatanzaro & Storey 1989). Since urine collected from males housed in proximity to females disrupts pregnancy with greater efficacy than urine collected from males housed alone, it can be concluded that social stimulation influences the chemical composition of urine in this context. Various accessory sex glands in male mice have the capacity to alter the chemical constituents of urine. Increased plasma testosterone can increase output of pheromones into the urine from male preputial glands (Ma, Miao & Novotny 1999, Pandey & Pandey 1985). These glands have been shown in males to secrete factors that can influence female mate selection (Ninomiya & Brown 1995) and are involved in intermale aggression (Bronson & Caroom 1971). The vesicular-coagulating complex is a testosterone dependent accessory gland involved with the production of copulatory plugs that are inserted into the vagina during mating (Jones & Nowell 1973). These glands

have also been found to influence aggression thorough modulation of pheromones released in the urine. It is possible that these glands are modifying the urine of males in the social context of females. This may explain why urine collected from males housed alone failed to produce this effect in previous trials of this experiment (deCatanzaro et al. 1995).

This experiment does not specifically differentiate between the exposure of novel males to females and the exposure of them to conspecifics in general. The exposure of the novel males to conspecifics may also have an influence on the composition of their urine since the sex accessory organs are also involved in the control of urine pheromones that alter aggressive behavior (Bronson & Caroom 1971). However, during the urine collection period, males in both conditions were housed in the upper portion of the exposure apparatus. This apparatus allows two males to be housed next to each other with a plastic partition separating them. This would put the males in both experimental and control conditions in olfactory proximity to other males while the urine was collected. This would control for any influence exposing males to other males would have on the properties of the collected urine.

The results from this experiment show that the loss of pregnancy in females that had urine painted on their noses was substantially lower than that found in the previous experiments when the males were actually housed above the females. This could be due, among other possibilities, to the absence of any behavioral interaction. When novel males are housed above a pregnant female, the female often climbs on the grid that separates them, which results in sniffing and mutual investigative behavior. This behavior may contribute to the loss of pregnancy since various behaviors have been

reported to disrupt pregnancy (deCatanzaro & Storey 1989). The absence of the novel males' feces may have also played a minor role in reducing the strength of the Bruce effect when urine is used alone. A final factor that may have played a role in the diminished effect is the volume of urine the females in this experiment were exposed to was much lower than that from 3 males.

In summary, these findings support the role of novel male urine in the transmission of the Bruce effect. It is required that the females physically contact these excretions for pregnancy to be disrupted. Also the greater amount of excretions a female encounters, the greater probability there is of pregnancy disruption. Novel male urine alone can induce the loss of pregnancy but not as efficiently as the presence of the male with both urine and feces present also. Feces do not significantly contribute to the transmission of the Bruce effect. Furthermore, in all experiments the differences in the number of pups per group were proportional to the number of females bearing litters since litter sizes among all groups were similar when nonparturient females were excluded. This indicates that there was no partial pregnancy disruptions and that this Bruce effect is an all or nothing effect.

These experiments have redefined some of the simpler parameters of the Bruce effect that may have been confounded in previous reports. In the present experiments, human handling of the inseminated females was kept to a minimum since this alone can induce the disruption of pregnancy. The females' cages were well ventilated and direct behavioral interactions, such as mating behavior between the inseminated female and the stimulus males, were prevented by a wire grid partition. To measure implantation success or failure, litters and pups were counted. These direct measures of pregnancy

outcome are more reliable than observing implantation sites or cell cornification used by other researchers. Counting implantation sites does not account for delayed implantation while measuring cell cornifications can introduce confounds since this requires vaginal stimulation and can disrupt implantation. These experiments have defined a workable and repeatable paradigm for the Bruce effect. This paradigm is used in subsequent studies in this thesis to study the transmission from male to female and transduction within the female of the Bruce effect.

TABLES and FIGURES

Chapter 2

Table 1: Mean (±SEM) number of pups per group in Experiment 1 including zero for nonparturient females after indirect exposure during the first five days of pregnancy to one, two or three novel HS males housed above.

Condition	N	Number Delivering	Mean Pups Per Group (±SEM)	Mean Pups Per Litter (±SEM)
Control	19	16	10.0 (±1.2)	11.9 (±0.6)
1 Male	15	8	6.9 (±1.8)	12.9 (±0.9)
2 Males	15	4	3.1 (±1.4)	11.6 (±2.5)
3 Males	15	2	1.8 (±1.3)	13.5 (±0.8)

Table 2: Mean (±SEM) number of pups per group in Experiment 2 including zero for nonparturient females after indirect exposure during the first five days of pregnancy to three novel HS males housed above or below.

Condition	N	Number Delivering	Mean Pups Per Group (±SEM)	Mean Pups Per Litter (±SEM)
Control	16	14	10.5 (±1.1)	12.0 (±0.7)
3 Above	16	3	2.2 (±1.2)	11.7 (±1.5)
3 Below	16	10	7.2 (±1.5)	11.5 (±0.6)

Table 3: Mean (±SEM) number of pups per group in experiment 3 including zero for nonparturient females, after indirect exposure during the first five days of pregnancy below two novel HS males. There was either a fine mesh screen to filter the feces or no screen.

Condition	n	Number Delivering	Mean Pups Per Group (±SEM)	Mean Pups Per Litter (±SEM)
Control	29	29	10.9 (± 0.4)	10.9 (±0.4)
No Screen (feces)	27	9	3.8 (± 1.1)	11.4 (±0.4)
Screen (no feces)	28	16	6.3 (± 1.1)	11.3 (±0.5)

Table 4: Mean (± SEM) number of pups born for all females in Experiment 4 including zero for nonparturient females. Females received water, unstimulated urine or stimulated urine painted on their snout from a novel HS male during the first five days of pregnancy.

Condition	n	Number Delivering	Mean Pups Per Group (±SEM)	Mean Pups Per Litter (±SEM)
Water	40	35	10.9 (±0.8)	12.5 (±0.5)
Stimulated Urine	41	24	6.3 (±0.9)	10.8 (±0.7)
Unstimulated Urine	28	22	9.3 (±1.0)	11.8 (±0.6)

Figure 1: Percentage of CF-1 females in Experiment 1 delivering litters after housing during days 1-5 of pregnancy below 1, 2 or 3 novel outbred HS males. Conditions with similar letters (i.e. a, b, c) are not significantly different from each other.

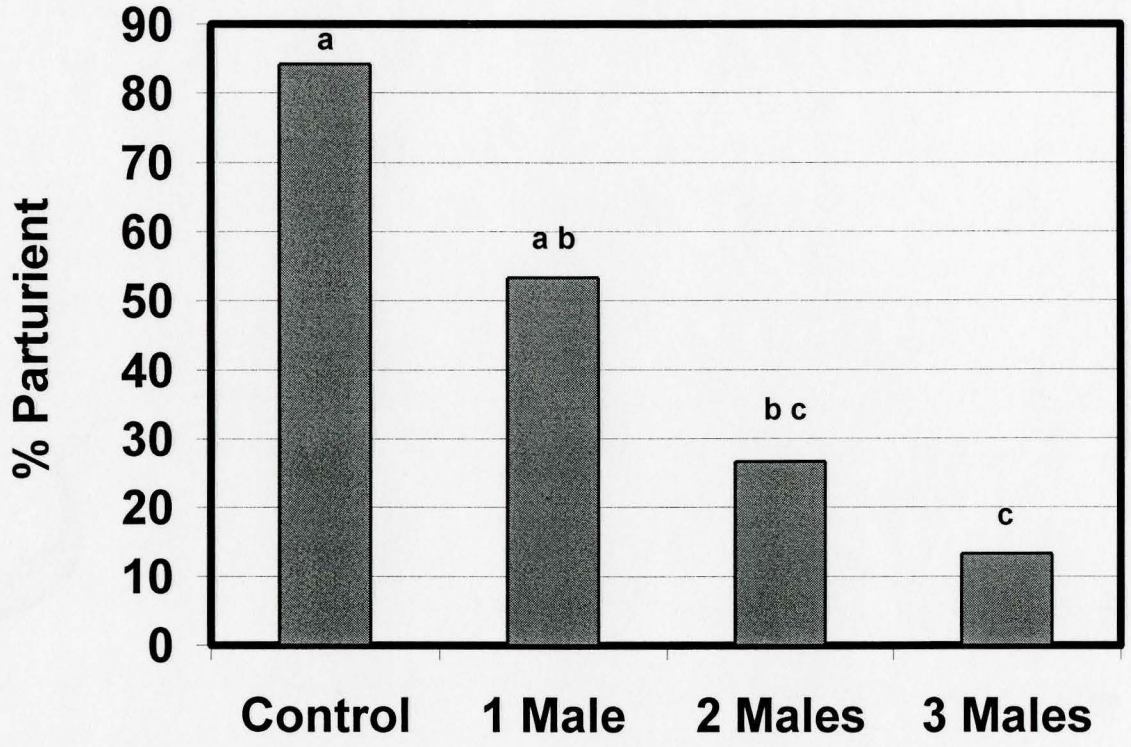


Figure 2: Percentage of CF-1 females in Experiment 2 delivering litters after housing during days 1-5 of pregnancy above or below 3 novel outbred HS males. An asterisk (*) indicates statistical significance.

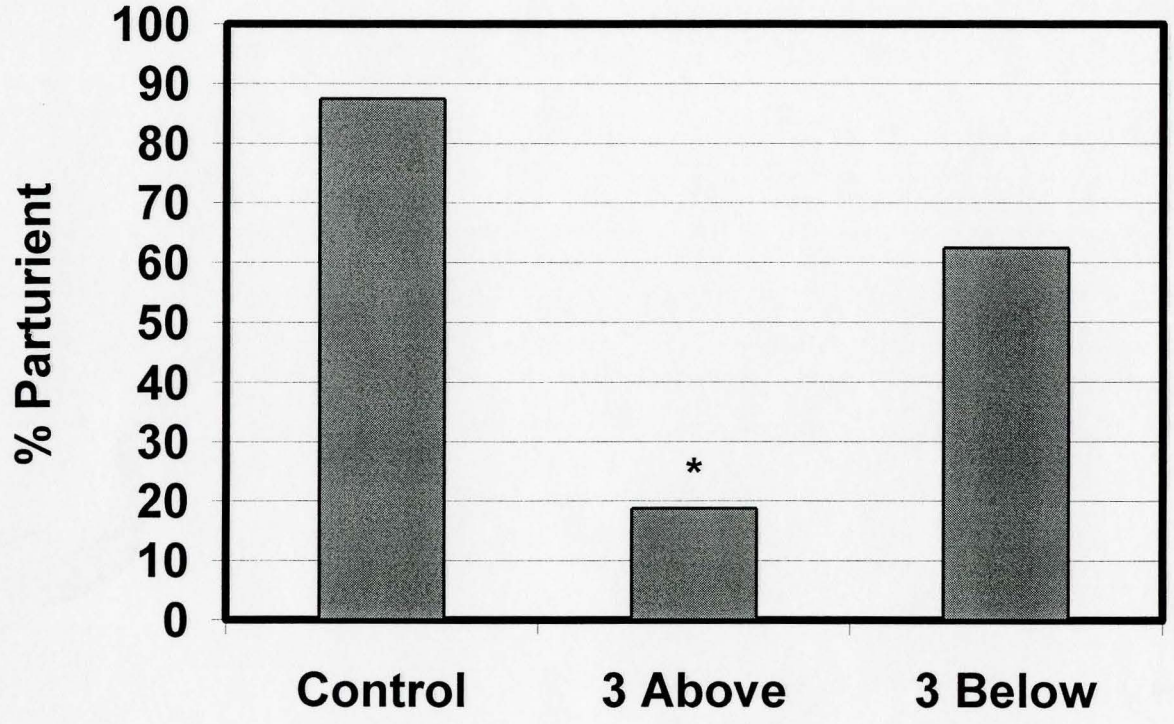


Figure 3: Percentage of CF-1 females in Experiment 3 delivering litters after housing during days 1-5 of pregnancy below 2 novel outbred HS males with either a fine mesh screen to filter the feces or with no screen. Conditions with similar letters (i.e. a, b, c) are not significantly different from each other.

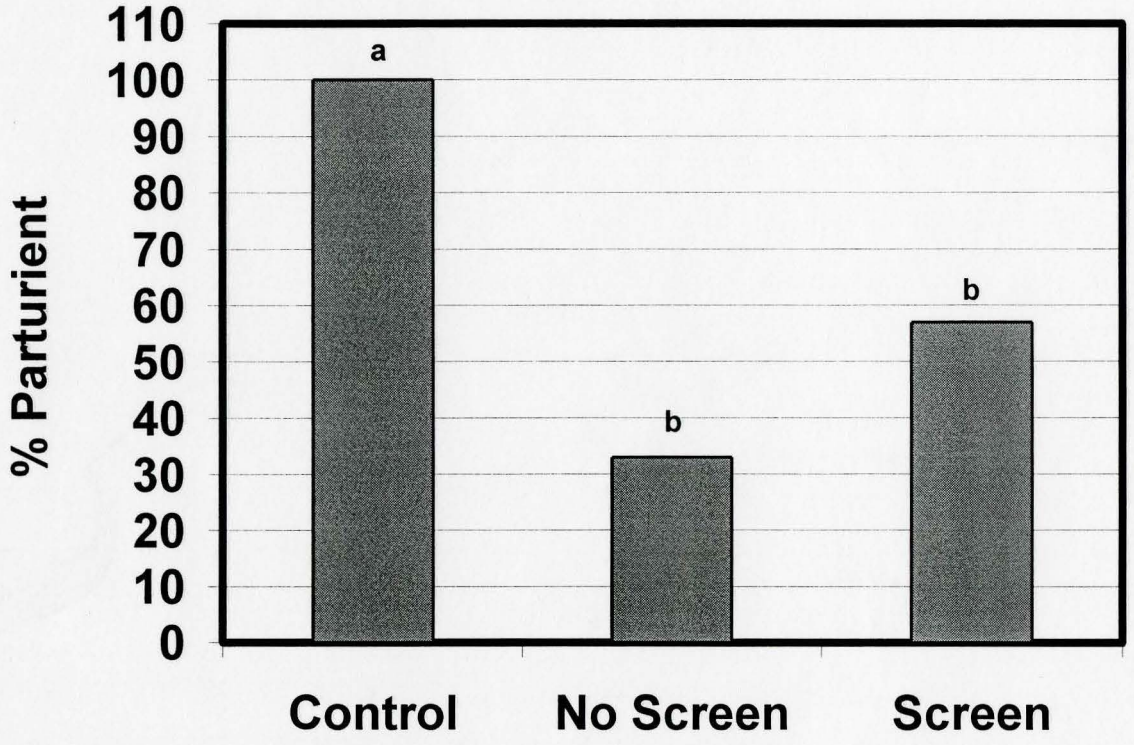
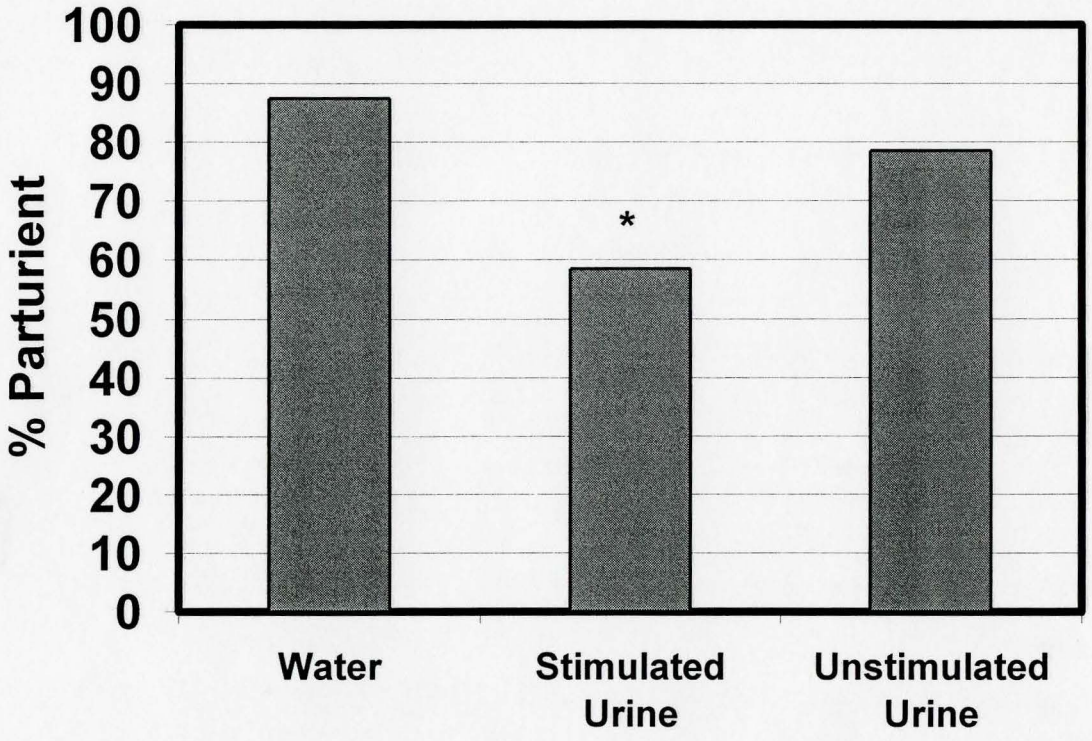


Figure 4: Percentage of CF-1 females in Experiment 4 delivering litters after having water, stimulated HS novel male urine or unstimulated HS novel male urine painted on their snouts during days 1-5 of pregnancy. An asterisk (*) indicates statistical significance.



Chapter 3

The preputial glands as a source of a potential pheromone mediating the Bruce effect

The purpose of this study was to localize the source of the pheromone released by the stimulus novel males that may be responsible for the transmission of the Bruce effect. It has been reported that a pheromone might be transmitted in an oily pungent smelling secretion from the stimulus males. This substance had been observed on the ventral surface near the genitals saturating the fur when two males are housed together above a female they cannot access (deCatanzaro, Muir, Spironello, Binger & Thomas 1999). This secretion also completely covered subordinate males when three males were housed together above a pregnant female. If the source of the secretion could be localized then it could be analyzed further. If a pheromone could be isolated then the physiological profile of its actions in the female when administered in a controlled fashion could be analyzed or it could be analyzed biochemically. This experiment involved the removal of the male preputial gland, which was suspected as a possible source of a pheromone. The preputial glands are thought to secrete signaling pheromones that attract and possibly stimulate estrus in females and cause intermale aggression (Jones et.al.1972).

Evidence suggests that specific urinary odors of mice arise from the preputial glands. Odor preference studies have been conducted to determine the effect of preputial secretions on mouse behavior. There is evidence that preputial gland odors of males are used for mate selection by females (Ninomiya & Brown 1995). Preference studies suggest that purified preputial gland constituents may play a wide-ranging role in the female recognition of sexually mature and socially dominant males (Jemiolo, Xie & Novotny 1991). Female mice prefer to investigate the urine of normal male mice as

opposed to that of castrated or preputialectomized males (Ninomiya & Kimura 1988). It has also been suggested that both urinary and preputial factors of males are involved in female attraction (Ninomiya & Kimura 1988).

Odors common to males and females emanating from preputial glands of the mother apparently influence early postnatal mice and determine their odor preferences in later life (Hayashi 1979). There is evidence that mice reared by mothers with preputial glands prefer females with preputial glands to females without preputial glands (Hayashi 1979). Furthermore, such females prefer odors from males with preputial glands to odors from males without them. Similarly, females reared by mothers without preputial glands prefer the odor of preputialectomized males and females to intact animals. There is also evidence that the urinary factors which influence female odor preference are androgen-dependent while preputial factors may be androgen-independent (Ninomiya, Kimura 1988). Pandey and Pandey (1985) found that the antiandrogen cyproterone acetate could inhibit the properties of the preputial glands that elicit attraction to the opposite sex in wild mice. This was thought to decrease production of a releaser pheromone from the male's preputial gland.

Biochemical studies show evidence that cohabitation with other mice can influence the chemistry of the glands. Two constituents, E,E,-alpha-farnesene and E-beta-farnesene, have been found elevated in dominant male urine when compared to subordinate or control males (Novotny, Harvey & Jemiolo 1990). These urinary compounds are the most prominent constituents of the preputial glands and not found in the bladder urine of males. Subordinate males who, normally investigate novel male urine extensively, are discouraged from prolonged investigations when these substances

were added to normal male urine. These males behave qualitatively similarly when presented with the urine of dominant males (Novotny et al. 1990). These major volatile constituents of the male mouse preputial gland have also been examined for their role in inducing estrous cycles in grouped female mice. When purified and added to control urine, these constituents have been found as effective as the homogenate of the intact preputial gland in inducing estrus. Extracts of preputial tissue from castrated males have no effect on estrus (Ma et al. 1999).

Histological studies show that social conditions can influence the size of the preputial glands. Preputial glands of males increase in size when housed beside a normal male across a barrier. The glands increase more substantially when there is periodic contact with a normal male but not with a castrated male. In contrast, contact with normal females decreases preputial gland weight in males, but contact with ovariectomized females does not (Hayashi 1986). It has been suggested that the nature of the interactions and the compounds found in the glands may play a wide-ranging role in the territorial-marking behavior of male mice.

Although there is no evidence that the preputial glands are involved in the induction of the Bruce effect, the source of the pheromone thought to induce the effect has never been identified. Based on evidence suggesting that the preputial glands are involved with various scent related effects, removal of the preputial glands in the novel stimulus males may reduce the Bruce effect. If these glands are relevant in the production of pheromones that cause the loss of early pregnancy, then females exposed to novel preputialectomized males should remain pregnant.

Experiment 5

This experiment was designed to isolate the source of a potential pheromone(s) involved with the transmission of the Bruce effect. The preputial glands were removed from the stimulus novel male mice, and the pregnancy outcome of females exposed to these males was compared to that of females exposed to intact males. It was hypothesized that the preputialectomized males would fail to disrupt pregnancy, since they would lack the preputial pheromones that are known to be involved with mediating other social sexual behaviors (deCatanzaro et al. 1996).

Preputialectomy

The subjects were of the same strain, age, and sexual experience as in the previous experiment. The preputial glands of the male mouse consist of a pair of leaf-shaped ectodermal exocrine glands opening to the exterior on either side of the urethral meatus. They are located bilaterally between the skin and the body wall anterior to the external genitalia. They are yellowish-brown in color, dorsoventrally flattened, measuring approximately 7×5 mm although quite variable in size among individuals. In order to remove the glands, the animals were anesthetized with sodium pentabarbitol. Two incisions each measuring approximately 5 mm were made on either side of the external genitalia, or, if possible, both glands were removed via a single incision. The glands were found immediately under the skin layer. Once located, they were snipped off at the stem. In some individuals, a thin membranous tissue was present either over or between the two glands; this was snipped as necessary. Once the glands were removed, the skin incision was closed by a wound clip and animals were kept warm on a heating

pad until normal activity returned. The wound clip was removed without anesthesia one week following surgery. For sham-preputialectomized animals, the procedures of anesthesia, skin incisions, and recovery were the same as those receiving the full surgery, but the preputial glands were left undisturbed. All surgically treated males were allowed at least two weeks recovery before being employed as stimulus animals in the experiments. After the experiments, a subset of preputialectomized males was sacrificed for histology at the site of surgery to confirm that the glands had not regenerated.

Experimental Procedure

The insemination procedure was the same as in the previous experiment. On day 1 of pregnancy, females were designated as either controls, which were inseminated and left undisturbed for the duration of the pregnancy, or experimental animals. In the experimental group females were either indirectly exposed to 2 sham-preputialectomized males (housed above the females for the first five days of pregnancy) or they were indirectly exposed to 2 preputialectomized males. The females in the control group were individually housed in standard clean cages and left alone for the entire pregnancy.

Pregnancy Outcome Measures

At the end of the five-day exposure period, the females were placed in a standard clean cage and left undisturbed for the remaining period of gestation. Litter checks began 18 days after the detection of the sperm plug and were conducted three times a day until day 30 or until all of the pregnant females had delivered. Pregnancy outcome was measured by the presence of litters and by the total number of pups born.

Results

Figure 5 shows the percent bearing litters and the number of pups in each condition. It appears that there was no difference in the ability to disrupt pregnancy between the preputialectomized males and the sham males. Figure 5 shows that 91 percent of the females in the control group produced litters, compared to 19 percent of those exposed to 2 preputialectomized novel males and 33 percent of those exposed to 2 sham preputialectomized novel males. A Chi-square test of association between conditions and the presence or absence of parturition showed significance, χ^2 (2) = 26.07, $p < 0.001$. Significance was also found between the control and preputialectomized groups, χ^2 (1) = 23.36, $p < 0.001$, and between the control and sham groups, χ^2 (1) = 15.94, $p < 0.001$. However there was no significant difference between the sham and preputialectomized groups. Table 5 presents data for the number of pups born in each condition as well as sample size. Analysis of variance on the number of pups born was significant, F (2, 62) = 16.81, $p < .0001$; and a multiple comparisons Newman-Keuls test ($p < 0.05$) revealed a difference between the control condition and each of the other two conditions, but not between the preputialectomized and sham conditions. The differences in the number of pups per group was proportional to the number of females bearing litters since litter sizes among all groups were similar when nonparturient females were excluded.

Histology

Ten animals that had been preputialectomized were sacrificed, four at two months after the surgery and six others at five months after the surgery. In each case, the former site of the preputial glands could be identified. In all cases, full removal of the preputial glands was confirmed, and in no case was there any sign of regeneration of these glands.

Discussion

These data show that removing the preputial glands from stimulus novel males does not diminish their capacity to disrupt pregnancy. Females in the control condition delivered significantly more litters than those exposed to preputialectomized males or to sham-preputialectomized males. Pregnancy rates in these two experimental groups did not differ from each other.

Several independent observers in this laboratory reported a subjectively pungent and distinct odor coming from the cages of the stimulus males. The sides of the Plexiglas cages were smeared with an oily secretion that was being secreted by many of the stimulus males. It was postulated that this secretion may have been of preputial origin and may be involved in the transmission of the Bruce effect. However, this secretion did not differ in magnitude between the preputialectomized and sham-preputialectomized males' cages.

These glands do not appear to be the source of the pheromones that are involved in the Bruce effect. It can be speculated that the source of the pheromone may be other glands. Data suggest that removing the coagulating gland and the vesicular gland, in the same paradigm did not diminish the ability of the novel males to disrupt pregnancy

(Zacharias, deCatanzaro & Muir 1999). These glands are also male sex accessory glands and some evidence suggests that pheromonal emissions from these glands are involved in sexual attraction and aggression (Jones et al. 1972). Other male glands could be the source of such pheromones, such as the bulbourethral glands, kidneys, or even salivary glands.

In summary, it is known that androgens in the male are critical for the transmission of the Bruce effect. The fact that the preputial glands are androgen dependent made them a likely candidate for the source of a pheromone that may be involved in the disruption of pregnancy. Removal of the male's preputial glands does not affect their capacity to disrupt pregnancy. It is more likely that some direct role of androgens is involved either in the urine of the stimulus males or within the female. Subsequent chapters in this thesis focus on this idea.

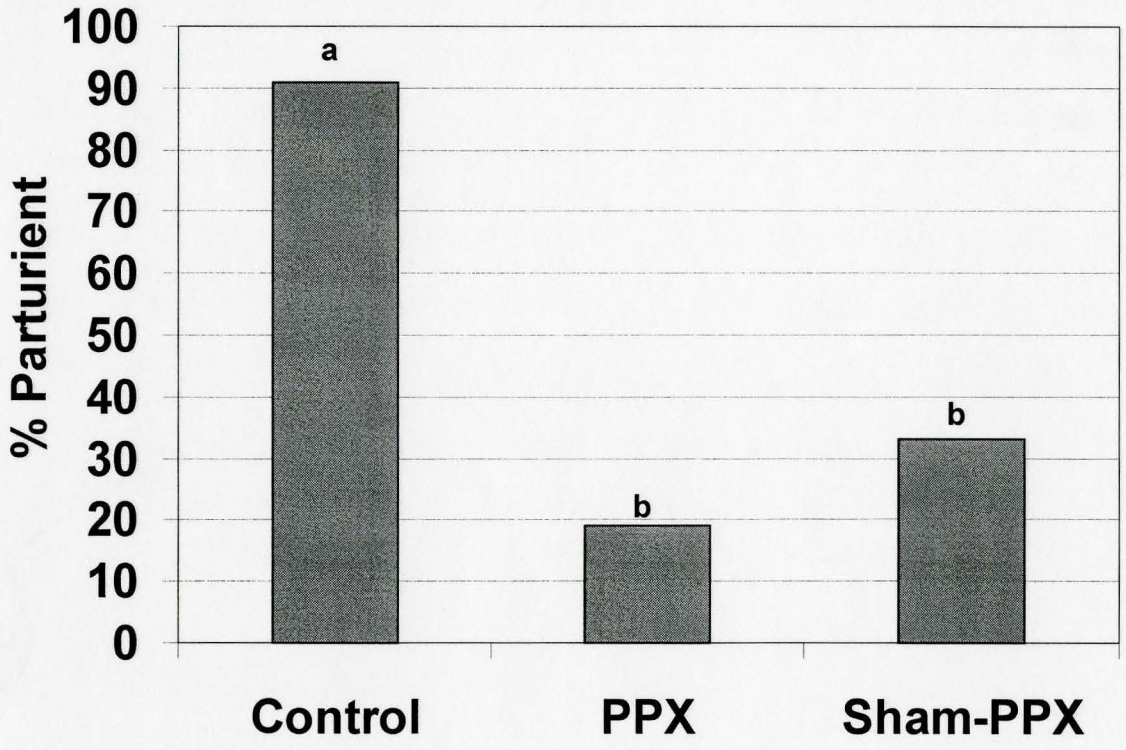
TABLES and FIGURES

Chapter 3

Table 5: Mean (±SEM) number of pups per group including zero for nonparturient females after indirect exposure during the first five days of pregnancy to 2 novel HS males either preputialectomized or sham-preputialectomized housed above.

Condition	n	Number Delivering	Mean Pups Per Group (±SEM)	Mean Pups Per Litter (±SEM)
Control	23	21	10.5 (±1.5)	11.6 (±0.5)
Preputialectomized	21	4	2.0 (±0.9)	10.5 (±0.9)
Sham-Preputialectomized	21	7	4.5 (±0.8)	13.5 (±0.8)

Figure 5: Percentage of CF-1 females delivering litters after housing during days 1-5 of pregnancy below 2 preputialectomized or sham-preputialectomized novel outbred HS males. Conditions with similar letters (i.e. a, b, c) are not significantly different from each other.



Chapter 4

The role of 17β-estradiol in the transmission of the Bruce effect

The focus of this chapter is the role of 17β-estradiol in the transmission of the Bruce effect from the male to the female and the transduction of the effect within the female. Increasingly, evidence indicates that elevated androgens and estrogens can disrupt early pregnancy. In mice (deCatanzaro et al. 1991), and rats (Harper 1967, 1969) during the implantation period, pregnancy can be disrupted by administering dehydroepiandrosterone (DHEA) and androstenedione. These steroids are produced in the adrenal glands under stressful conditions and are precursors for other androgens and estrogens. The final product in this metabolic pathway is 17β-estradiol, which is thought to be involved in the mediation of stress induced implantation failures. Early pregnancy can be disrupted by 17β-estradiol when administered daily during the implantation period at a fraction of the dose required by DHEA and androstenedione (deCatanzaro et al. 1991). Furthermore, radioimmunoassay of plasma estrogens in pregnant restraint stressed rats revealed elevated 17β-estradiol during their implantation period compared to unstressed controls (MacNiven, deCatanzaro & Younglai 1992). It has also been found that injections of monoclonal antibodies specific to 17β-estradiol given to restraint stressed pregnant female mice during implantation significantly increased their capacity to bear litters (deCatanzaro, MacNiven, Goodison & Richardson 1994). The idea that estrogens may play a role in the Bruce effect is investigated further in this chapter.

The deleterious effects of estrogens on implantation are well known. High plasma estradiol levels and the resulting elevation in the estradiol/progesterone ratio have been recognized to produce suboptimal endometrial receptivity (Suginami 1995).

Estrogens are known to control various mechanisms of progression and retention of ova through the fallopian tubes including muscular contraction, ciliary movement and flow of secretions. Manipulations in the time course of ovum transport in the oviduct may influence implantation by creating asynchrony between the embryo and the endometrium (Croxatto & Ortiz 1975). This may also create an inappropriate tubal environment for the early embryo (Land Evers, Boeckx & Brosens 1987). High plasma estradiol has been shown to decrease the rate of travel of fertilized ova in the fallopian tubes (Burdick & Whitney 1937, Pauerstein et al. 1976, Pauerstein & Weinberg 1980, Whitney & Burdick 1936) and can induce lysis of the corpus luteum (Greenwald 1964). It is suspected that elevated 17β-estradiol may be contributing to novel male induced implantation failure.

Sex steroids in the novel males are critical for the occurrence of the Bruce effect. Castration of the stimulus males eliminates their capacity to disrupt pregnancy when exposed to inseminated females but replacement testosterone restores this (deCatanzaro et al. 1995, Dominic 1965). Although male urine alone can induce pregnancy disruption (deCatanzaro, Muir, Sullivan & Boissy 1999), castrated male urine cannot (Bruce 1965, Dominic 1965). It has been found that males exposed to novel females have increased plasma testosterone (Macrides, Bartke & Dalterio 1975). The ability to disrupt pregnancy can be stimulated in females if given testosterone (deCatanzaro et al. 1995, Dominic 1965) or in castrated males if given depo-testosterone epiandrosterone, androstenedione. Each of these hormones metabolizes to testosterone (Hoppe 1975). It is a possibility that biologically active androgens or estrogens are being transmitted to the pregnant female through urine or feces and are being absorbed through the skin, lungs or

olfactory organs. This could unfavorably elevate estrogen levels in the inseminated females and disrupt pregnancy.

Testosterone is the precursor for 17β-estradiol and will readily metabolize to this steroid (Loraine 1971). Androgens that can potentiate the pregnancy-disrupting capacity in castrated males can also disrupt pregnancy when administered directly to inseminated females. This next experiment explores the possibility that exogenous 17β-estradiol might potentiate the disruption of early pregnancy in female mice, not through direct administration but through indirect administration of it to castrated novel males housed above them.

Experiment 6 – Exposing females to castrated novel males given 17β-estradiol

This experiment was designed to determine if administering 17β-estradiol to castrate stimulus males was sufficient to induce the disruption of pregnancy. Structurally similar testosterone given directly to castrated males can reinstate their capacity to disrupt pregnancy. Females were indirectly exposed to castrated males given 17β-estradiol or an oil vehicle injection and their pregnancy outcomes were compared to control females.

Methods

Female C57BL/6J strain mice were obtained from Charles River Breeding Farms, La Prairie, Quebec at 55-60 days of age or bred in this laboratory from the same stock. Prior to the experiment, females were housed in groups of three or four. All males were housed individually. All housing was in standard polypropylene cages measuring 28 ×

16 × 11 (height) cm with straight-wire tops allowing continuous access to food and water. The colony room was maintained under a reversed 14:10 light/dark cycle at 21°C.

Mature castrated HS males were prepared through bilateral removal of the testes via a single peritoneal incision under sodium pentobarbital anesthetic, with care not to remove sex accessory glands during the surgery. They were allowed to recover for at least 7 days and then each assigned randomly to one of four dosage groups: 0, 9, 27, and 81 μg of 17β-estradiol (Sigma) delivered subcutaneously in 0.05 cc peanut oil daily. The males had each received at least five such injections before being put into their experimental condition.

After a standard insemination procedure (see experiment 1), each female was housed in the lower compartment of a double-decker cage. Control females housed beneath an unfamiliar sexually experienced castrated HS male given an oil vehicle injection only. Experimental females were housed beneath novel castrated males given 9, 27 or 81 μg of 17β-estradiol. Females remained exposed in such manners to stimulus males or their effects for 5 days. Injections continued throughout the period in which the males were housed above the females.

Experimental females were continuously in that cage until 5-6 h after commencement of the dark phase of the light cycle on day 6 of pregnancy. At that point each female was directly transferred to a clean standard mouse cage and left undisturbed until pregnancy outcome measures began. Beginning at day 18 after the detection of the sperm plug, females were checked on two occasions each day for the birth of litters. Such inspections continued until 30 days after detection of the plug. Pregnancy outcome

was measured through the presence or absence of a litter and by counts of the number of pups born.

Results

Figure 6 summarizes the pregnancy outcomes from experiment 6. Females in the group exposed to castrated oil-vehicle treated males had the greatest percentage of litters followed by those exposed to males given 9 μg, 27 μg and 81 μg respectively. The mean number of total pups per group followed the same trend due to fewer litters in each successive group. Table 6 shows the mean number of pups per female. The number of pups per female bearing a litter was consistent across all groups. A Chi-squared test of association comparing groups to proportion pregnant showed significance, $\chi^2 = 15.26$, $p < 0.05$. Analysis of variance on the number of pups born to all females was also significant, $F(3, 76) = 5.34$, $p < 0.05$. Multiple comparisons (Newman-Keuls test, $p < .05$) showed that the females exposed to males in the 27 and 81 μg doses were significantly different from the oil-vehicle treated male group. The differences in the number of pups per group was proportional to the number of females bearing litters since litter sizes among all groups were similar when nonparturient females were excluded.

Discussion

This experiment was designed in order to determine whether 17β-estradiol administered to castrated males could restore their capability to disrupt pregnancy and to determine the dose that would most effectively accomplish this. The results suggest that a dose as low as 27 μg administered daily to males can reinstate their pregnancy

disrupting capacity. Females exposed to castrated males given no hormone had the highest number of litters. For the remaining groups, there was a significant trend suggesting that the more 17β-estradiol a male received the greater the disruption to pregnancy. This suggests that 17β-estradiol, like testosterone, can reinstate the ability for castrated novel males to disrupt pregnancy. The effective dosage was below the dosage of testosterone needed to reinstate the pregnancy-disruption capacity in castrated males. However, it was much higher than the 17β-estradiol dosage needed to disrupt pregnancy when directly administered to inseminated females (deCatanzaro et al. 1991).

It has been shown that exposure to novel female mice can increase plasma testosterone in male mice (Macrides et al. 1975). This same stimulation can also change the quality of male urine with respect to its pregnancy disrupting properties (deCatanzaro, Muir, Sullivan & Boissy 1999). Exogenous testosterone could modify the composition of male urine in a similar way and stimulate pheromone production. Similarly, 17β-estradiol might stimulate production of pheromonal products in castrated males in the same way that androgens may. In addition, 17β-estradiol may enhance the limited behavioral contact between the male and female. A final possibility is that biologically active 17β-estradiol or androgens may be directly transmitted in the male urine to the females' environment and is being absorbed by the female.

It is interesting that the steroid hormones that potentiate the transmission of the Bruce effect are the same as those that best disrupt pregnancy directly. It has been shown that 17β-estradiol is elevated in restraint stressed inseminated females (MacNiven et al. 1992) and when a 17β-estradiol antibody is administered to restraint stressed females this prevents pregnancy loss (deCatanzaro et al. 1994). It is possible that the adrenal glands

and the ovaries are responsible for the release of androgens and estrogens during restraint stress. Conceivably, adrenal androgens and/or estrogens are also mediating the loss of pregnancy in the Bruce effect.

Estrogens are known to have detrimental effects on implantation. Research has shown that the endometrium is less receptive when estradiol levels are high (Suginami 1995). High plasma estradiol has been shown to decrease the rate of travel of fertilized ova in the fallopian tubes (Burdick & Whitney 1937, Hodgson et al. 1980, Morris & VanWagenen 1973, Overstrom et al. 1980, Pauerstein et al. 1976, Pauerstein & Weinberg 1980, Whitney & Burdick 1936) and can also induce lysis of the corpus luteum (Greenwald 1964). It has also been suggested that the oviducts secrete factors that are toxic to embryo development after the period of normal tube transport, which can prevent ectopic pregnancy (Pauerstein & Weinberg 1980). Conversely, lowered estrogens during ovum transport can result in accelerated oviductal transport and expulsion of ova from the uterus (Bigsby et al. 1986). It is possible that these mechanisms are taking effect in inseminated females exposed to novel males

The role of potentially elevated 17β-estradiol has never been investigated in the Bruce effect. Research shows that early pregnancy can be disrupted by numerous stressors (deCatanzaro & MacNiven 1992). It has not yet been determined if the exposure to novel males disrupts pregnancy due to its "stressfulness" to females, triggering sympathetic and adrenocortical responses, or if the olfactory memory of pheromones from novel males trigger an unspecified cascade of neuroendocrine events that prevent implantation. Low dosages of exogenous androgens and estrogens administered directly to inseminated females can disrupt intrauterine implantation

(deCatanzaro et al. 1991, Harper 1967, 1969) and 17β-estradiol is higher in inseminated restraint stressed females (MacNiven et al. 1992). It can be hypothesized that this would also be the case in inseminated females that are exposed to novel males. This can be tested indirectly by administering 17β-estradiol antibodies to inseminated females exposed to novel males. If 17β-estradiol is higher in these mice and is causing the pregnancy loss then lowering 17β-estradiol with antibodies would allow implantation to take place.

Experiment 7 – Using antibodies to lower 17β-estradiol in females

This experiment was designed to test the hypothesis that inseminated females exposed to novel males have higher 17β-estradiol levels during their implantation period. DeCatanzaro et al. (1994) used an antibody specific to 17β-estradiol to lower a hypothesized increase in 17β-estradiol in inseminated restraint stressed mice. These mice were able to implant and remain pregnant. If the loss of early pregnancy in the Bruce effect is due to an increase in 17β-estradiol, then administering a monoclonal antibody specific to 17β-estradiol should lower the incidence of pregnancy loss. Females were indirectly exposed to novel males and given either 17β-estradiol antibodies or a vehicle injection and their pregnancy outcomes were compared to control females.

Methods

Mice and housing conditions were similar to those described in Experiment 1. Female subjects were inseminated by males of their own strain and randomly assigned on day 1 after the detection of a sperm plug. Experimental females were placed in the lower

compartment of a double-decker cage. An unfamiliar sexually experienced HS male was placed in the upper compartment. Females remained below them for the first five days of pregnancy. Control females were placed in a regular clean cage with fresh bedding.

On each of days 1 through 5 after insemination, females received an injection at 4h after the beginning of the dark phase of the light cycle. All injections were 0.05 cc 0.9 percent saline injected subcutaneously at the nape of the neck. Females received saline either alone or saline containing antibodies to 17β-estradiol. The antibodies were obtained in powder form from CIDtech Research Inc., Mississauga, Ontario, and had been prepared by injecting rabbits with 17β-estradiol as described elsewhere (Weilgosz et.al., 1980). They are marketed in vials containing 100ml of 1:90 dilution with a titer measured at 1:40000. The highest dosage involved addition of 1.67 cc saline to this vial, such that each injection contained 3 ml of the obtained powder. Remaining dosages were progressively diluted with 2 parts saline to one part of the previous dosage, producing nominal dosages of 3.0, 1.0, 0.33 ml on each of the five days. We initially measured smaller sample sizes in each dosage group, as well as controls not exposed to males and male exposed controls given saline vehicle only. As it emerged that the intermediate antibody dosage group had the greatest effect, we increased the sample size in this dosage group and the two control groups to increase confidence in the apparent effects.

On day 6 of pregnancy, about 4 h after the onset of the dark phase of the light cycle, each female was rehoused alone in a normal cage with clean bedding, where they remained undisturbed for the remainder of pregnancy. On day 18 after the detection of the sperm plug, females were checked on two occasions each day for the birth of litters.

Inspections continued until 30 days after detection of the plug. Pregnancy outcome was measured by the presence of litters and by the total number of pups born.

Results

Figure 7 shows the proportion of females bearing litters in this experiment. The females given only the saline vehicle and exposed to novel males had the fewest litters. The control females and the females given 1.0 μl antibody and exposed to novel males had a similar yet much higher occurrence of pregnancy than those given only saline. A Chi-square test of association, relating condition to the presence or absence of a litter, showed significance when all five conditions were compared, χ^2 (4) = 19.2, $p < 0.05$ or when just the three conditions replicated the most were compared, χ^2 (2) = 9.3, $p < 0.05$. Table 7 shows the number of pups born in each group. Analyses of variance for all females considering number of pups born was significant for all five conditions, F (4, 122) = 5.93, $p < 0.05$. When just the three groups were compared there was also significance F (2, 98) = 5.94, $p < 0.05$. A Newman-Keuls test for multiple comparisons ($p < 0.05$) showed that the control and 1.0 Ab group differed significantly from the lowest dose Ab group and the male exposed group given saline.

Discussion

The goal of this experiment was to determine if reducing a hypothesized increase in 17β-estradiol, with 17β-estradiol antibodies, would allow pregnant females to implant despite the exposure to novel males. The results suggest that this is at least partially effective. Females that were given the antibody had a similar occurrence of pregnancy to that of control females. These results are consistent with a previous report that 17β-

estradiol antibodies can prevent implantation failure in restraint stressed mice. The effective dosage of antibody in the two different paradigms is consistent. The manufacturer of the antibody has published data suggesting that the antibody does not cross react with other known steroids of similar structure.

These results provide confidence that unbound circulating estrogens may be at least partly responsible for pregnancy loss in this paradigm. The present data are concordant with other reports suggesting that endogenous estrogens may be mediating the implantation of fertilized ova during the exposure to stressful stimuli. However, this study does not directly address the hypothesis that estrogens are elevated. Furthermore, the origin of the elevated 17β-estradiol remains unclear. It is possible that the increase in estrogens is coming from the males' urine or feces, the adrenal glands, the ovaries or some combination of these.

In summary, these studies emphasize the importance of 17β-estradiol in the transmission and transduction of the Bruce effect. In castrated males exogenous 17β-estradiol can reinstate their capacity to disrupt pregnancy in the same way exogenous testosterone can. When a female is exposed to a novel male physiological events occur that lead to implantation failure. These studies indirectly show that elevated 17β-estradiol is important in the implantation physiology of the females and may be mediating the disruption of implantation.

FIGURES

Chapter 4

Table 6: Mean (±SEM) number of pups per group including zero for nonparturient C57BL/6J females after indirect exposure during the first five days of pregnancy to single novel HS castrated males housed above given various doses of 17β-estradiol.

Condition	n	Number Delivering	Mean Pups Per Group (±SEM)	Mean Pups Per Litter (±SEM)
Vehicle Control	23	18	5.3 (±0.7)	6.8 (±0.5)
9μg 17β-estradiol	20	12	4.3 (±0.9)	7.2 (±1.1)
27μg 17β-estradiol	21	6	1.9 (±0.7)	6.7 (±0.5)
81μg 17β-estradiol	15	4	1.8 (±0.8)	6.8 (±0.6)

Table 7: Mean (±SEM) number of pups per group including zero for nonparturient C57BL/6J females after indirect exposure during the first five days of pregnancy to single novel HS castrated males housed above. The females were given daily injections of antibody specific for 17β-estradiol in various doses.

Condition	n	Number Delivering	Mean Pups Per Group (±SEM)	Mean Pups Per Litter (±SEM)
Control + Vehicle	28	20	5.6 (±0.7)	7.9 (±0.4)
Male Exposed +Vehicle	37	14	2.7 (±0.6)	7.1 (±0.6)
Male Exposed +0.33 Ab	11	1	0.5 (±0.6)	6.0 (±0.0)
Male Exposed +1.0 Ab	36	24	5.31 (±0.7)	8.0 (±0.3)
Male Exposed +3.0 Ab	15	6	3.2 (±1.1)	8.0 (±0.4)

Figure 6: Percentage of C57BL/6J females delivering litters after housing during days 1-5 of pregnancy below single castrated outbred HS males given various daily doses of 17β-estradiol. Conditions with similar letters (i.e. a, b, c) are not significantly different from each other.

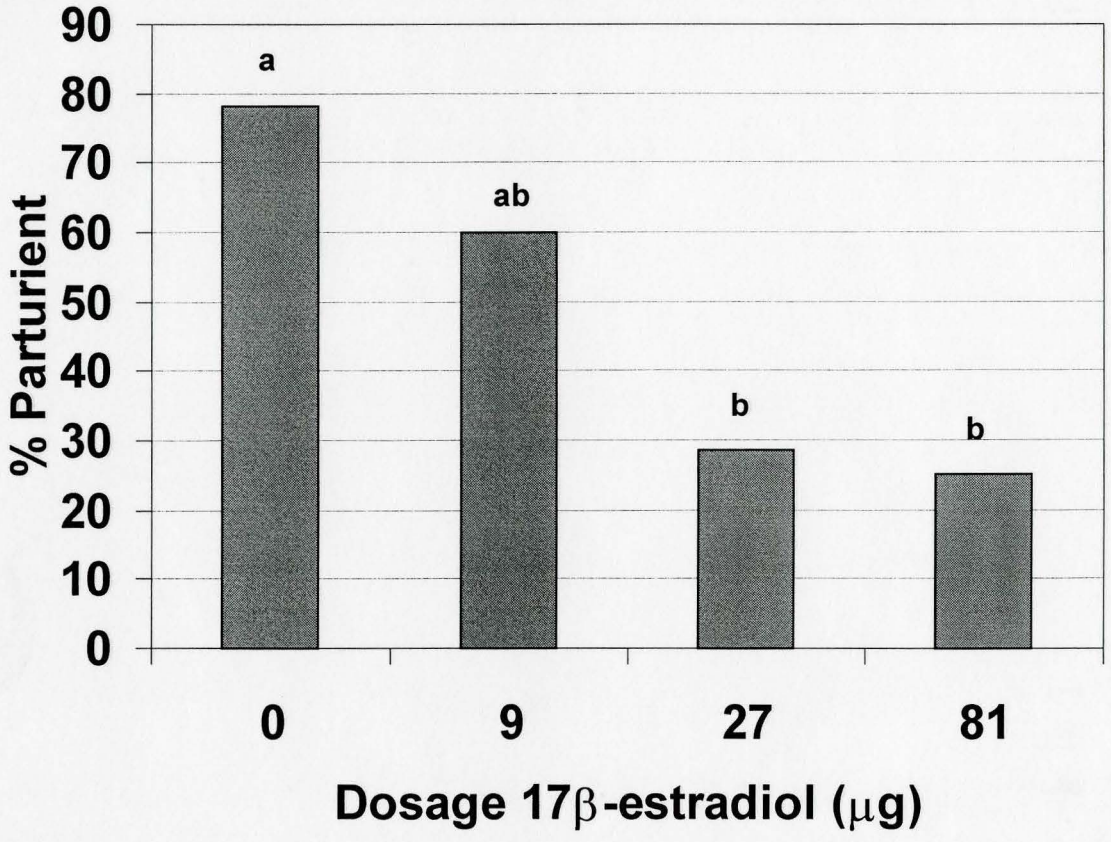
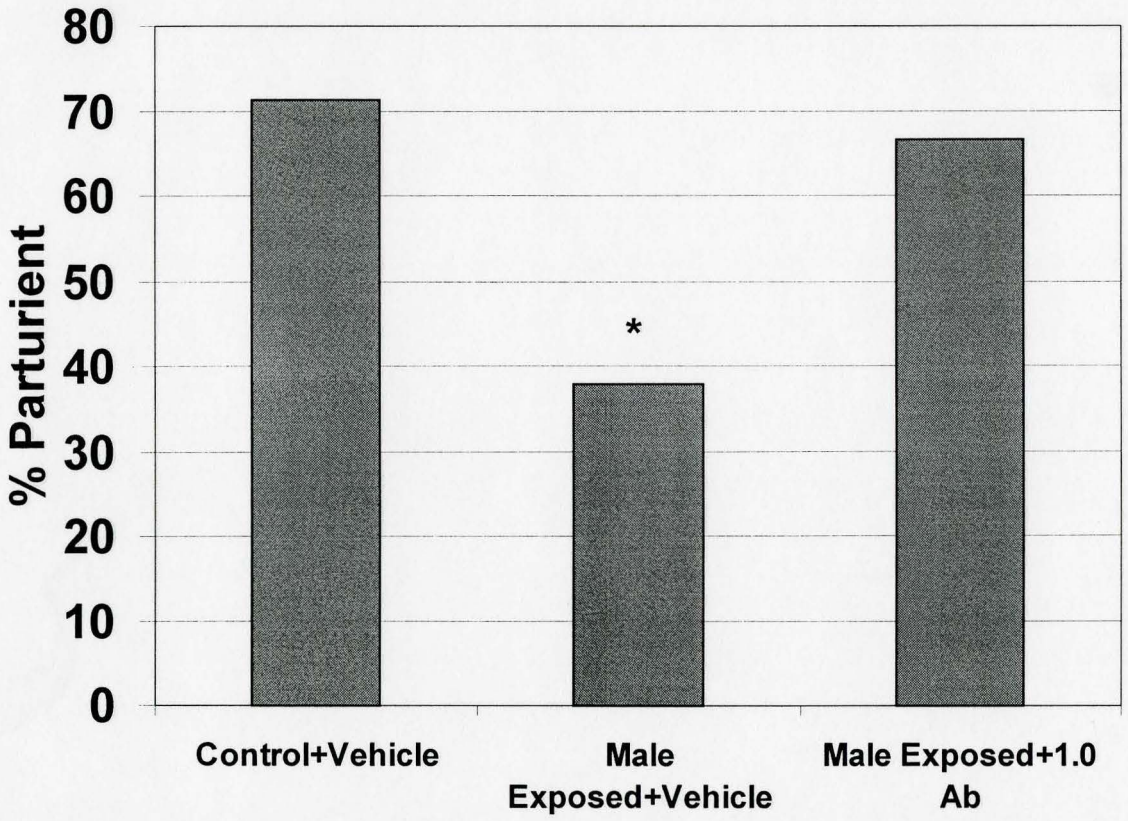


Figure 7: Percentage of C57BL/6J females delivering litters after housing during days 1-5 of pregnancy below single novel outbred HS males. The females were given daily injections of antibody specific for 17β-estradiol in various doses. An asterisk (*) indicates statistical significance.



Chapter 5

The measurement of testosterone, estrone conjugates and 17β-estradiol in the urine and feces of pregnant female mice exposed to novel males

Increasingly, evidence suggests that androgens and estrogens are important in both the stimulus novel males and the inseminated females in the Bruce effect. It has previously been demonstrated that these steroids disrupt implantation when administered to female mice in early pregnancy at physiologically low doses (deCatanzaro et al. 1991, Harper 1967, 1969,). It is also known that estradiol disrupts implantation by a number of mechanisms that involve the rate of travel of fertilized ova down the fallopian tubes (Burdick & Whitney 1937, Pauerstein et al. 1976, Pauerstein & Weinberg 1980, Whitney & Burdick 1936). It has now been shown that testosterone (deCatanzaro et al. 1995, Dominic 1965) and 17β-estradiol given to castrated novel males can reinstate their capacity to disrupt pregnancy. In addition, 17β-estradiol antibodies administered to recently inseminated females exposed to novel males can prevent implantation failure (Chapter 4). Although the use of antibodies is an indirect measure of elevated 17β-estradiol, measures that are more direct can now be taken of androgens and estrogens in inseminated female mice.

Assay technology has made possible the analysis of hormones in blood, urine and feces of various mammals. Measuring blood has the advantage of its rapid secretory dynamics but this can lead to inaccurate measures depending on the time of day of collection. In small fragile animals, such as the laboratory mouse, daily evaluations involving repeated measures are not possible due to the proportion of blood required for the assay. The amount of blood required to measure a single hormone for one day would be a substantial amount of the total blood volume of the animal. Furthermore, restraining

the animal to collect blood could stress the animal and potentially influence the secretion of the very hormones of interest. Recent advances in excretion assay technology allow for accurate assessment of endocrine status through fecal and urinary analysis (Munro & Lasley 1987). All steroid hormones secreted into the vascular space are cleared within minutes undergoing only modest structural changes (Lasley & Kirkpatrick 1991). The resulting metabolites are concentrated in either urine and/or feces and are relatively stable for prolonged periods. The excretia containing the metabolites can be collected and preserved indefinitely by freezing. Sufficient data now exist to suggest that excretion profiles of gonadal steroid metabolites reflect gonadal activity and provide an accurate assessment of overall endocrine status (Lasley & Kirkpatrick 1991).

Androgens and estrogens can be measured by an enzyme linked immunosorbent assay (ELISA) that I have recently developed (see appendix). It can be hypothesized that elevated androgens and/or estrogens relate to pregnancy loss in females in the Bruce effect. The hormonal dynamics of females exposed to novel males, over the first five days of pregnancy, can be analyzed. Testosterone 17β-estradiol and estrone conjugates can be quantified and correlated with pregnancy loss and the exposure to novel males.

This study was designed to measure testosterone, 17β-estradiol and estrone conjugates in females exposed to novel males. Because each of the experimental and control animals can remain pregnant or not, various hypotheses can be made about the steroid levels in each outcome group. It is hypothesized that these steroids will be elevated in females that are exposed to novel males. It is also hypothesized that these steroids will be elevated in females that do not bear litters regardless of their experimental condition. Because not all females exposed to novel males lose their

pregnancy, it can be hypothesized that steroid levels will be highest in females that are exposed to novel males *and* have a disrupted pregnancy. From this logic, females in the control condition that remain pregnant should have the lowest levels of these steroids.

Experiment 8

The loss of early pregnancy in the Bruce Effect is consistent with exposure to exogenous androgens and estrogens in both the stimulus males and the inseminated females. This study is designed to determine whether elevated androgens and estrogens in the inseminated females correlate with pregnancy loss, with exposure to novel males or with both. The urine and feces of inseminated females are collected for the first five days of pregnancy and measured for levels of testosterone, 17β-estradiol and estrone conjugates. The females are either exposed to novel males or housed alone.

Experimental Procedure

The subjects and insemination procedure were identical to that described for experiment 1. On the first day after the detection of the sperm plug, approximately 4 hours after the onset of the dark phase of the light cycle, inseminated females were put into one of two conditions. Control females were housed individually in a clean cage and left undisturbed for the duration of the experiment. Experimental females were put into the lower level of a double-decker cage with two novel males above. After the first five days of pregnancy, the females were individually housed in standard clean cages for the remaining gestation period.

Urine Collection

HS males were housed in pairs in the upper half of a clear double-decker Plexiglas cage (30×21×27 cm) with a wire grid floor and a double-layer opaque Plexiglas partition separating the males from each other. The pregnant females were housed beneath the males, on a wire grid floor. The entire apparatus was situated on fine wood-chip bedding in a stainless steel tray. When urine was collected, each section of cage was placed on a clean surface until 500μl of urine and 0.5-1g of feces from both males and females 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 collected for each animal for the first five days of pregnancy and stored without preservatives at -20°C until assayed for steroid content.

Pregnancy Outcome Measures

At the end of the five-day exposure period, the females were placed in a standard clean cage and left undisturbed for the remaining period of gestation. Litter checks began 18 days after the detection of the sperm plug. They were conducted three times a day until day 30, or until all of the pregnant females had delivered. Pregnancy outcome was measured by the presence of litters and by the total number of pups born.

Fecal extraction

Fecal samples were treated with 0.5 ml distilled water and 4.0 ml methanol and 1.0 g of aluminum oxide broken up and tumbled 360° in test tubes at 20rpm for 1 hour.

They were centrifuged at 2500 rpm for 15 min and the supernatant was poured off and stored at -20°C until assayed for steroid content.

Assay procedure

These assay procedures are similar to those described by Munro and Lasley (1988) and Munro et al. (1991). The creatinine, 17β-estradiol, testosterone and estrone conjugate standards were obtained from Sigma Chemical. All antibodies (anti-E2 R4972 anti-E1C R522 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.

Creatinine Assay

To account for variations in fluid intake and output concentration in the animals' urine creatinine is assayed and used as an index of urine production. Standard creatinine values of 100, 50, 25,12.5, 6.25 and 3.12 μg/ml are used and distilled water is used as zero. All urine samples are diluted 1:50. Using regular plates 50 μl per well of standard are added with 50 μl distilled water, 50 μl 0.75 N NaOH and 50 μl 0.4 N picric acid. The plate is then shaken and incubated at room temperature for 30 minutes. The plate is measured for optical density on a plate reader (Bio-tek instruments inc. EL 312E microplate reader) with a single filter at 490nm. Standard curves were generated, regression lines were fit and sample values were interpolated into the equation

Estrone Conjugate ELISA (Feces and Urine)

This assay was performed on both the urine and fecal samples. The assay was carried out on NUNC Maxisorb plates which were first coated with 50 µl of antibody stock diluted at 1:50,000 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 EIA phosphate buffer (0.1 mol/L sodium phosphate buffer, pH 7.0 containing 8.7g of NaCl and 1g of BSA per liter) per well was added.

Urine samples and fecal extracts were assayed undiluted and were measurable in the sensitive range of the standard curve. Two quality control urine samples were prepared by spiking mouse urine with estrone conjugates and diluting them to measure at 30% and 70% binding (the low and high ends of the sensitive range of the standard curve). To each well, 20µl of standard, sample or control was added along with 50µl estrone-glucuronide-horseradish peroxidase diluted at 1:50,000 in EIA buffer and the plates were incubated for 2 hours at room temperature. 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) (ABTS) 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 at 405nm 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 and all standards and unknown values were divided by this mean zero reading. This gives a percent binding value.

Estradiol ELISA for Feces

This assay was run using a similar protocol as estrone conjugates, except that the antibody stock was diluted at 1:10,000 before plate coating and the fecal extracts were diluted 1:4 in phosphate buffer before being added to the plate. The quality control fecal samples were prepared by spiking mouse extract with estradiol and diluting them to measure at the low (30% binding) and high (70% binding) ends of the sensitive range of the standard curve.

Testosterone ELISA for Urine and Feces

This assay was also run similarly to the estrone conjugate assay, except that the antibody stock was diluted at 1:10,000 before plate coating and only 30 minutes elapsed between the first plate wash the next day, and the addition of the standards and samples. The fecal extracts for these samples were diluted 1:16 while the urine was diluted 1:4 each in phosphate buffer. The quality control fecal samples were prepared by spiking mouse fecal extract with testosterone and diluting them to measure at the low (30% binding) and high (70% binding) ends of the sensitive range of the standard curve. Finally, 50µl of standard, sample or quality control samples were added to each well along with 50µl E2-horseradish peroxidase.

Results

In each of the two conditions, there were females that were either parturient or not. For the purposes of statistical analyses, the groups compared were control-pregnant,

male-exposed-pregnant and male-exposed-not-pregnant. There was a single female in the control condition that was not parturient and these data were excluded from the analyses due to sample size.

From the optical densities obtained, standard curves were generated; a regression line fit and samples interpolated into the equation to get a value in pg/well. From these values, values of ng/g for feces or pg/ng creatinine for urine were determined.

Testosterone

Figure 9 shows the means of each group over the first five days of pregnancy. Urinary testosterone levels were highest in male-exposed-non-pregnant females condition with a mean (± SEM) of 136.0 (± 14.7) pg/ng creatinine followed by male-exposed-pregnant females (mean 90.0 ± 7.5 pg/ng creatinine) and control pregnant (mean 58.9 ± 7.0 pg/ng creatinine). Analysis of variance among these three experimental conditions shows a significant difference in urine testosterone levels, $F(2, 16) = 5.83$ $p < 0.05$. Multiple comparisons (Duncan's Multiple Range test, $P < 0.05$) showed a difference between the male-exposed non-pregnant group and the control pregnant group. The greatest difference among groups appeared on day 2. A one-way ANOVA performed on the testosterone levels of the three groups on day 2 shows significance also, $F(2, 16) = 5.01$, $p = 0.02$. Multiple comparisons (Duncan's Multiple Range test, $p < 0.05$) revealed significant differences between the male-exposed non-pregnant group and the male-exposed pregnant groups as well as between the male-exposed non-pregnant and control pregnant groups.

Estradiol

Fecal estradiol levels were highest in females in the male-exposed non-pregnant group with a mean (±SEM) of 19.2±1.8 ng/g feces followed by the male-exposed pregnant (mean 15.5±1.0 ng/g feces) and control pregnant (mean 14.9±1.0 ng/g feces). Figure 8 shows the means of each group over the first five days of pregnancy. Analysis of variance among these three conditions fell short of the conventional significance level of .05, $F(2,16) = 2.71$ $p=0.09$. A post-hoc Duncan's Multiple Range test showed the biggest differences ($P<0.05$) between the control pregnant and male-exposed non-pregnant groups.

Estrone Conjugates

Estrone conjugates were measured in both urine and feces. There were no significant differences among the three groups. Control pregnant females had an average (±SEM) 6.35 ±0.61 pg EC/μg creatinine, the male-exposed pregnant had 6.01 ±0.48 pg EC/μg creatinine while male-exposed non-pregnant had 7.41 ±1.14

Discussion

The results from this experiment show that testosterone and 17β-estradiol were highest in females exposed to novel males that did not bear litters. Females exposed to novel males that had litters had intermediate levels while those in the control group that had litters had the lowest levels of steroids. Estrone conjugates did not differ significantly among the three groups. In the control condition, there was a single female that did not

have a litter. This subject was an extreme outlier with testosterone levels more than three times that for other animals. Based on the measure of testosterone and the infertility of this individual we support that it could have been a hermaphrodite. For statistical reasons, this subject was left out of the analysis.

In this experiment, there was not a significant Bruce effect. This could have been due to the urine and fecal collection procedure which involved removing the female from the males for 8 to 10 hours each day. This also could have been due to a small sample size. Since there was not a significant Bruce effect, no direct inferences can be made regarding the exposure to novel males being responsible for elevating these steroids. To interpret these data it is necessary to combine the outcome measure of pregnant or not pregnant with the *a priori* group designation of experimental or control. It is possible that exposure to novel males elevates testosterone and 17β-estradiol levels but pregnancy is only disrupted when a threshold concentration of these steroids is reached. According to this logic, this would suggest that females in the Bruce effect condition with no litters have higher concentrations than females in the Bruce effect with litters followed by control females with litters. The data clearly show this trend.

These data suggest that a certain threshold level of steroid be reached to disrupt implantation. It has previously been demonstrated that exogenous testosterone and 17β-estradiol, when administered during the implantation period, can prevent implantation (deCatanzaro et al. 1991, Harper 1967, 1969). In addition, it has been shown that antibodies specific for 17β-estradiol can prevent implantation failure in the Bruce effect. It is possible that these elevated steroids are hindering the travel of the fertilized ova down the fallopian tubes. This would create a mismatch of timing with the brief window

of receptivity of the endometrium and the arrival of the fertilized ova. If the ova were to arrive in the uterus once optimal conditions for ova survival have passed, then implantation would not take place and the mouse would return to estrus.

The nature of these steroids is also of interest. The elevations of 17β-estradiol and testosterone could be originating from a number of sources. Since 17β-estradiol and testosterone are structurally similar and can be excreted in urine and feces, it is possible that they are being transmitted in a biologically active form from the males above into the female's environment. If these hormones were entering the female's circulation by permeating the skin, nasal mucosa or by ingestion, then this would create an unfavorable uterine environment for implantation. It is also possible that these hormones are being produced by the adrenal glands in the female due to a simple stress response elicited by the novel males housed above. A third possibility is that there is a specific neuroendocrine response elicited through a pheromonally induced olfactory memory cascade that triggers irregular ovarian estrogen and progesterone secretions.

Unpublished findings from this laboratory show that 17β-estradiol and testosterone concentrations in the excretions of the stimulus males correlate with the loss of pregnancy in the female. This gives some support to the hypothesis that the elevated steroids in the female may be coming from the males. However, other unpublished data show that when testosterone in oil is painted on the noses of inseminated females, they do not lose their pregnancy. This could indicate that testosterone is either not being absorbed by the female or that testosterone does not behave similarly in oil as it does in male urine. Future research could clarify the origin of these steroids. This could include painting castrate testosterone/17β-estradiol treated novel male urine on the noses of

inseminated females. This would determine if the stimulus males were the source of elevated steroids in the females. Furthermore, in inseminated females exposed to novel males, the adrenal precursors of testosterone and 17β-estradiol could be inhibited with exogenous antibodies to determine if they are of adrenal origin.

FIGURES

Chapter 5

Figure 8: Fecal 17β-estradiol concentrations (±SEM) of CF-1 females for each of the first five days of pregnancy. Females were housed alone and parturient (Control), below two novel outbred HS males and parturient (Male exposed Pr), or below two novel outbred HS males and non-parturient (Male exposed N/Pr).

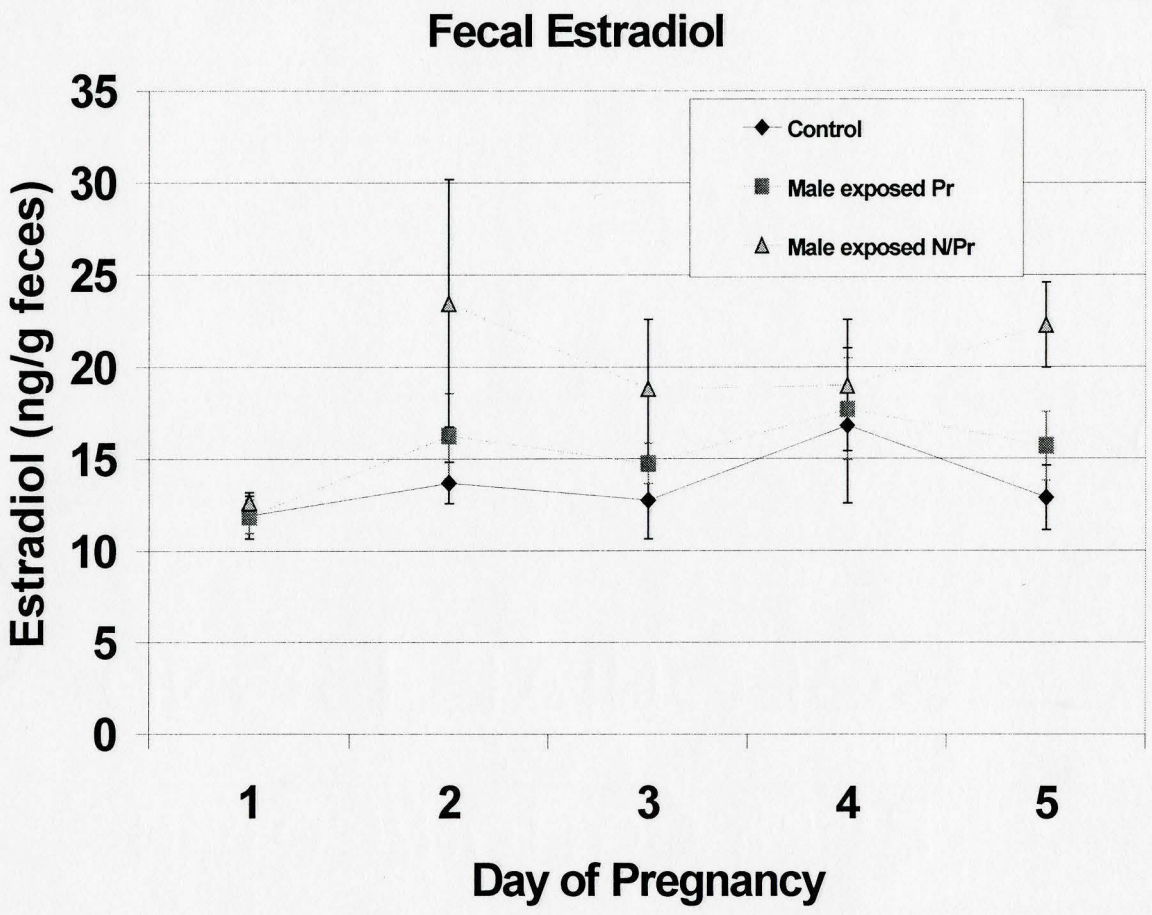
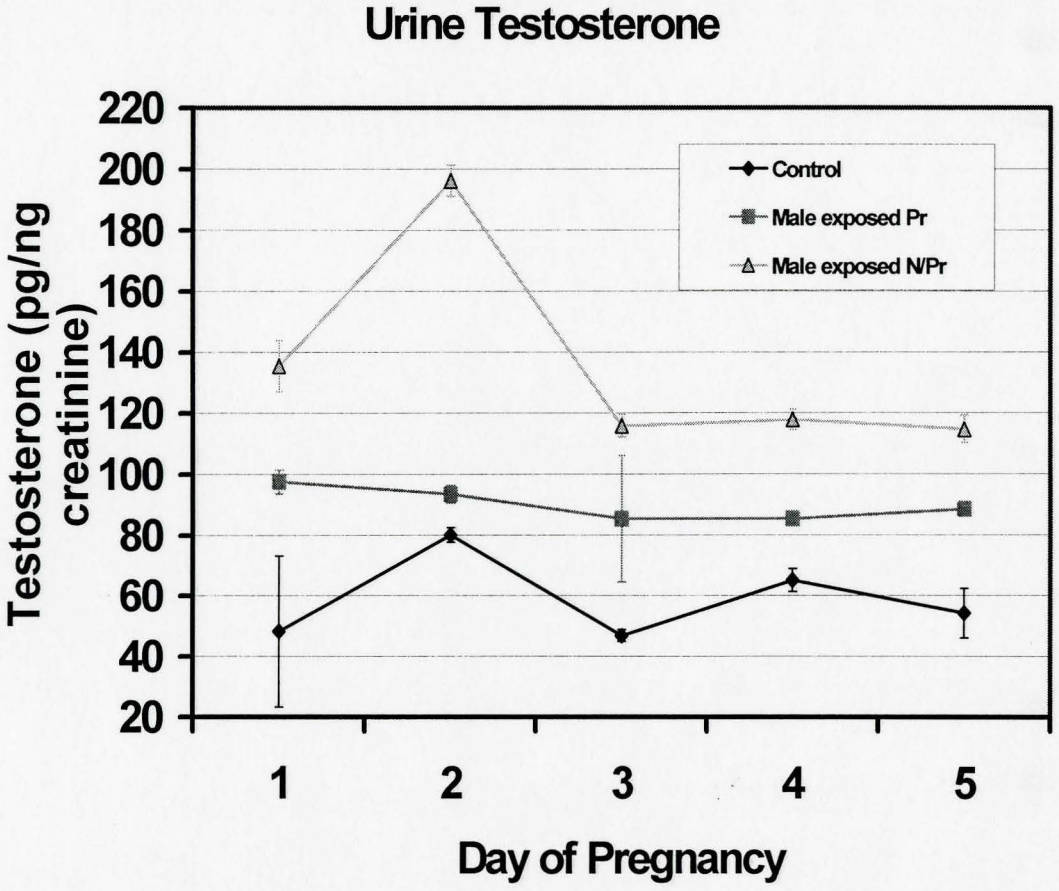


Figure 9: Urinary testosterone concentrations (±SEM) of CF-1 females for each of the first five days of pregnancy. Females were housed alone and parturient (Control), below two novel outbred HS males and parturient (Male exposed Pr), or below two novel outbred HS males and non-parturient (Male exposed N/Pr).



Chapter 6

General Discussion

General Summary of Thesis Findings.

The primary objective of this thesis was to gain a better understanding of the role of steroids in the chemical transmission, from male to female, and the physiological transduction within the female, in the Bruce effect. With the use of an enzyme immunoassay, I have been able to quantify for the first time, testosterone, 17β-estradiol and estrone conjugates in the urine and feces of mice. Urine and fecal levels of steroids reflect plasma levels of these steroids cumulatively for 8-12 hours in urine and 12-24 hours in feces (Peter et al. 1996). Using this assay, I have been able to take repeated measures over the female's implantation period, present a profile for these steroids, and contribute to the understanding of the role of steroids in the Bruce effect.

In study 1, in order to isolate the important components of the Bruce effect, our laboratory defined the parameters of a reliable and repeatable effect that minimized male and female behavioral interactions. This allowed for the isolation of the chemical transmission of the effect without confounding measures with behavioral interactions. It was found that females must contact the excretions of the males in order for pregnancy to be disrupted. This study also showed that excretions from three males disrupted pregnancy better than did that from two or one males. Urine alone was able to disrupt pregnancy provided it was collected from a male that was housed in the vicinity of a female that could not be accessed. Finally, when the females did not come in contact with the feces of the stimulus males the Bruce effect was diminished, but not significantly.

Study 2 investigated the role of the preputial glands in the stimulus males. It was hypothesized that these glands may have been a source for the pheromone responsible for the Bruce effect. These androgen dependent glands play a large role in producing chemicals released into the urine that are involved in chemical communication in mice. They release various chemosignals that promote aggression and may be involved with individual recognition. Surgically removing these glands did not diminish the males' capacity to disrupt pregnancy.

Study 3 investigated the role of 17β-estradiol in inseminated females and the stimulus males. Elevated 17β-estradiol is found in restraint stressed inseminated female rats and has detrimental effects on ova transport. It was found that decreasing 17β-estradiol in inseminated females exposed to novel males allowed the females to remain pregnant. Furthermore, data from this study provided evidence that administering 17β-estradiol to castrated males reinstated their ability to disrupt pregnancy. Structurally, 17β-estradiol is similar to testosterone, which is also known to potentiate the capacity to induce pregnancy disruption in castrated males.

Study 4 quantified the steroids testosterone, 17β-estradiol and its major metabolites the estrone conjugates in the urine and feces of inseminated females exposed to novel males or housed alone. Testosterone and 17β-estradiol can disrupt pregnancy when administered in physiologically low doses during the implantation period (Harper 1967, 1969 deCatanzaro 1991). Elevated 17β-estradiol has also been measured directly in both restraint-stressed female rats and indirectly in restraint stressed female mice in their implantation period. This study provided the first direct evidence that testosterone

and 17β-estradiol are elevated in females exposed to novel males that do not deliver litters.

Interpretation of the Role of Steroids in the Bruce Effect

These experiments have broken down the transmission of the Bruce effect into two individual components, behavioral and chemical. The behavioral component was kept to a minimum by the wire grid in the cage in order to investigate further the chemical transmission of the effect from the males to the females. The chemicals responsible for the transmission were found to be in the urine, and to a much lesser extent, the feces of the stimulus males. Effective chemical transmission is dependent on the proximity of the males to novel females when urination occurs. This is thought to be due to either testosterone contained in the urine, since testosterone levels are known to increase in males when they are exposed to unfamiliar females, or perhaps a testosterone dependent pheromone. Various other behavioral and endocrine changes can be induced by pheromones contained in male urine. The induction of estrus and puberty acceleration can be induced by novel males and have similar parameters to the Bruce effect (Vandenbergh 1967, Whitten et al. 1968). Contact with the urine must be made by the females for pregnancy to be disrupted.

In mice, various sex accessory glands are involved with the production of urinary pheromones that can induce social and endocrine changes in conspecifics. The preputial glands have been implicated in the production of pheromones responsible for strain recognition and mate choice. The size and chemistry of these androgen dependent glands can be altered when mice cohabitate with other males or females, but not gonadectomized conspecifics. These facts made the preputial glands likely candidates for the production

of pheromones involved with the transmission of the Bruce effect. However, surgical removal of these glands did not diminish the capacity for males to disrupt pregnancy. The vesicular/coagulating glands have similar social and endocrine properties but surgical removal of these glands from the stimulus males did not diminish the Bruce effect (Zacharias et al. 1999). Other male glands could be the source of such pheromones such as the bulbourethral glands, kidneys or even salivary glands. However, it is more likely that androgens play a direct role in the transmission of the Bruce effect in the urine of the stimulus males.

It is intriguing that testosterone plays such an important role in the male transmission of the Bruce effect while 17β-estradiol and testosterone can disrupt pregnancy at very low doses. These steroids are structurally very similar. This thesis demonstrates that these steroids are both excreted in unconjugated forms in the urine of males. Exogenous testosterone and 17β-estradiol can both reinstate castrated males' ability to disrupt pregnancy. This suggests that the production of a pheromone secreted by the male in the urine may be influenced by 17β-estradiol as well as testosterone. It also suggests that 17β-estradiol might be directly transmitted in the urine. Furthermore, inseminated females given antibodies specific to 17β-estradiol while exposed to novel males during implantation maintain their pregnancy. Although this is an indirect measure, it suggests that 17β-estradiol is elevated in these females.

An enzyme immunoassay of female feces and urine verified that testosterone and 17β-estradiol are elevated in females exposed to novel males during their implantation period. This elevation could be due to steroids passing directly from the male to the female in the male urine. If the female were to receive exogenous 17β-estradiol or

testosterone from the male urine this would be detrimental to the implantation process and pregnancy could terminate.

The idea of 17β-estradiol and testosterone in the male urine influencing the females' physiology does not exclude the possibility that an androgen-dependent pheromone in the males is influencing olfactory memory in the females. It also does not exclude the possibility that exposure to novel males is activating the adrenal glands in the females and a general stress response is elevating these steroids. There is no experimental evidence that indicates the origin of these steroids. This could be investigated in a number of ways. If the steroids are being transmitted in the urine of the males, the levels of these steroids could be measured and correlated with pregnancy loss in exposed females. Unpublished data from our laboratory suggests that this may be the case. Similarly, 17β-estradiol and testosterone could be put into castrated male urine and painted on the noses of inseminated females. This would test to see if these steroids are contributing to the pregnancy disruption directly or if they are altering some other chemicals in the urine of the males. Preliminary data from this laboratory suggest that when peanut oil containing 17ß-estradiol is painted on the noses of recently inseminated females pregnancy is disrupted.

Although much of the recent work on the physiological transduction in the Bruce effect has been focused on olfactory memory and pheromones, there has been no previous research on the possible role of steroids. These two lines of research do not seem to overlap or exclude one another. Olfactory stimulation causes a cascade of events in the vomeronasal organ through to the amygdala and ends with the suppression of prolactin by dopamine in the hypothalamus and pituitary (Brennan et al. 1990, Dominic

1966a, Kaba et al. 1994, Rajendren & Dominic1988). This is thought to inhibit the corpus luteum and terminate pregnancy. This theory does not account for the actions of steroids such as 17β-estradiol and testosterone. The steroid 17β-estradiol is known to be detrimental to implantation. Although these steroids have now been found elevated in these females, their origin is still in question. They could be arising from the adrenal glands or they could be absorbed into the female's circulation from the stimulus males' urine or feces. It is reasonable to assume that more than one of these mechanisms is possible. Most physiological systems that are of importance for survival and reproduction have multiple redundant mechanisms to ensure proper function of that system. It is also possible that the olfactory cascade pathway stimulates steroid production from the adrenal glands, the ovaries or both.

Appendix 1

The validation of an Enzyme-Linked-Immunosorbent Assay for the detection of testosterone, estrone conjugates, and 17β-estradiol in mouse urine and feces

During the last 20 years, with improvements in the production of antibodies, immunoassays have become the best methods for determining endocrine status in animals. Although not all species metabolize androgens and estrogens similarly, assaying for steroid hormones has broad applications to many species. This is possible due to the conservation of steroid hormone molecules across taxa and the stability of steroid hormone metabolites in measurable media such as excretions and blood (Lasley et al. 1991). However, the species-specific nature of steroid secretion patterns is a limiting factor for applying existing assays to all mammalian species (Lasley and Kirkpatrick, 1991). Numerous procedures for collecting and analyzing steroids and gonadotropins have been developed, although increasing evidence indicates that species differences necessitate customized approaches (Peter et al. 1996).

Existing serum assays can be easily transferred among species, but fecal and urinary assays must be carefully developed due to excretion differences of steroid hormones among species. A fundamental consideration in the process of developing a urinary or fecal assay for any species is that urinary and fecal hormone metabolites must reflect circulating biologically active compounds. There is often a delay in excretion of hormonal metabolites (e.g.: 24 hours in rhesus macaque) (Peter et al. 1996). In addition, one must be cautious when extrapolating information obtained from assays, even between related species, since excretion profiles are not always the same.

Interpretation of endocrine status is difficult without validation of a specific assay in a given species. Progestins are less conserved and are more distinct among species and therefore cannot be predicted easily in feces or urine (Graham et al. 1995). Some species may not produce similar metabolites in the urine and feces but may produce several similar immunoreactive metabolites that may reflect ovarian function. Fortunately, the presence of these metabolites may correlate with ovarian cyclicity (Kirkpatrick et al. 1990). The main objectives in most research have been to develop and validate methods of extracting and measuring fecal estradiol and progestins and to use fecal and urinary analysis as a noninvasive means for monitoring ovarian steroids. However, this type of steroid measurement has not previously been adapted for the common laboratory mouse.

Advantages and Disadvantages of Urinary and Fecal ELISAs

Many researchers have converted already existing radioimmunoassays (RIAs) for feces and urine, to enzyme-linked immunosorbent assays (ELISAs), which have several advantages. When converting RIA to ELISA the only requirement is the attachment of the detector enzyme, horseradish-peroxidase (HRP), to the inactive moiety of the detectable antigen. This allows the active site of the antigen to retain its immunoreactive properties. The relative small size of HRP and the location on the antigen will allow the antigen to still bind to the same antibody used in RIA (Czekala et al. 1986).

There are many advantages of ELISA over RIA that are mostly of environmental and safety concerns. RIAs require the use of radioactive material as a detector and use organic solvents for extracting steroids from feces. The primary goal of making the conversion from RIA to ELISA is to simplify the extraction procedures for feces and

develop a method that would limit the use of organic solvents. However, when using ELISA, solubilization with an aqueous buffer does not remove steroid conjugates like organic solvents do (Lee et al. 1995). The solubilization of feces with a modified phosphate buffer containing 20% methanol essentially expands the water compartment already present in the feces, allowing most of the particulate matter to be removed by centrifugation. Because methanol at such a low concentration does not adversely affect the binding of most antibodies, this approach permits the direct assay of the supernatant by ELISA (Shideler et al. 1993). In addition, the fecal ELISA eliminates the need for hydrolysis with the use of organic solvents and extraction of free steroids. ELISAs involve only simple solubilization and measures whole steroids and steroid conjugates (Lee et al. 1995). ELISA also avoids the special requirements involved in using radioisotopes that can be cumbersome to dispose of and require special licensing (Peter et al. 1996). Finally, ELISA makes possible the development of nonlaboratory tests or noninstrumented tests that can be used in the field.

There are also many benefits for testing urine and feces, as opposed to blood, by way of immunoassay. Fecal and urinary analysis eliminates human handling or restraint, and avoids the use of chemical immobilization in animals. This is beneficial because protocols can be imposed for long periods without stress to the animal (Lasley et al. 1991). Daily samples of urine and feces can be taken while for blood, this is not always possible; and blood can not always be taken from small fragile animals. As with plasma samples, urine and fecal samples can be frozen and stored for long periods without preservatives. However, the advantage of blood is its rapid secretory dynamics and the ability to measure momentary changes in hormones (Peter et al. 1996). Non-specific

antisera can be used for total excreted hormone or a more specific antibody can be used for known major conjugates. In addition, steroid concentrations are often 2-4 times higher in urine and fecal samples than in blood (Munro and Lasley, 1987, Peter et al. 1996). Typically, a close relationship exists between plasma estradiol and urinary estrone conjugates.

Measuring hormone levels in urine and feces also has some disadvantages. Since the entire amount of steroid may be in a combination of both urine and feces, one must first learn the excretory route of the steroid of interest. The investigation of the route of excretion can be done by recovering and measuring radiolabeled exogenously administered steroid (Schille et al. 1984). One must also know all free forms and all metabolites of the steroid of interest as well as different cross-reactivities for the antibodies to be used. Problems can also arise in assessing urine since urine production is not constant over time. Total hormone production can only be assessed by total urine collection. However, an alternative is to index urinary creatinine concentrations in small samples. Because the total daily production and excretion of creatinine is relatively constant, the ratio of creatinine to hormone in urine should remain constant unless hormone production changes (Peter et al. 1996). The lack of this type of indexing compound in feces can make fecal measurements less reliable.

Technical Design and Troubleshooting

One potential problem is the binding of the solid phase (antibody) to the plate since it must not only remain bound to the plate but, once bound, must also retain its

immunological properties. Since the mechanism by which proteins such as immunoglobulins adhere to plastic has much to do with hydrophobicity and charge, maintaining an optimized pH in the coating buffer and ensuring proper quality of the distilled water are important. Other problems may also arise involving variations in the time taken to coat the plate or the volumes of the reagents used. Also of importance is the temperature in which the reactions take place, precise incubation time, purity of sample, maintenance of reactivity of the labeled detector, and the plates and their batch-to-batch variations in binding (Kemeny, 1991).

To detect the presence or absence of unlabeled antigen there needs to be competition with a detector antigen with a physically measurable quality; in this case, a colour or optical density. HRP is an enzyme label that labels the detector molecule, in this case, a steroid molecule. For competitive assays, the detector has specificity for the plate-bound antibody and is used to detect unused antibody binding sites (Tijssen, 1985). When using labeled antigens, it is sometimes difficult to assess the effect of conjugation on the antigen (Munro and Lasley, 1987). To accommodate this, small molecules such as haptens, such as biotin, are used as labels and have less effect on the antigen's ability to bind antibody.

The competition between the sample and the detector provides a measurable optical density which, by itself, has no quantitative properties and must be compared to a reference. The reference is simply a curve that provides a range of optical densities that are reflective of the range of equivalence between the known standard, and the known-labeled antigen, competing for antibody sites (Kemeny, 1992). Results are plotted on a log concentration vs. a linear optical density graph and are read by taking the mean

optical density of the sample and looking across to the reference curve (Kemeny, 1991). The lower limit of the assay is often 1.5 times the background value. This can be defined either as a well containing all reagents except the sample, or from a well containing a negative sample. However, the working range of the assay will be inadequate if the background non-specific binding is high. The end-point can be defined as the smallest quantity of sample that can be reliably detected by the assay. If the end-point is too close to the background non-specific binding then measurement is unreliable since very small differences in concentration give equally small changes in color (Kemeny, 1992).

The fact that the test sample must be run at the same volume as the reference sample assumes that the reference and sample give parallel dilution curves. There are many reasons why a reference curve may not show a linear relationship between color and concentration. The assay itself may have deficiencies, e.g. too little antigen or antibody on the plate or there may be too little detector. In addition, the sample itself may not be heterogeneous (see parallelism). Since the relationship between the color generated and the concentration of the sample is not usually linear, a reference is needed (Kemeny, 1991).

In order to determine if the steroids extracted behave immunologically in a similar manner to steroid standards, a test for parallelism can be performed. This test will indicate primarily if the antibodies or antigens of interest are present in measurable quantities, and if they react with appropriate antibodies in a predictable fashion. A parallelism is simply a serial dilution that will generate percentage-binding data to compare to a standard curve when plotted against their logarithmically transformed doses

on the x-axis (Kemeny, 1992). If a sample runs parallel to a standard curve, then it can be assumed that there is enough sample antigen or antibody to be measured.

To demonstrate the efficacy of the assay for a particular antigen, it must be demonstrated that the assay does not detect other known compounds that might interfere through cross-reactivity (Mares et al. 1995). Since there are certain advantages in sensitivity when the cross-reacting compounds are all metabolites of one parent circulating bioactive steroid molecule, cross-reactivity is not necessarily a weakness. By evaluating a range of samples, the number of cross-reacting substances and their relationship to a physiological event can be determined.

If an assay is to be considered valid, sensitivity and precision also need to be determined. Sensitivity can be defined as the least amount of hormone that can be distinguished from zero concentration of standard. This can be calculated from the 95% confidence limits at the zero point of the standard curve (Munro et al. 1991). The precision of the assays can be assessed by determination of an intraplate coefficient of variation (CV). This includes a CV for antibody binding variation within a plate (intraplate) and a CV for between plate variation (interplate) (Munro et al. 1991). The intraplate coefficient of variation measures the variation within a single plate and can be determined by running 40 identical aliquots of the same sample within the same plate (Munro et al. 1991). To determine the interplate CV, control samples are run during each assay that have been calibrated to measure a predetermined percent binding. These samples are often chosen to bind consistently within the range of the standard curve at the high and low end (30% and 70%).

The formula for this is:

$$CV = \frac{\text{Standard deviation}}{\text{Mean}} \times 100$$

where the standard deviation and mean are for the optical densities of the control samples for the interplate CV, and are for each of the 40 aliquots for the intraplate CV. If the results of these samples fall outside the acceptable limits of the assay (10-20%) then there has been a problem with the binding properties of the antibodies or standards.

Other tests of accuracy can be performed in the final phases of extraction and assay development that help to keep the consistency of measure. In order to determine the efficacy of an extraction of hormone from fecal samples, a recovery study can be completed. Recovery curves can be generated by adding steroid standards to a sample in increasing amounts and extracting them for measurement. The amount recovered should include what was added plus the amount in the original sample. The measure of this should be a percentage of what was added.

The following experiments were run in order to fulfil the published standard requirements of validating fecal and urinary enzyme immunoassays. Pooled samples of non-pregnant female urine and feces were used for each of the validations.

Methods

The subjects were sexually inexperienced female CF1 strain mice weaned at 30 days of age, bred in this laboratory from stock originally obtained from Charles River Breeding Farms, La Prairie, Quebec. Prior to insemination the females were housed in groups of four or five in standard polypropylene cages measuring 28×16×11 (height) cm with wire bar tops allowing constant access to food and water.

Fecal extraction

Fecal samples were treated with 0.5 ml distilled water and 4.0 ml methanol and 1.0 g of aluminum oxide, then broken up and tumbled 360° in test tubes at 20rpm for 1 hour. They were centrifuged at 2500 rpm for 15 min and the supernatant was poured off and stored at -20°C until assayed for steroid content.

Assay procedure

These assay procedures are similar to those described by Munro and Lasley (1988) and Munro et al. (1991). The creatinine, 17β-estradiol, testosterone, and estrone conjugate standards were obtained from Sigma Chemical. All antibodies (anti-E2 R4972 anti-E1C R522 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.

Creatinine Assay

Since there are variations in fluid intake and output in the animals, and the concentration of urine is not always constant, an assay is performed on creatinine. Creatinine is a metabolite consistently found in urine and can act as an index of urine concentration.

Standard values of 100, 50, 25,12.5, 6.25, and 3.12 μg/ml are used and distilled water is used as zero. All urine samples are diluted 1:50. Using Dynatech Immulon plates, 50 μl per well of standard are added with 50 μl distilled water, 50 μl 0.75 N

NaOH and 50 µl 0.4 N picric acid. The plate is then shaken and incubated at room temperature for 30 minutes. The plate is measured for optical density on a plate reader (Bio-tek instruments inc. EL 312E microplate reader) with a single filter at 490nm. Standard curves were generated, regression lines were fit and sample values were interpolated into the equation.

Estrone Conjugate ELISA (Feces and Urine)

Estrone conjugates (E1C) collectively reflect plasma estrogens and include estrone sulfate and estrone glucuronide. This assay was performed on both the urine and fecal samples. The assay was carried out on NUNC Maxisorb plates which were first coated with 50 µl of antibody stock diluted at 1:50,000 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 EIA phosphate buffer (0.1 mol/L sodium phosphate buffer, pH 7.0 containing 8.7g of NaCl and 1g of BSA per liter) per well was added.

Urine samples and fecal extracts were assayed undiluted and were measurable in the sensitive range of the standard curve. Two quality control urine samples were prepared by spiking mouse urine with estrone conjugates and diluting them to measure at 30% and 70% binding (the low and high ends of the sensitive range of the standard curve). 20µl of standard, sample or control were added to each well along with 50µl estrone-glucuronide-horseradish peroxidase diluted at 1:50,000 in EIA buffer and 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) (ABTS) 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 at 405nm 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 and all standards and unknown values were divided by this mean zero reading. This gives a percent binding value.

Estradiol ELISA (Feces and Urine)

This assay was run using a similar protocol to that for estrone conjugates except the antibody stock was diluted at 1:10,000 before plate coating and the fecal extracts were diluted 1:4 in phosphate buffer before being added to the plate. The quality control fecal samples were prepared by spiking mouse fecal extract with a known amount of 17β-estradiol and diluting it to measure at the low and high ends of the sensitive range of the standard curve (30% and 70% binding).

Testosterone ELISA (Feces and Urine)

This assay was also run similarly to the estrone conjugate assay, except the antibody stock was diluted at 1:10,000 before plate coating and only 30 minutes elapsed between the first plate wash the next day and the addition of the standards and samples. The fecal extracts for these samples were diluted 1:16 while the urine was diluted 1:4 each in phosphate buffer. The quality control fecal samples were prepared by spiking mouse fecal extract with testosterone and diluting them to measure at the low and high ends of the sensitive range of the standard curve. Finally, 50μl of standard, sample, or

quality control (as described above) were added to each well along with 50µl E2-horseradish peroxidase.

Assay Validation

A test for parallelism was performed for estradiol, testosterone and estrone conjugates in order to determine if they behave immunologically in a similar manner to the steroid standards. This is simply a serial dilution of samples that are compared to a standard curve when plotted against their logarithmically transformed doses. This test indicates primarily if the steroids of interest are present in measurable quantities, and if they react with appropriate antibodies in a predictable fashion (Kemeny, 1991) (Figures 10-12).

In order to demonstrate the efficacy of the assay for a particular antigen, it must be demonstrated that the assay does not detect other known compounds that might interfere through cross-reactivity. The precision of the assay was determined by an intraplate and an interplate coefficient of variation (CV). The intraplate CV was determined by running 40 aliquots of the same sample (at 50% binding) on the same plate coated with its corresponding antibody. The interplate CV was determined by running quality control samples on every plate. These quality controls were run in each assay to account for inter assay and plate coating variations. This was done by running specially calibrated samples in each assay, which were measurable in the upper and lower parts of the standard curve. It is common practice to run samples in each assay that have been calibrated to bind at 30% and 70% consistently. The sensitivity of the assay was determined by measuring the least amount of hormone standard consistently

distinguishable from the zero concentration of standard using a t-test ($p<0.05$). An extraction efficiency measure can account for procedural losses of hormone. Recovery curves were generated by adding steroid standards to pooled samples in increasing amounts and extracting them for measurement. The amount recovered includes what was added plus the amount in the original sample. The measure of this is a percentage of what was added

.

Assay Characterization

Figures 10-12 show dose response curves and parallelisms for each of the hormones validated. All samples measured could be serially diluted in parallel to their corresponding standard dilutions.

Estrone Conjugates

The E1C standards have a range of 100-0.39 pg/well with a sensitivity of 19.5pg/ml urine or 175.5pg/g feces. The interplate coefficient of variation for the anti-E1C R522 antibody for the high and low urine samples was 5.2 and 2.3 respectively while the intraplate CV for the medium concentration sample was 8.3%. The extraction efficiency (±SEM) for the estrone conjugate assay was 31.5% (±8.7). The cross reactivities for the E1C R522 antibody are: estrone-3-glucuronide, 100.0%, estrone-3-sulfate 66.6%, estrone 238.0%, estradiol-17β 7.8%, estradiol-3-glucuronide 3.8%, estradiol-3-sulfate, 3.3% and all other structurally similar steroids <0.1%.

Testosterone

The testosterone standards have a range of 312.5-2.4 pg/well with a sensitivity of 48 pg/ml urine and 432 pg/g feces. The interplate coefficient of variation for the anti-testosterone R156/7 antibody for the high and low urine samples was 8.8 and 4.8 respectively while the intraplate CV for the medium concentration sample was 7.1%. The extraction efficiency for the testosterone assay was 99.0 ±5.8%. Cross reactivities for antibody R156/7 are as follows: Testosterone 100%, 5α-Dihydrotestosterone 57.4%, androstenedione 0.27%, and androsterone, DHEA, Cholesterol, β-estradiol, progesterone, and pregnenolone<0.05%.

17β-Estradiol

The estradiol assay had a range of 250-0.49 pg/well with a sensitivity of 219.6 pg/g feces. The interplate coefficient of variation for the anti-E2 R4972 antibody for the high and low urine samples was 6.1 and 9.0 respectively while the intraplate CV for the medium concentration sample was 8.7%. The extraction efficiency for the E2 assay was 88.9 ±8.1%. The cross reactivities for anti-E2 R4972 were: estradiol-17β 100%, estrone 3.3%, progesterone 0.8%, testosterone 1.0%, and androstenedione 1.0%, while all other steroids were less than 0.1%.

Figure 10: Female fecal and urine samples serially diluted to run parallel when measured against 17β-estradiol standards. This indicates that 17β-estradiol is present in the sample in measurable quantities and reacts with the anti-E2 R4972 antibody with the same binding properties as standardized 17β-estradiol.

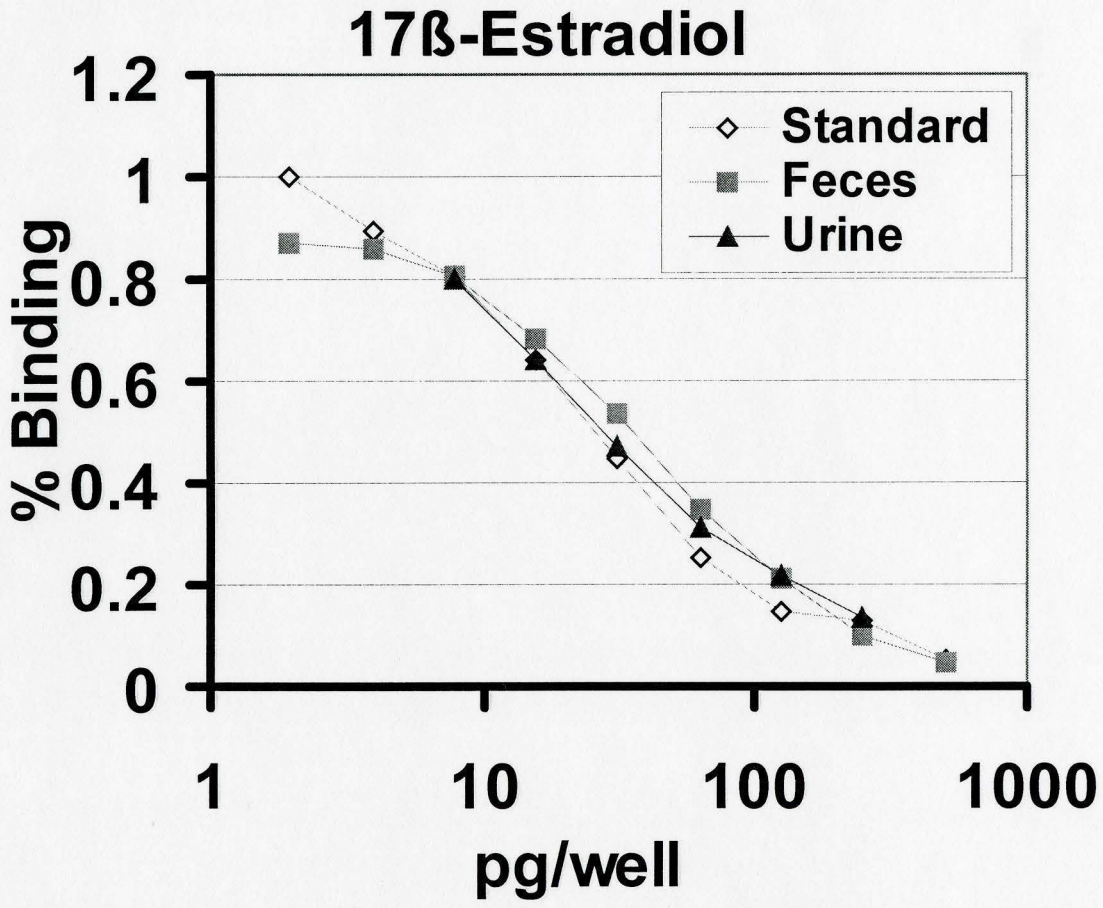


Figure 11: Female fecal and urine samples serially diluted to run parallel when measured against estrone conjugate standards. This indicates that estrone conjugates are present in the sample in measurable quantities and react with the anti-E1C R522 antibody with the same binding properties as standardized estrone conjugates.

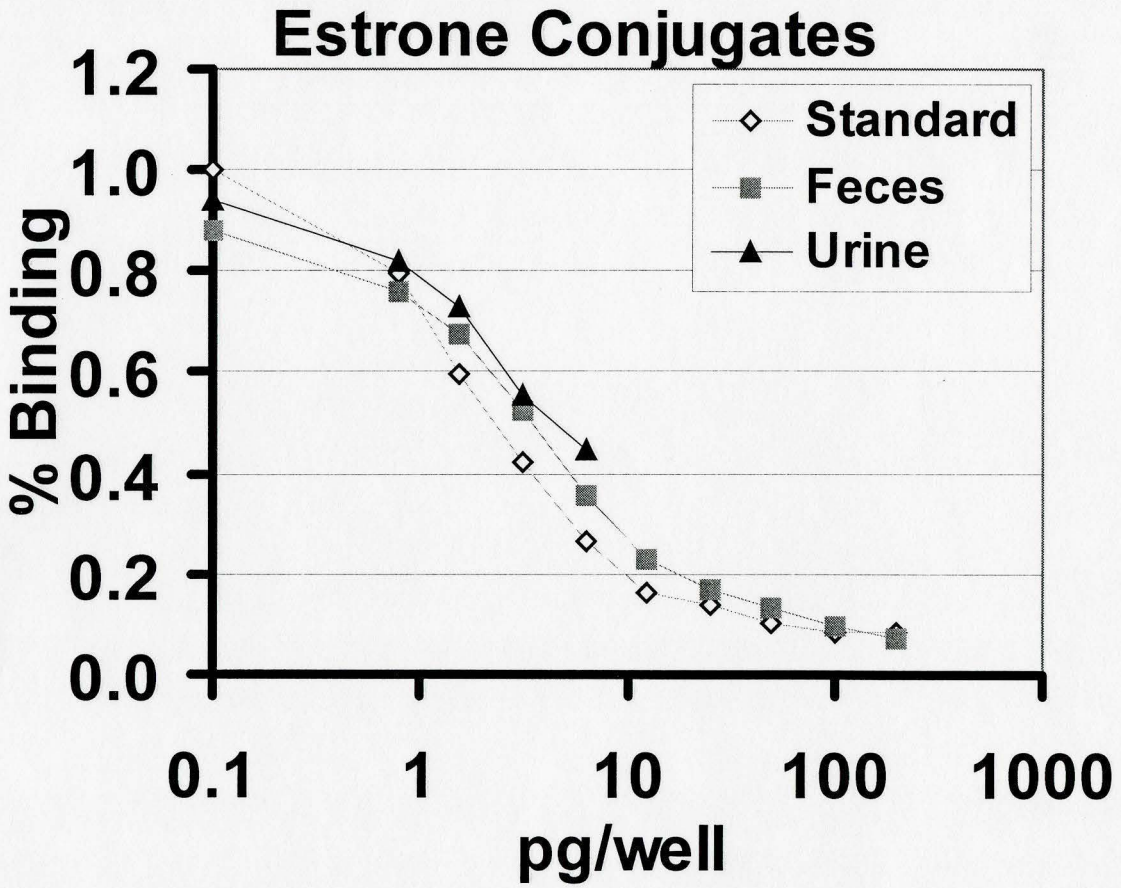
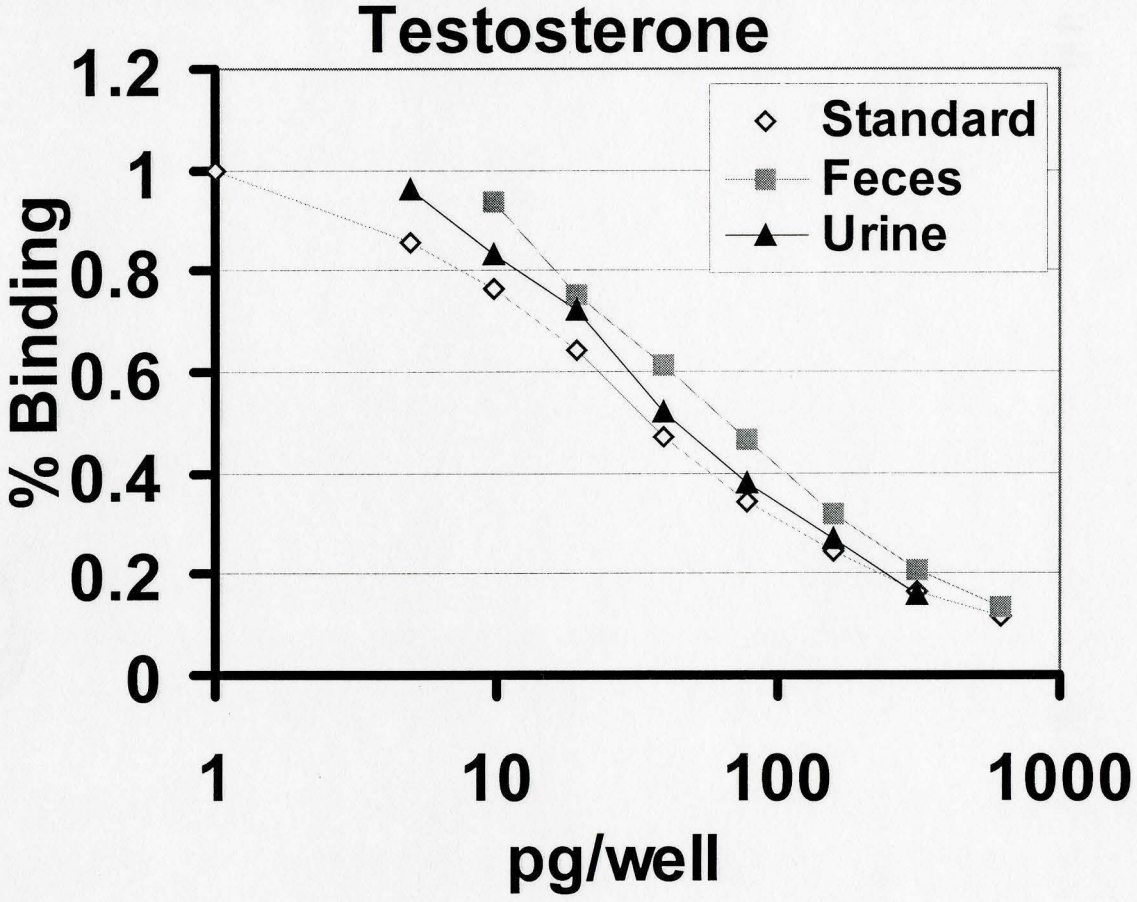


Figure 12: Female fecal and urine samples serially diluted to run parallel when measured against testosterone standards. This indicates that testosterone is present in the sample in measurable quantities and reacts with the R156/7 antibody with the same binding properties as standardized testosterone.



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