# EFFECT OF GLAND REMOVAL ON PREGNANCY DISRUPTIONS IN MICE

# THE EFFECT OF SEX-ACCESSORY GLAND REMOVAL ON STRANGE-MALE-INDUCED PREGNANCY DISRUPTIONS IN MICE

By

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## ABSTRACT

Early pregnancy in mammals can be disrupted by numerous stimuli. In particular, exposure to males which did not sire the litter disrupts early pregnancy in previously inseminated female mice. This is known as the Bruce effect. Evidence suggests that this effect is mediated by chemical emissions (pheromones) from the males. Castration of the males eliminates the effect whereas testosterone replacement restores it. This has suggested that androgen-dependent male accessory glands might be responsible. In particular, the preputial, vesicular and coagulating glands seem likely candidates for subserving the Bruce effect since they have been implicated in a variety of social behaviors.

In these experiments, inseminated females were each housed below either 1) two males which had undergone preputial gland removal or, 2) two males which had undergone vesicular-coagulating gland removal or, 3) two males which had undergone preputial, vesicular and coagulating gland removal or 4) two males which had undergone sham surgery. In each case, males which had undergone gland removal disrupted pregnancy in inseminated females to the same extent as did intact males. Histology showed no regeneration of the glands. These results suggest that none of these major androgen-dependent male accessory glands is responsible for pheromonal emissions involved in the Bruce effect.

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# INTRODUCTION

## Early pregnancy disruption

Mammalian reproduction is a complex phenomenon which is sensitive to a variety of psychophysiological events. It is well known that certain environmental stimuli are able to disrupt the reproductive ability of female mammals (deCatanzaro & MacNiven, 1992). These stimuli disrupt the timing and occurrence of specific neuroendocrine events which are essential for reproductive success. Females are probably more sensitive to the effects of stress than are their male counterparts (Liptrap, 1993). In this context, a stressor, in its broadest meaning, is any influence on an organism's homeostatic state which requires adjustment or adaptation (Hinkle, 1977). This term is completely non-specific and encompasses both the stimulus and its effects. In pregnant female rodents these stressors could include human handling (Runner, 1959), exposure to predators (deCatanzaro, 1988), chronic physical restraint (Euker & Riegle, 1973) and extreme temperature (Wettemann & Bazer, 1985; Castro-Vazquez, Esquivel, Martin & Rosner, 1975). As well, nutritional stress (Archunan & Dominic, 1989), swimming stress (Guo, Nappi, Criscuolo, Ficarra & Amram, 1993) and a variety of environmental and social changes (Bronson, Eleftheriou, & Garick, 1964) have been known to disrupt early pregnancy. These external stressors appear to be disrupting pregnancy at the phase of

intrauterine implantation of fertilized ova. The psychogenic pregnancy disruptions which occur during this period are believed to be induced by chronic stress exposure (deCatanzaro & MacNiven, 1992).

The hormonal reaction which occurs in mammals under stress is stereotypical. During acute stress, a series of events is activated in the sympathetic division of the autonomic nervous system. This is often referred to as the fight-or-flight system because it mobilizes the body to handle extreme situations such as fear, exercise or rage. Afferent impulses travel to the hypothalamus of the central nervous system. These messages then synapse in the paravertebral ganglia with the greater splanchnic nerve. The fibers of the splanchic nerve pass through the celiac ganglion without synapsing and terminate by synapsing with the hormone producing medullary cells of the adrenal gland. When stimulated by the preganglionic fibers by the neurotransmitter acetylcholine, the medullary cells secrete norepinephrine and epinephrine (also called noradrenaline and adrenaline, respectively) into the blood. For this reason, the adrenal medulla is often referred to as an overgrown sympathetic ganglion since it is directly innervated and its hormone-releasing cells are stimulated to release catecholamines via incoming nervous messages. Following this, there is an immediate increase in arousal and muscular activity.

During chronic stress, the hypothalamic-pituitary-adrenal axis (HPA) is activated. The hypothalamic neurons release a factor which stimulates the

anterior pituitary gland to release adrenocorticotropic hormone (ACTH). This peptide hormone then travels through the bloodstream to act on the adrenal cortex. The adrenal cortex is the outer portion of the adrenal gland which is situated on top of the kidney. It is made up of glandular tissue and is not innervated. ACTH stimulates the cortex to release glucocorticoids, and in some circumstances also androgens and estrogens. Steroids released by the adrenal cortex target the reproductive organs as well as the limbic system in the brain. This steroid release facilitates the availability of energy over more prolonged periods. This neuroendocrine response is clearly adaptive as internal resources may be mobilized for action which, during rest, remain unavailable (Selye, 1973).

The physiological mediators of early pregnancy disruption have not been determined. Are the neuroendocrine events underlying early pregnancy disruption similar to those events involved in the stereotypical stress response or is there a unique mechanism by which the internal reproductive environment becomes disrupted? Many stress-related paradigms have been used to study early pregnancy disruptions. Although these stimuli vary in form and time course, most have been shown to elicit physiological responses characteristic of stress. More specifically, the role of the stress response has been studied, including adrenal corticosteroids and catecholamines. As well, the involvement of estrogens and progesterone has been investigated. Several experiments

have investigated an increase in activity in the HPA. With respect to pituitary gland output, earlier studies report that exogenous ACTH, administered to rats in the implantation period, reduces uterine capacity for pregnancy (Velardo, 1957). However, corticosterone administration during the implantation period failed to induce spontaneous abortion in C57 and HS-strain female mice (deCatanzaro, MacNiven & Ricciuti, 1991). It is known that the adrenal medulla releases epinephrine under diverse forms of stressful stimulation (Kvetnansky, Sun, Lake, Thoa, Torda & Koppin, 1978). Therefore, it is plausible that this hormone may play some role in such stress-induced reproductive failures. However, deCatanzaro and Graham (1992) found that although administration of exogenous epinephrine dampened sexual receptivity, such administration did not exert strong influences over the outcome of early pregnancy.

Administration of glucocorticoids also can inhibit uterine growth and preparation for implantation (Szego, 1952; Velardo, Hisaw & Bever, 1956). However, it has not been established whether major glucocorticoids like cortisol and corticosterone directly inhibit pregnancy, or whether any adrenal activity directly mediates the block of pregnancy induced by psychological variations. In order to pursue this question further, adrenalectomy was performed upon those female subjects who underwent the strange-male-exposure procedure. Although some found that adrenalectomy diminished pregnancy disruption (Snyder &

Taggart, 1967), others reported a failure of this surgery to affect early pregnancy disruption (Sahu & Dominic, 1981).

Certain lesser known steroids produced in the adrenals are known to have detrimental effects on pregnancy. Harper (1967, 1969) reported that androstenedione and dehydroepiandrosterone (DHEA) administration disrupts early pregnancy in rats. Studies in this laboratory (deCatanzaro *et al.*, 1991) confirm these findings. These steroids are produced in the adrenals under stressful conditions and are precursors to estrogens. Harper suggested that these steroids may be metabolized to estrogens and that estrogens mediate early pregnancy disruptions. Studies conducted in this laboratory have revealed the powerful effect of estrogen administration on the disruption of early pregnancy. More specifically, 17β-estradiol can reliably disrupt early pregnancy at a dose that is one thousandth that of DHEA and one five-thousandth that of androstenedione required to disrupt pregnancy with the same efficacy (deCatanzaro *et al.*, 1991). Radioimmunoassay in restraint stressed rats, which were in the implantation period of pregnancy, revealed a significant increase in 17β-estradiol compared to unstressed controls (MacNiven, deCatanzaro & Younglai, 1992). A subsequent experiment revealed that injections of monoclonal antibodies specific to 17β-estradiol, given daily to restraint stressed mice in the implantation period, significantly increased the capacity for them to bear litters, compared to restrained controls who were not given the antibody

(deCatanzaro, MacNiven, Goodison & Richardson, 1994). These experiments support the role of estrogens in the mediation of stress induced early pregnancy disruptions.

The role of progesterone in early pregnancy disruptions has been investigated and its dynamics may also be significant in this context. Some researchers have found that progesterone levels rise during stress due to an increase in ACTH (Piva, Gagliano, Motta & Martini, 1977), while others have shown that levels of this hormone decrease during stress (Wiebold, Stanfield, Becker & Hillers, 1986). More recently, MacNiven and deCatanzaro (1990) injected restraint stressed mice, in their first five days of pregnancy, with progesterone, finding a small reduction in the number of pregnancies disrupted. Also, MacNiven *et al.*, (1992) performed radioimmunoassays on restraint stressed pregnant rats during their implantation period and found significantly elevated levels of progesterone compared to controls. Because of the incongruity of these recent reports there is still much research needed in determining the role of progesterone in the disruption of early pregnancy.

Such hormonal dynamics have been studied in several paradigms which elicit early pregnancy disruption. However, the focus of this thesis is upon early pregnancy disruption which occurs as a result of strange male exposure. It is well documented that when a newly inseminated female mouse is removed from the stud male and exposed to another male, pregnancy fails and the female

returns to estrus as if mating had not occurred (Bruce, 1960a). A strange male is any male which did not inseminate the female and, therefore, is not the sire of the litter. Accordingly, this phenomenon is considered to be an adaptive response for the female since it is known that males will destroy offspring which they did not sire. Strange male induced pregnancy disruption is known as the Bruce effect (Bruce, 1959, 1960a, 1960b, 1963). It is not known if this type of pregnancy disruption is specifically mediated by the effects of the organism's stress response. It may be that certain pheromones and/or behavioral interactions between the newly inseminated female and the strange male are involved. One possible neuroendocrine mechanism of the male-induced disruption may be the depression of hypophysial prolactin secretion resulting in the failure of the functional development of the corpora lutea (Dominic, 1966b). However, an examination of the distinct components of the strange male stimulus which may be eliciting a unique neuroendocrine response in the female is necessary in order to understand the mechanism of the Bruce effect. The goal of this thesis is to investigate the physiological origin of the potential pheromone which may be mediating the effect.

The fact that other pheromonal effects can be observed in mammals strengthens the hypothetical position that the Bruce effect may be pheromonally mediated. The first described pheromonal effect in mammals was the stimulation of ovulation in adult female mice by the presence of male mice

(Whitten, 1956). Male chemosignals also accelerate puberty of female mice (Vandenbergh, 1969). These chemosignals are present in male urine and their production is under androgen control (Dominic, 1965; Bronson & Whitten, 1968; Vandenbergh, 1969). Furthermore, when juvenile females are treated with the excreted urine of intact females, puberty is delayed (Drickamer, 1983). It has been shown that adrenalectomy (Drickamer & McIntosh, 1980) but not ovariectomy (Drickamer, McIntosh & Rose, 1978) abolishes the biological activity of excreted urine to delay puberty in juvenile mice. Novotny (1995) has effectively identified the male-to-female puberty accelerating pheromone to be 6-hydroxy-6-methyl-3-heptanone. The structural features of this compound are consistent with its strong binding affinity to the major urinary protein. Although there is evidence indicating a variety of pheromonal effects in laboratory rodents, very little is known about the anatomical site of pheromone synthesis.

Pheromonal mediation of strange male induced pregnancy disruption is supported by several findings. Furthermore, the pheromone involved in the Bruce effect appears to be androgen dependent. Experiments show that if the stimulus animal is a female or a castrated male, pregnancy will not be disrupted in the inseminated female. Interestingly, testosterone replacement restores the pregnancy disrupting ability of the castrated males, and androgenized females also elicit a Bruce effect (deCatanzaro, Wyngaarden, Griffiths, Ham, Hancox & Brain, 1995). Dominic (1965, 1966a, 1966b) suggests that this type of

pregnancy disruption is caused by an androgen-dependent gland in the male mouse, and reports that strange male urine alone can disrupt pregnancy. As well, Marchlewska-Koj (1977, 1981) reported that urinary proteins salted out of male urine could disrupt pregnancy. In contrast, some evidence suggests that behavioral interactions between the strange male and the inseminated female play a role in mediating such early pregnancy disruption. Sexual activity was correlated with pregnancy outcome, and intromissions in particular are correlated with pregnancy disruption (deCatanzaro & Storey, 1989; Storey & Snow, 1990). Furthermore, some studies, such as that by deCatanzaro *et al.*, (1995), failed to show that urine alone was able to disrupt pregnancy. DeCatanzaro and MacNiven (1992) raised several concerns about the indirect assessment of pregnancy in these urine-alone experiments. In these studies pregnancy was assessed by the cornification of cells taken from vaginal smears approximately 4-7 days following insemination. Also, there was an extensive amount of human handling and female genital stimulation of the subjects. Furthermore, preliminary results from experiments conducted in this laboratory suggest that inseminated female mice experience urine-alone-induced pregnancy disruption only when this urine is collected from female-stimulated males. In summary, several researchers speculate that the Bruce effect is pheromonally mediated (Parkes & Bruce, 1962; Dominic, 1965; Marchlewska-Koj, 1977,1981). If so, removal of the pheromone source from the strange male

mouse should interfere with its capacity to disrupt early pregnancy in inseminated females.

The Bruce effect is thought to act through olfactory stimulation (Bellringer, Pratt & Keverne, 1980; Rajendren & Dominic, 1984). It also acts through contact and is thought to be non-volatile (Rajendren & Dominic, 1985). Bruce and Parrott (1960) reported that pregnancy disruption did not occur in those females who had undergone surgery to have their olfactory bulbs removed. Rajendren and Dominic (1984, 1985) reported that the accessory olfactory system, namely the vomeronasal organ is involved in the pheromonal stimulus which mediates the Bruce effect. Implantation failure does not occur by the reintroduction of the stud male to the female as the female is thought to have imprinted an olfactory memory formation (Bruce 1960b). Furthermore, Parkes and Bruce (1961) reported that pregnancy disruption is significantly reduced in those females which are exposed to a novel male in the presence of the stud male. It appears that a stud male affords some protection to the newly inseminated female against the incidence of the Bruce effect. By contrast, newly inseminated females housed with a familiar male during exposure to alien males exhibited a high rate of pregnancy disruption (Thomas & Dominic, 1989). Familiar males were defined as those males which were housed with the female at least seven days before presentation of stud male. These results suggest that the protective effect of the stud male on implantation is not because of the familiarity of the

female with his odor cues. These results are consistent with the view that the newly inseminated female mouse identifies her coital partner as an individual because she becomes 'imprinted' with his odor during the precopulatory period (Thomas & Dominic, 1989). Interestingly, this protective response seems to act through the main olfactory system and not the vomeronasal (accessory olfactory system). Thomas and Dominic (1988) showed that surgical ablation of the vomeronasal organ did not prevent the stud-male-exposure-induced pseudopregnancy in pregnancy-blocked females, whereas, pregnancy-blocked females made anosmic by intranasal irrigation with $ZnSO_4$ solution failed to exhibit pseudopregnancy following re-exposure to the stud males. Also, olfactory preference studies have shown that pregnant female mice are attracted to the soiled bedding of the stud male over clean bedding of a novel male (Drickamer, 1989). The pheromones which initiate this effect selectively activate the vomeronasal receptors rather than the main olfactory system and this memory is established about four hours after mating (Lloyd-Thomas & Keverne, 1982). It is thought that this memory is dependent on intact noradrenergic projections from the locus coeruleus to the accessory olfactory bulb which are active during the imprint phase and are alpha-adrenoceptor mediated (Selway & Keverne, 1990).

Earlier studies by Dominic concentrated physiologically on the descending pathway; that is from the level of the vomeronasal organ through to

the pituitary and the subsequent effects on uterine receptivity. He suggested that this pheromone induces implantation failure by depressing hypophysial prolactin secretion leading to the failure of the development of a functional corpora lutea (Dominic, 1966b), since prolactin is known to have luteotrophic effects. It is known that high estrogen blood levels stimulate prolactin inhibiting hormone (which is thought to be dopamine) and this acts at the anterior pituitary to prevent the release of prolactin. While low estrogen blood levels are known to stimulate prolactin releasing hormone and thus prolactin (Marieb, 1989). A study by Rajendren and Dutta (1988) showed that the Bruce effect could be inhibited if newly inseminated female mice were injected with haloperidol. Haloperidol induces hypophysial prolactin release by inhibiting the dopaminergic neurons of the hypothalamus. In this way, the inhibition of the Bruce effect appears to be due to stimulation of prolactin release which prevents luteal failure in strange-male exposed females.

Studies conducted in this laboratory have involved an indirect-exposure paradigm that produces reliable pregnancy disruption. This procedure involves a double-decker cage system which eliminates most of the behavioral interaction occurring between the inseminated female and the stimulus males. Males housed above the inseminated female and separated from her by a wire mesh grid disrupted pregnancy more reliably than did stimulus males housed below (deCatanzaro, Zacharias & Muir, 1996). It appeared that something was filtering

down from the male and being either ingested by the female or absorbed into her skin. Subjective observation in this laboratory indicated a greasy substance with an aversive, pungent odor deposited on the upper as well as the lower cage walls. This excretion was speculated to be involved in the mediation of the Bruce effect. Several of these experiments concentrated physiologically on the mechanisms of the adrenals and the ovaries on uterine receptivity and ova viability. Earlier work demonstrated that the Bruce effect is dependent on androgens in the stimulus males. Castration of the male eliminated the capacity to produce the Bruce effect but the effect was reinstated when the castrated males were given exogenous testosterone or 17β-estradiol (deCatanzaro, Smith & Muir, 1995). These results in particular point to the possibility of the involvement of androgen-dependent glands in the pheromonal mediation of the Bruce effect. The focus of this thesis was the removal of several of these androgen-dependent glands in order to observe the possible altered mediation of strange-male-induced pregnancy disruption.

In order to explore pheromonal mediation as a component of the stimulus complex mechanism of induced pregnancy blocks, an examination of several male mouse sex-accessory glands appears reasonable. Many of these glands are known to be involved in pheromone production mediating aggression, reproductive behavior, maternal care and intersexual attraction. More specifically, the preputial, coagulating and vesicular glands (Figure 2) have been

implicated in a variety of social behaviors. Therefore, they seem likely candidates for subserving the Bruce effect. The hypothesis presented in this thesis is that these glands, either individually or synergistically release pheromones that might mediate strange-male-induced pregnancy disruptions. If so, removal of any or all of these glands should interfere with the Bruce effect.

**Preputial glands**

The preputial glands are prominent sex-accessory scent marking glands in the male mouse (Figure 1) which are androgen-dependent (Mugford & Nowell, 1971). They are specialized sebaceous glands situated between the skin and body wall anterior to the external genitalia (Mugford & Nowell, 1971). They are prominent in male mice, but are normally vestigial in females where they are sometimes referred to as clitoral glands (Mugford & Nowell, 1971; Raso, Timar & Lapis, 1992). Although a considerable amount of literature has been devoted to their histology and endocrine relationships, the function of these glands is not certain. Some indication was provided by McKinney and Christian (1970), who found that removal of preputials from one of a pair of opponent mice had a significant effect upon the aggressive behavior of both animals. Bronson and Caroom (1971) and Jones and Nowell (1973) have suggested that the male mouse preputial glands release a pheromone which promotes inter-male aggression. Mugford and Nowell (1970, 1971) reported that this aggression-

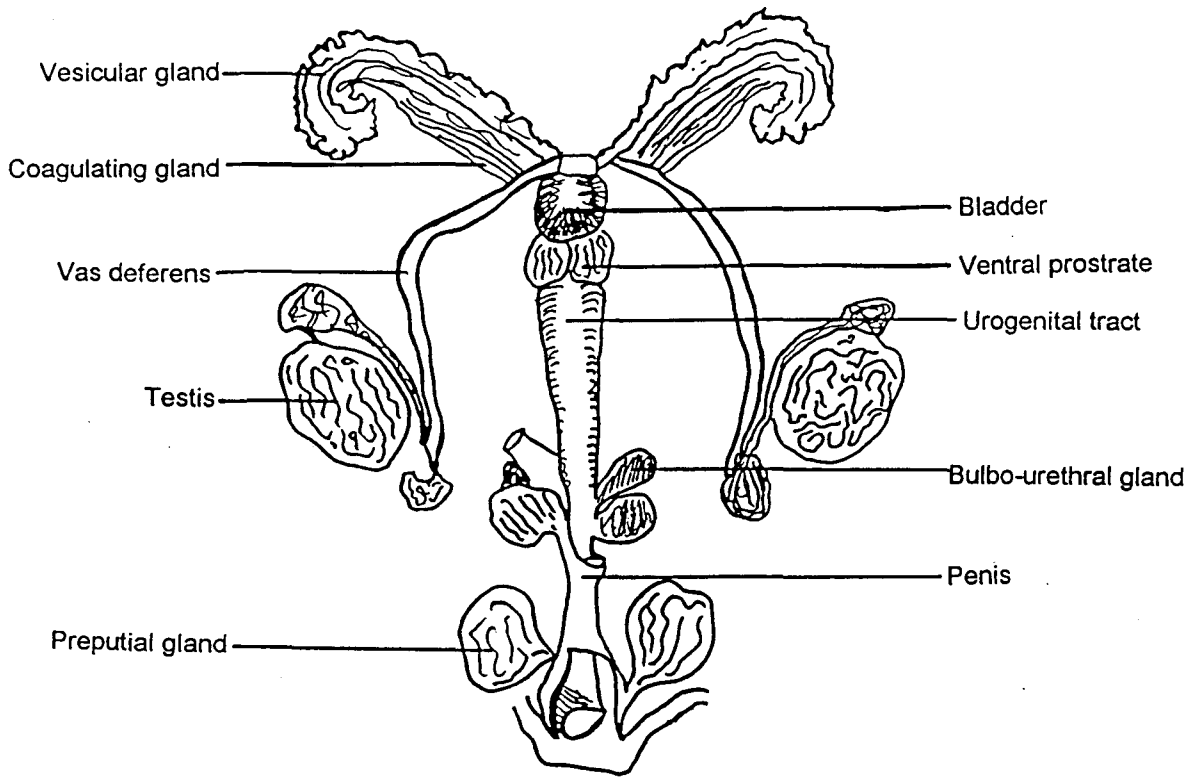


**Figure 1**: Drawing of the male genital system, ventral view

promoting pheromone is evidenced by an increase in fighting behavior between male mice. The absolute weight of these glands is related inversely to density in fixed population of both albino and wild-stock male house mice (Christian, 1955). Hucklebridge, Nowell & Wouters (1972) reported that isolation, which is known to increase aggressiveness, resulted in an increase in preputial gland weight. Those animals rendered aggressive by isolation always had the higher preputial gland weight. Mugford and Nowell (1971) reported that injection of testosterone propionate (TP) also induces a hypertrophy of the female's preputial glands. Since injection of TP into female mice increases aggressive attacks from males, and also increases the size of their preputial glands, Mugford and Nowell (1970) suggested that the preputial glands might be the source of urinary aggression-promoting odors in androgenized females. They concluded that androgens can stimulate the release of aggression-eliciting pheromone from two sources, one being the preputial gland which may act as a social signaling device during agonistic encounters.

Other evidence indicates that, in the rat, preputial gland secretions are attractive to animals of the opposite sex (Noble & Collip, 1941; Orsulak & Gawienowski, 1972). Thody and Dijkstra (1978) concluded that ovarian steroids, as well as controlling receptivity in the female rat, appear to control the production of sex attractants in the preputial gland. A further finding was that there was not a relationship between the size of the preputial glands and their

ability to attract male rats, which suggests that preputial gland growth and production of sex attractants are not under the same hormonal control. Other researchers (Gawienowski, Orsulak, Stacewicz-Sapuntzakis & Joseph, 1975; Gawienowski, Orsulak, Stacewicz-Sapuntzakis & Pratt, 1976) have tentatively identified the sex attractants in the rat preputial gland as C7 and C8 aliphatic alcohols. Bronson and Caroom (1971) indicated that the male preputial gland apparently secretes a signaling pheromone which, acting olfactory, attracts females, at least under the restricted condition of laboratory testing. They showed that the preputial gland factor was attractive to female mice whether it was contained in urine, in the form of a saline homogenate, or occurred as a lipid extract. This finding led to their conclusion that the same pheromone which carried information leading to aggression when perceived by males (McKinney & Christian, 1970; Mugford & Nowell, 1970) served as an attractant when perceived by females.

Aside from the attraction-promoting and aggression-eliciting properties of preputial gland pheromones, several studies provide further insight into the properties of these glands. De and Banerjee (1992) purified a highly active soluble peroxidase (donor: H202 oxidoreductase EC 1.11.1.7) from the preputial gland of the rat by hydroxylapatite chromatography. Geursen and Grigor (1980) compared the properties of phosphofructokinase from a mouse preputial gland tumor with those of the enzyme from other mouse tissues. They reported that

the tumor may provide a suitable model for studying the metabolism of sebaceous glands. Marchlewska-Koj, Pochron & Sliwowska (1990) reported that the preputial glands as well as the salivary glands of male mice are a source of estrus-stimulating pheromone in female mice. Furthermore, Brouette-Lahlou, Amouroux, Chastrette, Cosnier, Stoffelsma and Vernet-Maury (1991) found that a chemical agent contained in the rat pup preputial gland was found to regulate anogenital licking. This is a fundamental pattern of maternal care which is crucial to the survival of the newborn pups. Their experiments also indicate that the olfactory cues are sufficient to bring about this behavior. The preputial glands were also established by Moore and Samonte (1986) as providing chemosignals for maternal discrimination of a pup's sex.

**Coagulating-Vesicular glands**

The vesicular glands of the male mouse (Figure 1) contain the milky white substance which produces the white copulatory plug that is inserted into the female's vaginal opening immediately following insemination. This copulatory plug remains in the vagina several hours after copulation. Speyer (1959 as cited in Hart & Greenstein, 1968) identified the seminal vesicle secretion as coagulinogen, which helps form the coagulated protein when reacted with coagulase. Coagulase is a heat-labile protein that initiates the diphasic coagulation reaction (Speyer, 1959).

The coagulating glands are male sex-accessory glands (Figure 1) which are also involved in semen coagulation (Drori, Amir & Folman, 1968). Walker (1910) identified coagulating gland secretion as containing the enzyme vesiculase, the agent responsible for the initiation of semen coagulation in the rat and guinea-pig (Camus & Gley, 1899). Vesiculase is a heat-labile protein which reacts with seminal vesicle secretion to produce a coagulant (Gotterer, Ginsburg, Schulman, Banks & Williams-Ashman, 1955). Vesiculase converts procoagulase to coagulase. Procoagulase is heat-labile and its conversion to coagulase can be inhibited by metal chelating agents such as versene (Gotterer & Williams-Ashman, 1957 as cited in Hart & Greenstein, 1968). Additional research by Beil and Hart (1973) confirmed that coagulating gland substance contains vesiculase and that vesiculase is the potentiating factor of rat semen coagulation. They suggested that the mechanism of coagulation may involve an "interaction reaction" where vesiculase from the coagulating glands is reacting with the substrate from the seminal vesicles in the intermediary step towards semen coagulation. More specifically, a transamidase reaction may be occurring prompted by the vesicular gland enzyme, vesiculase. Other researchers (Greenstein & Hart, 1964 as cited in Hart & Greenstein, 1968) supported these findings by stating that semen coagulation resulted from the mixing of male sex accessory gland secretion, particularly the coagulating glands. Price and Williams-Ashman (1961) indicated that semen coagulation

required the action of the seminal vesicles. Hart (1970) reported that the clottable substrate from the seminal vesicles of the rat for each of the coagulation reactions has been purified and has shown to be a basic protein with a mol weight of 40 000.

The size and fructose content and concentration of the coagulating glands are inversely related to the male's frequency of mating (Drori, Amir & Folman, 1968). In order to demonstrate this, Drori and Folman (1964) raised low- and medium-frequency mating male rats and ran a variety of experiments with them, using non-mated males as controls. At the end of their experiments, the males were killed and the coagulating glands were removed, weighed and frozen. High-frequency mated males had a reduced fructose content and concentration in their coagulating glands as compared to low-frequency mated males. Interestingly, if these males were left unmated for 7 days before being killed, the fructose content and concentration rapidly increased. This inverse relationship is due to the frequent evacuation of the secretion and the preponderance of fructose in the secretion rather than in the glandular tissue (Drori & Folman, 1964). At the same time, the content and concentration of fructose in the coagulating glands was highest in the groups mated at low frequency as compared to non-mated males. Since ejaculation evacuates glands, one would expect to find less fructose in the glands of mated males if the rate and fructose production in mated and unmated males were equal. However,

since male rats mated at low frequency had consistently more fructose in their coagulating glands, it appears that they produce more fructose than unmated males (Drori & Folman, 1964).

A common finding in the literature is that male accessory reproductive gland size is related to androgenic activity. Drori and Folman (1964) observed that mated male rats have larger accessory reproductive organs than unmated males and attributed this to increased androgenic activity. They conducted experiments where male rats were raised in cohabitation with sexually receptive females and compared these with unmated males. Following this, they compared the size of several accessory reproductive organs. They found that the males raised with receptive females had larger accessory reproductive organs and kidneys. At 286 days of age, a degeneration of the coagulating glands was observed. Their findings suggest that in the male rat cohabitation is necessary for the proper maintenance of testicular androgen secretion. This observation confirms the original claim of Steinach (1936 as cited in Drori & Folman, 1964). Steinach stated that male rats housed alone and denied heterosexual contact suffered atrophy of the testes and the accessory reproductive organs and consequent loss of libido.

Christian (1955) also reported that the absolute weight of preputial and vesicular glands declines with increasing population size. This effect was observed after correction for body weight. Christian proposed two mechanisms

for this weight decline. Firstly, it could be attributed to decreased androgen activity or, secondly, to a specific interference with organ activity. Mann and Parsons (1947) also reported that fructose formation in the male sex-accessory organs is the result of androgenic activity. Furthermore, Mann and Parsons (1950) specifically reported that the coagulating glands increase in a linear fashion under the influence of testosterone.

Haug (1971 as cited in Jones & Nowell, 1973) suggested that the coagulating gland is involved in the scheme of pheromonally mediated aggression. More specifically, Haug reported that secretions from the coagulating gland inhibit aggression. However, further work conducted by Jones and Nowell (1973) did not support this finding. Jones and Nowell (1973) suggested, however, that although coagulating gland secretions do not independently affect aggressive behavior, coagulating gland secretion combined with bladder urine may neutralize the aggression-promoting properties found in bladder urine alone. Furthermore, their study indicated that preputial gland secretions can elicit aggression without the involvement of urine.

The bulbo-urethral gland is a male sex-accessory gland (Figure 1) which is also active in semen coagulation. Hart (1968) presented evidence for the mechanism of action through which bulbo-urethral gland secretion coagulates rat semen. The reaction appears to be non-enzymatic, involving an ionic interaction between bulbo-urethral gland secretion and substrate. Hart and

Greenstein (1968) reported that in the rat, mouse and hamster but not in the guinea pig, bulbo-urethral gland secretion reacts in vitro with vesicular gland secretion to produce a coagulant.

**Purpose of the Present Study**

The current study is an attempt to examine the effect of the removal of the preputial glands, the vesicular-coagulating gland complex, as well as the combination of the preputial, vesicular and coagulating glands upon strange-male-induced pregnancy disruption. Since it is thought that the strange-male-induced pregnancy disruption is at least partially governed by pheromones, it follows that the potential source of these pheromones needs to be investigated. With a bulk of the evidence indicating that androgen-dependent glands such as the preputial, vesicular and coagulating glands play a role in social behavior, it is plausible that these glands are involved in the failure of ova implantation in inseminated female mice. Therefore, this study is an attempt to examine whether or not these glands are responsible for the pheromonally-mediated component of the pregnancy disruption.

The experiments were conducted by indirectly exposing inseminated female mice to either 1) males which had their preputial glands removed or 2) males which had their vesicular-coagulating gland complex removed or 3) males which had their preputial, vesicular and coagulating glands removed. This

exposure occurred during the first five days following insemination. For the purposes of control, sham surgeries were also performed on other stimulus males. Inseminated females were exposed to either sham or fully-operated males. It is assumed that, if these glands are the source of pheromones mediating such pregnancy disruptions, those strange males which have undergone gland removal will fail to disrupt pregnancy in previously inseminated females insofar as the odor/pheromone source has been removed.

## EXPERIMENT 1

This experiment was conducted in collaboration with Cameron Muir and was designed to isolate the source of the pheromone(s) involved with the transmission of the Bruce effect. The preputial glands were removed from a subset of novel male mice to which the females were exposed, and the efficacy of the pregnancy disruption was compared to that induced by intact males. It was hypothesized that the preputialectomized males would fail to disrupt pregnancy insofar as they would lack the preputial pheromones which are known to be involved with mediating other social sexual behaviors (deCatanzaro, Zacharias & Muir, 1996).

### METHODS

#### Animals and insemination procedures

The experimental subjects and inseminating males were CF-1 strain mice (*Mus musculus*) bred in the McMaster Psychology Department from stock originally obtained from Charles River Breeding Farms, La Prairie, Quebec, Canada. The subjects were sexually inexperienced females weaned at 30 days of age. Prior to insemination, the females were housed in groups of four or five. The inseminating males were each housed individually in the same type of cages as the females, and were all sexually experienced and had been selected

for their sexual vigor. All housing was in standard propylene cages measuring 28x16x11 (height) cm, filled with approximately 0.5 L fine wood chip bedding, with wire grid tops allowing continuous access to food and water. The animal colony room was maintained at 21 degrees Celsius under a reversed 14 hour light:10 hour dark cycle.

The novel males were HS (heterogeneous strain), bred in the McMaster Psychology Department from stock originally obtained from the Department of Zoology at the University of Toronto (Toronto, Ontario, Canada). All males were housed individually. Those selected for surgery underwent preputial gland removal two weeks before the onset of the experiment. Sham-operated males were also prepared. These males experienced identical anesthetization, incision and recovery procedures, however their preputial glands were left intact.

When the females were between 65 and 100 days old, at the onset of the dark phase of the light cycle, they were each placed in an inseminating male's cage. The males had been deprived of access to females for at least 14 days. Three times a day during the dark phase, each female's hindquarters were inspected for the presence or absence of a vaginal copulatory plug. At the end of the dark phase of the cycle, all females with obvious copulatory plugs were identified as subjects; the day was designated as Day 0 of pregnancy. Each female remained housed with the inseminating male until the morning after

detection of the vaginal copulatory plug, about the start of the dark phase of the animals' lighting cycle.

**Indirect exposure paradigm**

Double-decker cages were constructed from clear Plexiglas which allowed exposure of an inseminated female to the effects of two males housed directly above (Figure 2). These males were separated from each other by a Plexiglas barrier to prevent behavioral interactions between them. The males' upper compartment was separated from the female's lower compartment by a wire mesh to prevent mating behavior. Each cage measured 30 x 27 x 21 cm, and the two compartments measured approximately 30 x 13 x 21 cm. These compartments were separated by a wire-mesh grid with 1 $cm^2$ square openings. The upper compartment was covered with a standard straight-wire mouse cage lid that provided the male with continuous access to food and water. The lower compartment was filled with approximately 0.5 L fine wood-chip bedding and had a water spout protruding through one wall. Food was delivered to the female in the lower compartment through a cylindrical plastic cup (4 cm in diameter and 5 cm deep); the open end of the cup provided access to the food through a wire mesh, and the cup was oriented to prevent urine and feces from the animal above from contacting the food.

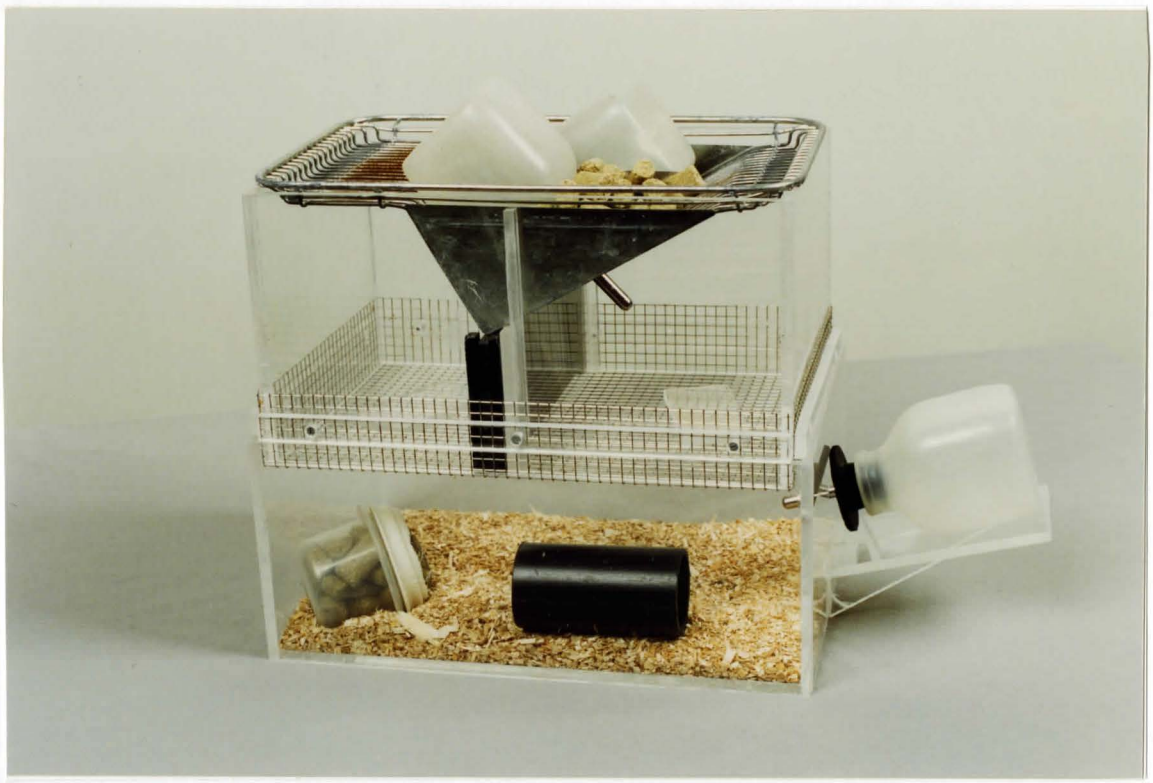

**Figure 2**: Photograph of double-decker cage system

CF1 females were inseminated as described above, and each was randomly assigned to one of three conditions: no male above, two novel preputialectomized males above or two novel sham preputialectomized males above. Throughout subject assignment, the date of insemination was counterbalanced across the three conditions. Inseminated females were placed in the lower unit of the double-decker cage within 2-4 hours after commencement of the dark phase of the lighting cycle on Day 1 after detection of the copulatory plug. They were continuously in that unit until 5-6 hours after commencement of the dark phase of the light cycle, 6 days after the identification of the copulatory plug. Then females were each directly transferred to a clean standard mouse cage and left undisturbed until standard pregnancy outcome measures began.

**Method of Preputialectomy**

The preputial glands of the male mouse consist of a pair of unusually enlarged modified leaf-shaped ectodermal exocrine glands opening to the exterior on either side of the urethral meatus (Figure 1) (De & Banerjee, 1992; Brouette-Lahlou et al, 1991; Raso, Timar & Lapis, 1992). The preputials are specialized sebaceous glands located bilaterally between the skin and the body wall anterior to the external genitalia (Mugford & Nowell, 1971). They are very

prominent in male mice. They are yellowish-brown in color, dorsoventrally flattened, measuring approximately 7 x 5 mm.

In order to remove the glands, the animals were anaesthetized with sodium pentabarbitol. Two incisions 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 the glands had been located, they were snipped off at the stem. Normally this did not produce much bleeding, therefore it was not necessary to ligate the glands at their base. The largest glands were found in those males which appeared to be extremely masculine (i.e. swollen testicle area). The glands seemed to be enveloped by a thin membranous transparent sheath. This sheath had to be snipped away or torn with tweezers in order to gain access to the preputial glands for subsequent removal. Sometimes a membranous tissue was present between the two glands which appeared to connect them to each other. This connective tissue was easily torn, however if left intact it was possible to slide both glands out of the same incision as both glands were being held together. Following preputial gland removal, the skin layer was closed with two woundclips. All animals were then placed on a heating pad and allowed to recover. Once normal activity resumed (0.5-1 hour post-suture), they were placed in a clean cage with fresh bedding and allowed continuous access to food and water for the duration of a two week recovery period.

**Pregnancy Outcome Measures**

At the end of the 120 hour period of exposure to novel males, each female was housed individually in a clean cage with fresh bedding and was left undisturbed for the duration of gestation. From the 18th day after the detection of the copulatory plug, females were checked on three occasions each day for parturition. Such inspections continued until 30 days after detection of the plug. Pregnancy outcome was measured by counts of the number of pups born and the duration of gestation. The number of pups three days after birth, cannibalizations and stillbirths were also recorded.

## RESULTS

Table 1 shows the number of females in each group bearing litters as well as the sample sizes of each group. These data compare the influences of no-male exposure to preputialectomized-male exposure to sham-preputialectomized male exposure. Most females (21 of 23) in the control group produced litters, but substantially fewer did so in either the preputialectomized novel male condition (4 of 21) or the sham-preputialectomized novel male condition (7 of 21). A test of association between conditions and the presence or absence of parturition showed significance ($\chi^2(2) = 26.06739$, $p<0.001$). A test of association comparing just the control and preputialectomized groups was similarly significant ($\chi^2(1) = 23.36$, $p<0.001$) as was one comparing the control

and sham-operated groups ($\chi^2(1) = 15.94$, $p<0.001$) but not comparing the preputialectomized and sham-operated groups. More pups were born to control females (10.56±0.81) than to either females in the preputialectomy-exposure condition (2.00±0.93) or to females in the sham-preputialectomy exposure condition (4.52±1.45). Analysis of variance on the number of pups born was significant, $F(2,62) = 16.81$, $p<0.0001$. Neumann-Keuls multiple comparison ($p<0.05$) test identified a difference between the control condition and each of the other two conditions, but not between the preputialectomized and sham conditions. The number of pups three days after birth was also recorded. This was done because newborn mice may not survive to day three because of cannibalization by the mother, or otherwise ill health. The trend in the measure of number of pups three days after birth showed essentially the same trend as number born. Cannibalizations showed no consistent trend. Figure 3 illustrates the data for pregnancy outcome as recorded in Table 1.

**Histology**

Ten animals which 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.

**Table 1**

Means (±SE) of Measures of Pregnancy Outcome after Indirect Exposure to 2 Preputialectomized (PPX) Males

| GROUP | N=65 | No. Females Delivering | No. Pups Born |
|---|---|---|---|
| Pure Control | 23 | 21 ( 91.3%) | 10.57±0.81 |
| Sham PPX | 21 | 7 (33.3%) | 4.52±1.45 |
| PPX | 21 | 4 (19.0%) | 2.0±0.93 |

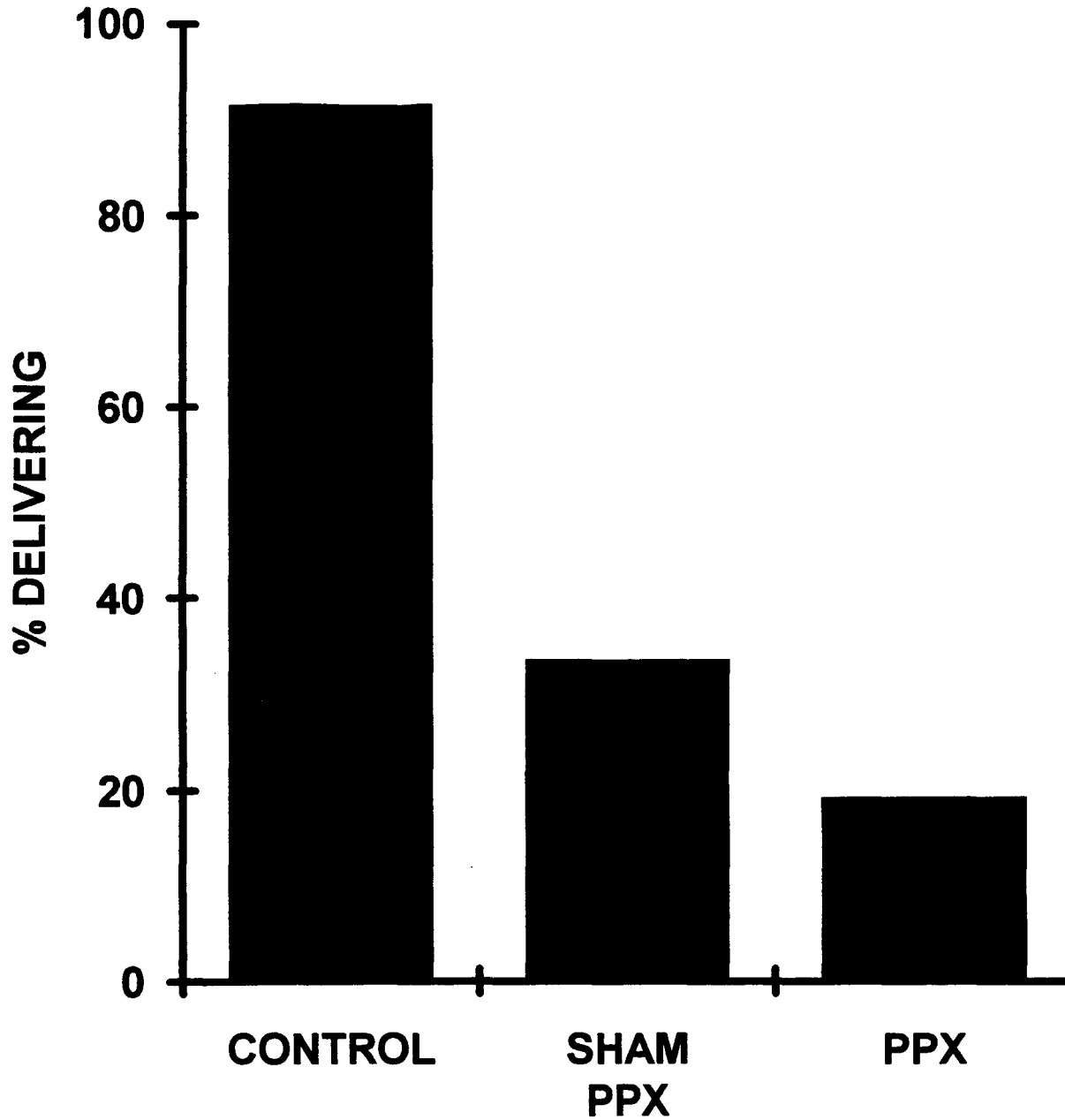


**Figure 3**: Illustration of data showing that males without preputial glands (PPX) disrupt pregnancy similarly as intact males

## EXPERIMENT 2

Experiment 1 demonstrates that there was no difference in the ability to disrupt pregnancy between the preputialectomized males and the intact males. Accordingly, it does not appear that the preputial glands are the source of pheromones involved in the mediation of the Bruce effect. As a result of these findings, the present experiment was designed to continue the search for the pheromone source. The coagulating-vesicular glands have been implicated in the mediation of intermale aggression (Jones & Nowell, 1973) so they seem likely candidates for subserving the Bruce effect. The vesicular and coagulating glands were removed from the novel male mice to which females were exposed, and the efficacy of the pregnancy disruption was compared to that of intact males. It is hypothesized that those males without their vesicular and coagulating glands would fail to disrupt pregnancy if these glands were responsible for pheromones subserving the Bruce effect.

### METHODS

The subjects and insemination procedures were the same as in Experiment 1. As well, the indirect-exposure paradigm utilized in this experiment was identical to that of Experiment 1. The novel males selected for surgery were

of the same strain and breeding stock as in Experiment 1. However, these males underwent either 1) surgical removal of their vesicular and coagulating glands or 2) sham surgery.

**Method of Vesicular-Coagulatingectomy**

The vesicular glands of the male mouse (Figure 2), otherwise known as the seminal vesicles, are a pair of translucent elongated sacs which appear bright white in a recently anaesthetized animal. They are the largest gland in the male genital system, measuring approximately 15 mm (length) x 3-6 mm (width) x 1-2 mm (depth) and are dorsoventrally flattened. They are situated at the top of the male genital tract with the proximal base attached to the tract just above the Vas deferens and the urinary bladder. The vesicular glands stretch longitudinally from this junction point for approximately 15-20 mm where they form a large curl at their distal end. The glands are most swollen at this distal end.

The vesicular glands contain the milky white substance which produces the white copulatory plug that is inserted into the female's vaginal opening immediately following insemination. During vesicular gland removal, it is possible to apply pressure to the distal end of an excised gland, thereby evacuating the gland of its milky white substance. The two glands together

contain approximately 0.5 mL of this opaque white fluid. The contained fluid is odorless and much more viscous than water.

The coagulating glands are situated directly beneath the vesicular glands and consist of a pair of thick transparent tissue-like membranes (Figure 2). Their form is continuous with the structure of the vesicular glands. To the naked eye the vesicular glands can only be distinguished from the coagulating glands by their non-white transparent appearance. The coagulating glands are much smaller than the vesicular glands and seem to fold up into the vesicular glands and therefore remain hidden until they are delicately teased away from the bright white vesicular glands with tweezers. They measure approximately 6 mm (length) x 2 mm (width) x 0.5 mm (depth) and are dorsoventrally flattened. When pressure is applied to the coagulating glands a transparent liquid is squeezed out. This liquid is odorless to humans and has a viscosity similar to water.

For the purposes of removal, the vesicular glands and the coagulating glands were removed together as the vesicular-coagulating gland complex. Individual removal of these glands would have been extremely difficult as they are enmeshed together and removal of either one would have caused tissue damage to the other. In order to remove the glands, the animals were anaesthetized with sodium pentobarbital. One 5 mm incision was made approximately 5 mm above the external genitalia. Once the outer skin had been cut, a second 5 mm incision was immediately made in the muscle wall. Extreme

caution was taken so as not to puncture the bladder which was located directly beneath the site of incision. The glands were found on either side of the bladder tucked in behind the genital tract. The bright white color of the coagulating glands aided in identifying the vesicular-coagulating gland complex. Once the glands had been located, they were snipped off at the stem. Bleeding was minimal or nonexistent, therefore it was not necessary to ligate the glands. Once again, as in the preputialectomy, the largest glands were found in those males which appeared to be extremely masculine (i.e. swollen testicle area). The glands were easily lifted away from the body cavity once snipped off. Following this, the muscle wall was sutured closed and the skin layer was closed with one or two woundclips. All animals were then placed on a heating pad until normal activity resumed, usually in about 0.5 -1 hour. At this point they were placed alone in a clean cage with fresh bedding and allowed continuous access to food and water for the duration of a two week recovery period.

Pregnancy outcome was measured as described in Experiment 1.

## RESULTS

Table 2 gives data for the proportion parturient in each condition of the experiment comparing the influences of two vesicular-coagulatingectomized males and two sham vesicular-coagulatingectomized males. The control group had the highest proportion of females delivering litters (41 of 51). A similar

proportion of females delivered litters in the vesicular-coagulatingectomized group (12 of 39) as compared to those females in the sham vesicular-coagulatingectomized group (15 of 41). A test of association between the conditions and the presence or absence of parturition showed significance ($\chi^2(2) = 27.41268$, $p<0.001$). A test of association comparing just the control and vesicular-coagulatingectomized groups was similarly significant ($\chi^2(1) = 22.47835$, $p<0.001$) as was one comparing the control and sham-operated groups ($\chi^2(1) = 18.31194$, $p<0.001$) but not comparing the vesicular-coagulatingectomized and sham-operated groups.

The number of pups born to control females was 8.41±0.68; for females exposed to vesicular-coagulatingectomized males, 3.31±0.84, and; for those exposed to sham males, 3.93±0.84. Analysis of variance on this measure was significant ($F(2,128) = 13.57$, $p<0.0001$) and multiple comparisons identified a difference between the control condition and each of the other two conditions, but not between the vesicular-coagulatingectomized and sham conditions. The number of pups three days after birth was recorded and showed essentially the same trend as number of pups born. Cannibalizations showed no consistent trend, and therefore, this measure was not submitted to analysis. Figure 4 illustrates the data for pregnancy outcome as recorded in Table 2.

## Histology

Seven animals which had been vesicular-coagulatingectomized were sacrificed at five months after the surgery. In each case the former site of the vesicular-coagulating complex could be identified. In all cases, full removal of the vesicular and coagulating glands was confirmed, and in no case was there any sign of regeneration of these glands.

**Table 2**

Means (±SE) of Measures of Pregnancy Outcome after Indirect Exposure to 2 Males which have undergone removal of Vesicular and Coagulating Glands (VSX)

| GROUP | N=131 | No. Females Delivering | No. Pups Born |
|---|---|---|---|
| Pure Control | 51 | 41 (80.4%) | 8.41±0.68 |
| Sham VSX | 41 | 15 (36.6%) | 3.93±0.84 |
| VSX | 39 | 12 (30.8%) | 3.31±0.84 |

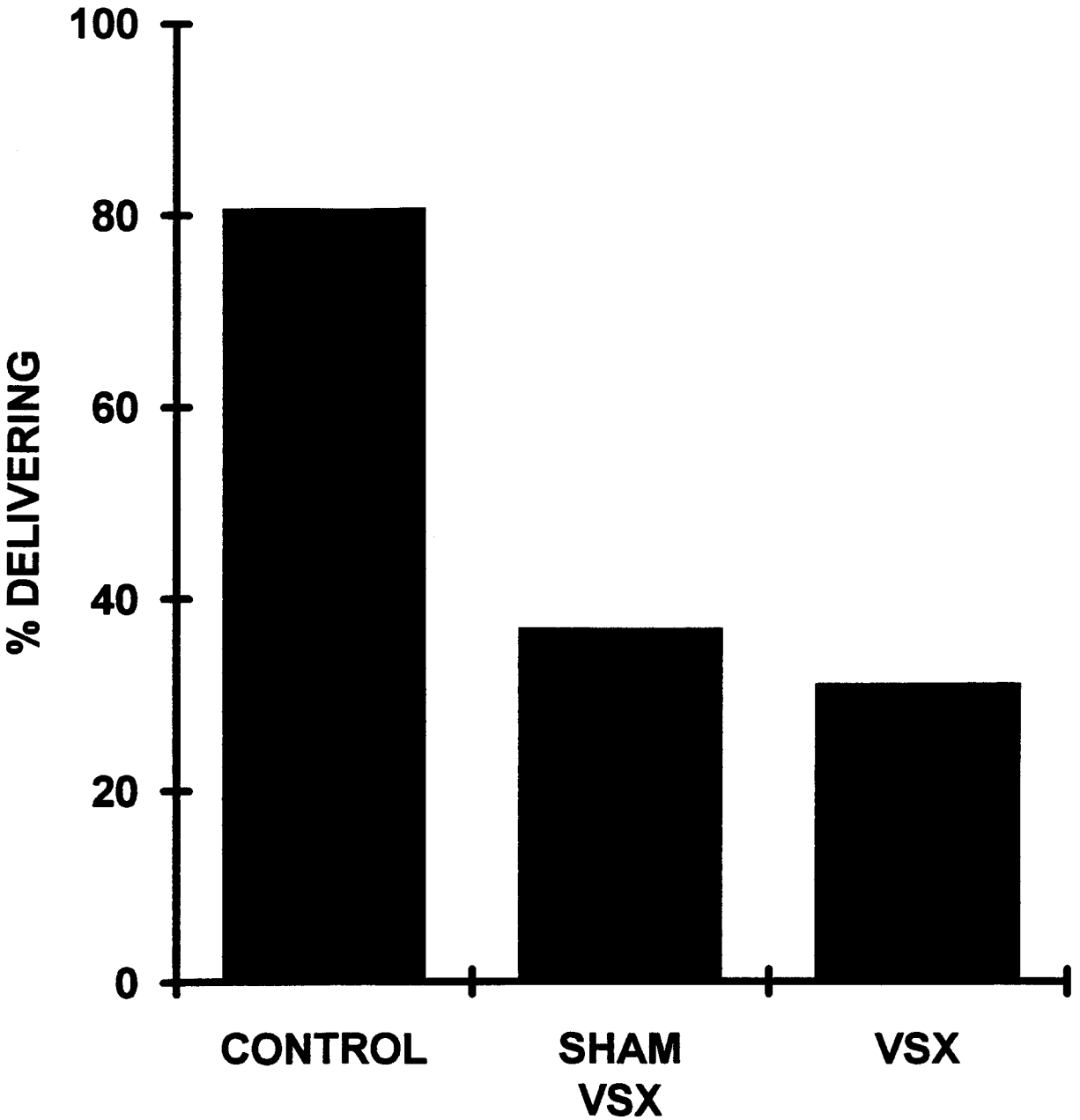


**Figure 4**: Illustration of data showing that males without vesicular-coagulating gland complex (VSX) disrupt pregnancy similarly as intact males

## EXPERIMENT 3

Experiments 1 and 2 demonstrate that individual removal of the preputial glands and individual removal of the vesicular-coagulating glands do not interfere with the capacity of the strange male to disrupt pregnancy. It remains conceivable that it is an either/or situation and that synchronous removal of these glands would interfere with the Bruce effect. Since the preputial and vesicular-coagulating glands are synergistically involved in mating behavior and share aggression-mediating properties, it is possible that they interact in the mediation of the Bruce effect. Therefore, the present experiment was designed to examine the effects of removal of the preputial, vesicular and the coagulating glands in order to be sure that these glands are not involved in an interaction. It is hypothesized that those strange males without their preputial, vesicular and coagulating glands would fail to disrupt pregnancy in newly inseminated female mice if such an interactive effect were responsible for the Bruce effect.

### METHOD

The subjects and insemination procedures were the same as in the preceding experiments. As well, the indirect-exposure paradigm utilized in this

experiment was identical to that described for Experiment 1. The novel males selected for surgery were of the same strain and breeding stock as in Experiments 1 and 2. However, these males underwent surgical removal of their preputial, vesicular and coagulating glands. A sham group was not included in this experiment since the effect of sham-exposure had already been well established in Experiments 1 and 2.

**Method of Preputial, Vesicular and Coagulating Gland Removal**

The method of removal of all three glands is the same as in the previous experiments. However, for this experiment, the glands were removed simultaneously. A single incision was made 10 mm above the external genitalia. The preputial glands were usually the first to be removed since they are located just under the skin layer. Once they had been snipped off at the stem and removed, the muscle wall was cut. The two vesicular-coagulating gland complexes were located on either side of the bladder (Figure 1), and their removal did not cause internal bleeding. All six of the glands were easily lifted away from the body cavity once snipped off. Following this, the muscle wall was sutured closed and the skin layer was closed with one or two woundclips. Animals were then allowed to recover on a heating pad until normal activity resumed. Histology was not performed on the males which had undergone preputial, vesicular and coagulating gland removal. Histological results in the

previous experiments confirmed that regeneration of these glands does not occur.

Pregnancy outcome was measured as described in the previous experiments.

## RESULTS

Table 3 summarizes the number of females bearing litters in each condition; no-male exposure and exposure to novel males which had undergone removal of the preputial glands as well as the vesicular-coagulating gland complex. Nearly all of the females in the control condition delivered litters (25 of 26). Comparatively, the number of parturient females which had been exposed to preputial-vesicular-coagulatingectomized males was greatly reduced (5 of 25). A test of association between the conditions and the presence or absence of parturition showed significance ($\chi^2(1) = 30.52$, $p<0.001$). Accordingly more pups were born to females in the control condition (10.85±0.61) as compared to females which were exposed to those novel males which had undergone preputial-vesicular-coagulating gland removal (2.24±0.93). Analysis of variance on this measure was highly significant ($F(1,49) = 60.87$, $p<0.00001$). Figure 5 illustrates the data for pregnancy outcome as recorded in Table 3.

**Table 3**

Means (±SE) of Measures of Pregnancy Outcome after Indirect Exposure to 2 Males which have undergone removal of Preputial, Vesicular and Coagulating Glands (PPX-VSX)

| GROUP | N=51 | No. Females Delivering | No. Pups Born |
|---|---|---|---|
| Pure Control | 26 | 25 (96.0%) | 10.85±0.61 |
| VSX-PPX | 25 | 5 (20.0%) | 2.24±0.93 |

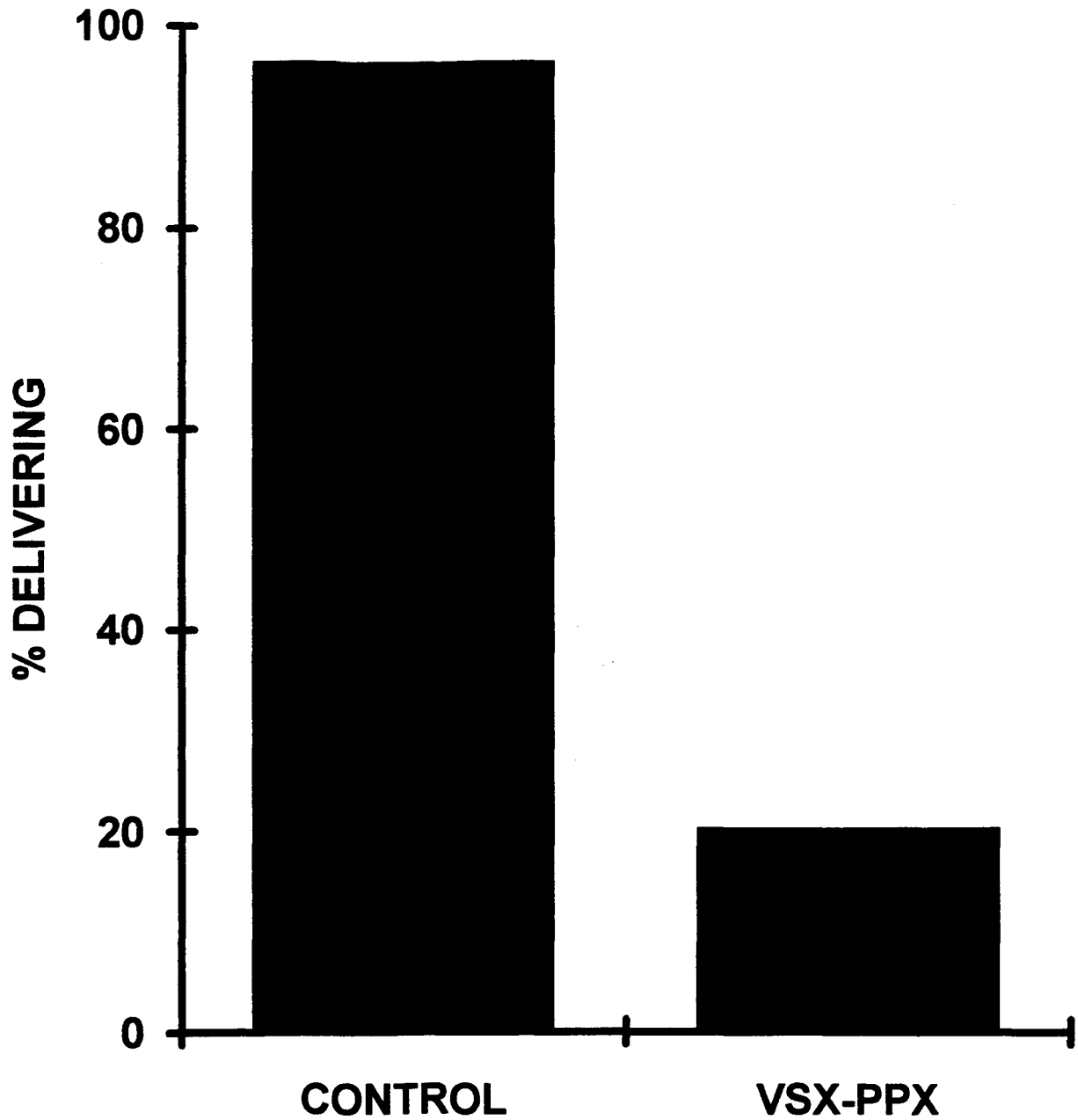


**Figure 5**: Illustration of data showing that males without preputial, vesicular and coagulating glands (VSX-PPX) disrupt pregnancy similarly as intact males

## DISCUSSION

These experiments demonstrate that removal of the preputial glands and/or the vesicular-coagulating glands does not diminish the capacity of strange males to disrupt pregnancy. The presence or absence of litters and the number of pups born, assigning zeros to nonparturient females, were used as two different measures of pregnancy disruption. It was found that effects were all-or-none and probabilistic, given that some control females did not produce litters and some females exposed to novel males did produce litters, while litter size of parturient females was in the normal range regardless of condition. Experiment 1 demonstrates that preputialectomy fails to interfere with the strange male's ability to disrupt pregnancy. Compared to the high percentage of control females producing litters (91.3%), both the females exposed to preputialectomized strange males and those females exposed to sham-preputialectomized males produced fewer litters, 19.0% and 33.3% respectively. The difference in the proportion delivering litters between the control group and each of the experimental groups was significant, whereas the difference between the preputialectomized-exposed and the sham-preputialectomized-exposed groups was not. As well, using an additional measure of pregnancy outcome,

undergone sham surgery was not significant. These data demonstrate the irrelevance of the vesicular-coagulating glands with respect to the ability of the strange male to induce pregnancy disruption in newly inseminated females.

Experiment 3 was conducted in order to discover if a possible interactive effect existed between the preputial and vesicular-coagulating glands which mediated the strange male induced pregnancy disruption. The strange males used in this experiment underwent surgery to have both their preputial and vesicular-coagulating glands removed. Results from this experiment demonstrate that the simultaneous removal of both the preputial as well as the vesicular-coagulating glands fails to interfere with the strange male's ability to disrupt pregnancy. All but one female in the control condition delivered litters, which converts to a percentage of 96.2%. This was compared to the percentage of females who delivered litters which had been exposed to males which had undergone removal of their preputial, vesicular and coagulating glands (20.0%). A sham-operated group was not included in this experiment as the sham surgery would have been identical to the sham surgery performed in Experiments 1 and 2. There were significantly more pups born to control as opposed to experimental females.

These results clearly indicate that the preputial glands as well as the coagulating and vesicular glands are irrelevant with respect to the ability of the strange male to disrupt pregnancy. Histology showed no imperfection in the

surgery, confirming that these glands do not regenerate with time. Although these findings do not disprove that the Bruce effect is pheromonally mediated, they do demonstrate the fact that neither the preputial, vesicular nor the coagulating glands are the source of the pheromone(s) involved in the Bruce effect. It is noteworthy that several researchers state that the Bruce effect is pheromonally mediated (Parkes & Bruce, 1962; Dominic, 1965; Marchlewska-Koj, 1977, 1981), and that the pheromone involved is androgen dependent (deCatanzaro *et al.*, 1995; Dominic, 1965, 1966a, 1966b). It follows that the preputial, vesicular and coagulating glands which are androgen-dependent glands involved in the pheromonal mediation of other mating behaviors (i.e. intermale aggression and intersex attraction) and are very prominent in the male mouse reproductive system would be likely candidates for subserving this effect. However, these results confirm that they do not mediate the Bruce effect. Further research is needed either to isolate the source of the pheromone(s) involved in the Bruce effect or to examine the possibility of non-pheromonal mediation.

Pregnancy disruption is observed when the strange male is housed with the female immediately following insemination (deCatanzaro, Zacharias & Muir, 1996). However, this direct-exposure method involves the complication of sexual interactions which occur between the strange male and the newly inseminated female. Prior work (deCatanzaro, Zacharias & Muir, 1996)

indicated that strange males, whether intact or preputialectomized, showed significantly more sexual behavior towards the previously inseminated females than did the males which sired the litter. Earlier research demonstrated that sexual interactions between the males and the inseminated females was an important component of the induction of pregnancy disruption. Such activity affected the female's reproductive physiology and thus may have played a crucial role in disrupting pregnancy. For example, deCatanzaro & Storey (1989) suggested a role for sexual activity in male-induced pregnancy disruptions, based on evidence that mating behavior between strange males and inseminated females correlated with pregnancy outcome. As well, several studies suggested that oxytocin was released during mating behavior (Fox & Knaggs, 1969; McNeilly & Ducker, 1972; Garcia-Villar, Schams, Alvinerie, Laurentie & Toutain, 1985). Oxytocin is a hormone which triggers uterine contractions in association with sexual activity (Fox, Wolff & Baker, 1970). In a purely physical sense, such uterine contractions may impede fertilized ova from implanting into the uterus, thus inhibiting pregnancy. Furthermore, deCatanzaro and Storey, in unpublished work, found that exogenous oxytocin blocked implantation, suggesting that endogenous oxytocin could be among the hormonal factors that mediate pre-implantation loss of pregnancies due to strange-male exposure. Therefore, behavioral interactions may have been

playing a significant role in the induced pregnancy block in direct-exposure paradigms.

In order to eliminate the complication of direct behavioral contact, the indirect-exposure paradigm was adopted for these experiments. A double-decker cage system was used which separated the strange males from the female by housing them above her, separated from the compartment below by a wire grid (Figure 2). This method eliminated any behavioral interaction between the strange males and the newly inseminated female. As well, there was no doubt as to the paternity of the litter since reinsemination was impossible. Furthermore, the males were also separated from each other by way of a Plexiglas material, so as to eliminate any behavioral interaction between them. Prior observation in this laboratory indicated highly aggressive interaction between males housed above a newly inseminated female. Subsequently, the paradigm was improved by complete separation of the males, thereby eliminating the intermale aggression. This change in the indirect-exposure paradigm did not affect the reliability of pregnancy disruption observed. Accordingly, the present experiments were designed using the indirect-exposure paradigm, eliminating the complication of behavioral interactions in order to focus on isolating the pheromone source involved in the Bruce effect.

During these experiments, several human observers have independently noted that a subjectively foul and distinct odor is present in the experimental

room when inseminated females are in the double-decker apparatus with males, and that the walls of the males' compartments are smeared with an oily emission during this exposure. There is no apparent difference in the magnitude of these observations with preputialectomized versus intact males. However, in Experiments 2 and 3, a decreased amount of this substance was deposited on the double-decker cage walls when the animals housed above were males without vesicular-coagulating glands compared to preputialectomized or intact males housed above. It appears that the removal of the vesicular-coagulating glands decreases the amount of emission coming from the males. It is possible that the milky-white substance contained in the vesicular glands, which is used to produce the copulatory plug, is the same substance which is being deposited on the cage walls. Therefore, when the glands have been removed from the strange male, the cages are less cloudy. This speculation is not completely supported, however, since this substance, during vesicular gland removal is odorless. It is subjectively apparent that the odor remains when females are exposed to males which have undergone removal of their vesicular and coagulating glands. It may be that the odor is not present in the material which is clouding the cages, but may be from another source. Further research is needed here. Additional experiments could involve the collection and subsequent analysis of the substance which is clouding the cages in order to determine its origin and effect.

Since the preputial, vesicular and coagulating glands are not the source of the pheromone(s) mediating the Bruce effect, further research is needed in order to locate a possible pheromone source. Perhaps the bulbo-urethral glands are involved. The bulbo-urethral glands (also called Cowper's glands) aid in the formation of the copulatory plug along with the coagulating and vesicular glands and are thus involved in the subsequent fertility in several rodent species (Hart & Greenstein, 1968). They are androgen-dependent organs which lie lateral to the urethra and are connected to it by a short duct (Cooke, Young & Cunha, 1987). They are paired structures lateral to the junction of the membranous urethra and penis. The main body of the gland is at the side of the urethral diverticulum buried in the bulbocavernosus muscle, the tail is between the diverticulum and the ischiocavernosus muscle, and the duct enters the urethra immediately anterior to the diverticulum (Figure 1). In rodents, the bulbo-urethral glands show histochemical characteristics typical for mucous glands. Accordingly, they secrete mucin which aids in semen coagulation(Geuze & Slot, 1976).

Geuze and Slot (1976) studied the discharge and synthesis of rat bulbo-urethral gland secretory product in relation to the normal copulatory process. They took measurements of the macromolecularly-bound hexose contents in the glands and revealed that immediately after the start of sexual intercourse, the glands emitted secretory product independently of the occurrence of ejaculation. Depletion of the glands was completed after 4 hours (64% loss of hexose

contents), concurrently with an ending of mating. Following this depletion, the glands refilled gradually with hexose. After one week, the hexose contents had returned to the control level. Also, they showed that glycoprotein synthesis increased immediately and peaked 6 hours and 13 hours after the start of copulation. After about 4 days, synthesis was again near the level of glycoprotein synthesis observed in the control males (those males kept separately from females during the experiment). These trends were accompanied by impressive morphological alterations. In control glands the lumen was filled with secretory product and the cells were studded with secretory granules and contained only scarce cytoplasm. Within 4 hours after the start of the intromissions the glandular lumen was empty and the development of cell structures indicated active formation of secretory product.

Studies by Cooke, Young and Cunha (1987) demonstrated the role of testosterone in the normal growth and epithelial morphogenesis of the bulbo-urethral gland. They found that this gland in the mouse differentiates from the urogenital sinus on day 17 of gestation (day 0 = vaginal plug, day 19 = birth), and continues to enlarge until about day 6 after birth. Their results show that if mice were castrated at birth or castrated and then treated with cyproterone acetate, an antiandrogen, over the first 6 days of life, bulbo-urethral gland growth was reduced by 80%, but not abolished. Thus, during early neonatal life, the growth of the bulbo-urethral gland is partially independent of androgens.

However, if mice were castrated as neonates, morphogenesis of the bulbo-urethral gland epithelium was completely abolished. Testosterone replacement given to neonatally castrated mice during days 0-6 restored development to normal. Testosterone injections also reinitiated growth and morphogenesis in developmentally retarded bulbo-urethral glands from 6-day-old neonatally castrated mice.

The prostrate gland may also play a role in the Bruce effect. The dorsal and ventral prostates are sex accessory glands bilaterally located directly beneath the urinary bladder, at the anterior end of the urethra (Figure 1). The dorsal prostate has many ducts, some entering the urethra lateral to all other ducts. The ventral prostate has several ducts entering the ventral walls of the urethra. Similar to the bulbo-urethral glands, the prostate is partially dependent on androgens for growth. However, in contrast to the bulbo-urethral glands, neonatal castration reduces but does not abolish epithelial morphogenesis of the prostrate, whereas in the neonatal bulbo-urethral gland, androgen deprivation completely abolishes epithelial morphogenesis (Cooke, Young & Cunha, 1987).

Although sex-accessory glands are potential candidates for subserving the Bruce effect, it has not been proven that they are the source of the causitive agent involved. Perhaps glands are not involved and males are producing a substance via another mechanism and excreting it through their skin or sweat glands. This excretia may have certain chemical properties which disrupts

pregnancy when absorbed or ingested by newly inseminated females. Prior work in this laboratory demonstrates that males housed above newly inseminated females disrupt pregnancy, whereas males housed below do not (deCatanzaro, Zacharias & Muir, 1996). It is plausible that the non-volatile component involved in the disruption of pregnancy contains estrogens and/or androgens. As this excretion is absorbed into or ingested by the female, her pregnancy becomes disrupted. The intrauterine implantation period is very sensitive to increased androgen and estrogen concentrations (deCatanzaro, MacNiven & Ricciuti, 1991). These hormonal excretions may be secreted via the skin of the strange male. Alternatively, these estrogenic and/or androgenic compounds may be bound to the major urinary proteins which are being excreted by the male in his urine. Marchlewska-Koj (1977, 1981) reports that urinary proteins salted out of male urine could disrupt pregnancy. Although there are several methodological concerns with this study (see deCatanzaro & MacNiven, 1992), it does raise the issue of urinary proteins being involved in the mediation of the Bruce effect. It is noteworthy, however, that proteins are large molecules in comparison to steroid hormones, such as androgens and estrogens. It may be that these large proteins are the carriers of compounds which are mediating the effect and not the source of the mediation. DeCatanzaro *et al.* (1995), report that androgens are involved in the capacity of strange males to induce early pregnancy disruption. Castration eliminates the

effect, whereas testosterone administration restores it. Thus, it may be that excretions of androgens and estrogens by novel males help to mediate the Bruce effect.

Aside from the pregnancy-disrupting influence of exogenous estrogens and androgens, evidence suggests that endogenous androgens and estrogens may be mediating stress-induced early pregnancy disruptions (deCatanzaro & MacNiven, 1992; deCatanzaro *et al.*, 1994). DeCatanzaro *et al.* (1991) report that 17β-estradiol can reliably disrupt early pregnancy at a dose that is one thousandth that of DHEA and one five-thousandth that of androstenedione required to disrupt pregnancy with the same efficacy. As well, radioimmunoassay in restraint stressed rats, who were in the implantation period of pregnancy, revealed a significant increase in 17β-estradiol compared to unstressed controls (MacNiven, deCatanzaro & Younglai, 1992). A subsequent experiment revealed that injections of monoclonal antibodies specific to 17β-estradiol, given daily to restraint stressed mice in the implantation period, significantly increased the capacity for them to bear litters, compared to restrained controls who were not given the antibody (deCatanzaro *et al.*, 1994). These findings support the hypothesis that estrogens, endogenous to the female may be mediating stress induced early pregnancy disruption.

It still remains to be shown, however, if similar mechanisms are mediating pregnancy disruption in the Bruce effect as in other stress-related paradigms. A

variety of environmental stimuli such as extreme temperature, physical restraint, overcrowding and predator exposure are able to disrupt pregnancy in female mammals (deCatanzaro & MacNiven, 1992). These stimuli have been shown to elicit physiological responses characteristic of stress. The stress response has been studied and more specifically, the increase in HPA activity has been investigated. Does strange male exposure elicit an increased firing of the HPA, thereby stimulating an increased steroidal output of the adrenal glands? If so, do these steroids produced by the adrenal glands under stress mediate pregnancy disruption? Harper (1967, 1969) reported that administration of adrenal steroids, namely androstenedione and DHEA, disrupts early pregnancy. DeCatanzaro *et al.* (1991) report similar results when C57- and HS-strain mice were injected with these steroids at 500μg per day. It is important to note that androstenedione and DHEA are precursors to estrogens. Harper suggested that these steroids may be metabolized to estrogens and that estrogens mediate early pregnancy disruptions. According to these findings, it is reasonable to hypothesize that strange male exposure may stimulate the HPA, thereby increasing the output of certain steroids which are metabolizing to estrogens. These endogenous estrogens may be the causative agents involved in the mediation of strange male induced pregnancy disruption. However, additional research is required in this area before such a mechanism can be proven.

Alternative notions have been proposed in explanation of the Bruce effect. Bellringer, Pratt and Keverne (1980) state that the Bruce effect might be subserved in the female by pheromone-altered prolactin concentrations. Rosser, Remfry and Keverne (1989) present further evidence which indicates that exposing mice to primer pheromones coincides with a surge of prolactin levels and this leads to pregnancy disruption by changing hypothalamic dopamine release. A study by Drori and Folman (1964) suggests that the kidneys may be involved. They reported that cohabitation of male rats with sexually receptive females affects the size of the males' testes, several accessory reproductive organs and kidneys. More specifically, they show that the males raised with receptive females had larger coagulating glands, testes and kidneys than did unmated males. These and other findings must be examined in order to better understand a possible mediating mechanism of the Bruce effect.

It has long been held that the strange male induced pregnancy disruption in mice is under the mediation of chemical cues of the strange male (Parkes & Bruce, 1962). This study was designed to remove the source of these chemical cues by removing the preputial glands, the coagulating and the vesicular glands from the strange male. This was a reasonable attempt at localizing the pheromone source as many report that pheromones coming from the preputial and vesicular-coagulating glands have a significant effect on the behavior of

mice (McKinney & Christian, 1970; Noble & Collip, 1941; Orsulak & Gawienowski, 1972; Bronson & Caroom, 1971; Haug, 1971 as cited in Jones & Nowell, 1973; Jones & Nowell, 1973). The androgen-dependent nature of these glands made them likely candidates for subserving this effect. However, the findings presented in this thesis clearly demonstrate the irrelevance of the preputial, vesicular and coagulating glands in the mediation of the Bruce effect. Additional research is needed in order to discover the pheromone source or the possible non-pheromonal mechanism of strange male induced pregnancy disruption in mice.

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