THE SIRE PROTECTION EFFECT

SIRE BEHAVIOUR IN THE CONTEXT OF NOVEL MALES: THE SIRE PROTECTION EFFECT

By

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Abstract

Female mammals are known to exhibit pregnancy failure when exposed to various stimuli during the implantation phase of pregnancy. When newly inseminated female mice are exposed to novel males in the absence of the sire, implantation is disrupted. This phenomenon is known as the Bruce effect. When females are exposed to novel males in the presence of the sire, pregnancy failure does not occur. This latter effect has been referred to as the *sire protection effect.* In these studies we examine the nature of female and sire behaviour in the context of novel males. Female interactions with novel males significantly decrease in the presence of the sire. This reduction in female-novel male interaction in seen irrespective of whether sires are free or confined within the female's cage. Novel-male exposed females exhibit pregnancy failure and this pregnancy block is removed when either free of corral-confined sires remain present in the cage. Finally, sires are highly motivated to engage in aggressive conflicts with novel males both in the presence and absence of the pregnant female. Sires were observed to behave aggressively towards novel males through a wire-mesh grid and in this context were able to inflict severe wounding upon novel males through the wire-mesh partition. In a direct exposure paradigm, sires were also witnessed to exhibit this aggression and were found to initiate and win all conflicts with novel males.

The sire protection affect is likely to involve a complex of both pheromonal and behavioural cues. Sire aggression towards novel males, pheromonal communication and limited behavioural interaction between the sire and the female, and reduced female-novel male interaction are all likely components of the effect.

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INTRODUCTION

Pregnancy Disruption

Pregnancy in female mammals can be affected by a variety of psychophysiological events. Exposure to certain environmental stimuli can disrupt specific neuroendocrine events that occur during normal reproduction (deCatanzaro and MacNiven, 1992). For example, pregnancy in female rodents can be affected by various environmental stimuli including high ambient temperatures (Hsu, 1948), human handling (Runner, 1959), chronic physical restraint (deCatanzaro et al., 1994; Weibold et al., 1986), exposure to predators (deCatanzaro, 1988 and 1985), and various environmental and social changes (Bronson, Eleftheriou, & Garick, 1964). These stimuli are believed to affect the homeostatic state of an organism and are often referred to as *stressors* (Hinkle, 1977).

The period of pregnancy that is the most sensitive is the *implantation* phase of pregnancy and occurs when the fertilized ova implant into the uterine walls. Females may exhibit increased sensitivity to various environmental stimuli at this time (Liptrap 1993). Such stimuli can directly or indirectly cause minute changes in the hormonal dynamics of a pregnant female and can lead to the failure of successful implantation.

Bruce (1960) discovered that when a newly inseminated female mouse is removed from the stud or sire male and placed in the context of novel males, implantation is disrupted and pregnancy is terminated. The female mouse then returns to estrus (Bruce, 1960a). This phenomenon, known as the *Bruce Effect*, is thought to be mediated

through pheromonal and behavioral interactions that trigger a stimulus-specific neuroendocrine response leading to implantation failure.

The Bruce Effect

The Role of Pheromonal Communication in the Bruce Effect

Several findings suggest that the Bruce effect is mediated by pheromonal transmission between novel males and inseminated females. When inseminated females are exposed to cages that have been sufficiently soiled by novel males and in which air exchange is minimized, pregnancy is terminated (Parkes & Bruce, 1962; deCatanzaro et al., 1999). It has also been found that inseminated females prefer the soiled bedding of stud males over that of novel males (Drickamer, 1989). Although indirect contact such as this can lead to pregnancy disruption, direct exposure between novel males and females does increase the effect size considerably (deCatanzaro et al., 1995; deCatanzaro et al., 1996). Bruce (1962) was unable to establish a Bruce effect when inseminated females were exposed to soiled cages recently vacated by novel males. However, a sufficient pregnancy block was established when females were housed in glass jars, with restricted ventilation, on cloth bedding that was highly retentive of animal odours, and when the soiled container was renewed twice daily for a 3-day exposure period (Parkes & Bruce, 1962). Such a pregnancy blocking effect required that as many as five novel males of an alien strain soiled the bedding. Such a high degree of soiling is far smaller than would be present in the case of a single novel male housed directly with an inseminated female.

These findings lend support to the theory that the Bruce effect is mediated by a complex of stimuli, novel male-originating pheromonal cues being only one of them.

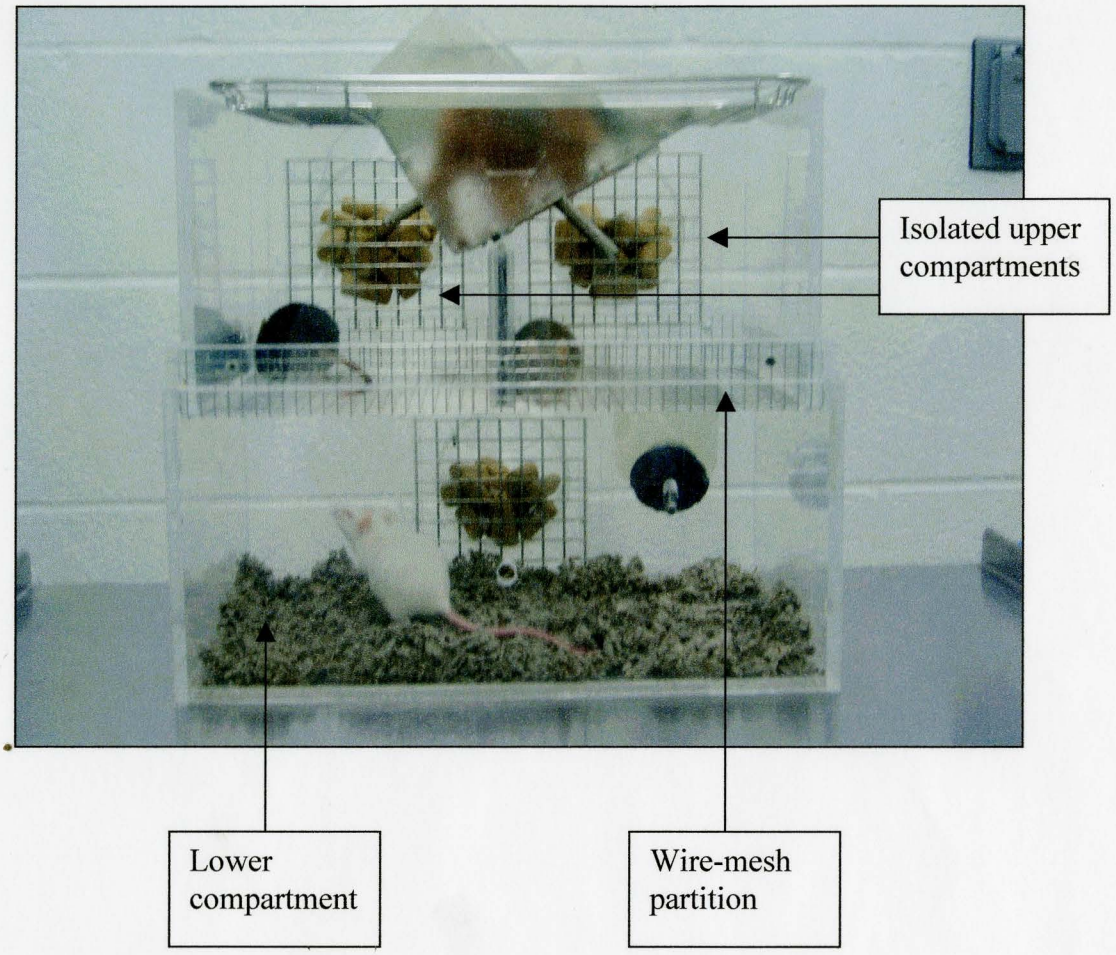
Studies by deCatanzaro at al. (1995) lend further support the theory that pheromonal communication alone might not be sufficient to induce the Bruce effect. The first of these studies showed that only direct placement of a novel male with an inseminated female caused a pregnancy block, whereas exposing the female to large amounts of novel male urine, either by injecting it into the female's bedding or by dripping it down through a funnel, had no apparent effect on pregnancy outcome. Other experiments have shown no substantial effect of urine painted directly on the female's nasal area. Based on these studies deCatanzaro et al. (1995) concluded that interactions of contact and odour cues are necessary for the Bruce effect to occur.

DeCatanzaro and Storey (1989) found that novel male sexual activity plays a role in the Bruce effect. When novel males and females are housed together, males either re-inseminate the female or simply terminate the existing pregnancy. Testosterone-treated castrates also engage in sexually motivated behaviours towards inseminated females and are able to induce a substantial pregnancy block. Not only is sexual activity correlated with pregnancy outcome, but novel male intromissions in particular are correlated with pregnancy disruption (deCatanzaro & Storey, 1989; Storey & Snow, 1990). These findings provide evidence that direct contact between novel males and females is an important component of the Bruce effect. It is well known that vaginal stimulation in rodents causes neuroendocrine changes that influence female physiology and induce

estrus (Komisaruk & Steinman, 1986); and it may be that sexual activity after insemination is incompatible with pregnancy maintenance.

The Bruce effect has been studied using a *double-decker apparatus* in which novel males are housed above an inseminated female (Figure 1). The upper and lower compartments of the apparatus are separated using a wire-mesh grid so that novel male excretions can fall below onto the female. The grid also serves to minimize behavioural interactions between the novel males and females although the animals can make some contact with one another through the square openings in the wire-mesh. Using this paradigm, it has been found that pregnancy is more reliably disrupted with the presence of an increasing number of novel males. The least number of pregnancy disruptions is seen when only one novel male is housed in the upper chamber of the apparatus. When two novel males are used, a significant Bruce effect can be reliably produced. These results indicate that the effect is quantitative; the greater the amount of novel male originating pheromone, the larger the proportion of females that lose their pregnancies (deCatanzaro, Zacharias, & Muir, 1996).

Figure 1: Double-decker Apparatus



The spatial configuration of the novel males with respect to inseminated females is also important. When novel males are housed below the inseminated female as opposed to above her, the Bruce effect is significantly diminished (deCatanzaro, Zacharias, & Muir, 1996). This suggests that the pheromone involved is non-volatile.

Many studies have been conducted with the aim of better understanding how pheromonal cues are transmitted at the anatomical level in the inseminated female. When the olfactory bulbs (Bruce & Parrot, 1960) or vomeronasal organs (Bellringer et al., 1980; Lloyd-Thomas & Keverne, 1982) of inseminated females are lesioned, the Bruce effect can not be established. Similarly, if the inseminated female's olfactory tract is lesioned, pregnancy is no longer disrupted (Rajendren & Dominic, 1986). These results indicate that the pheromones involved in the Bruce effect may be transmitted via olfactory pathways.

A significant albeit small Bruce effect can be established if the urine of novel males is painted directly on the nose of inseminated females, however the urine collected must be from novel males that have been housed in the context of females (deCatanzaro et al., 1999). Urine collected from isolated novel males does not elicit a Bruce effect (Bruce & Parkes, 1962). This suggests that males secrete a specific substance when they are housed in the context of females and that this substance is involved in mediating the Bruce effect.

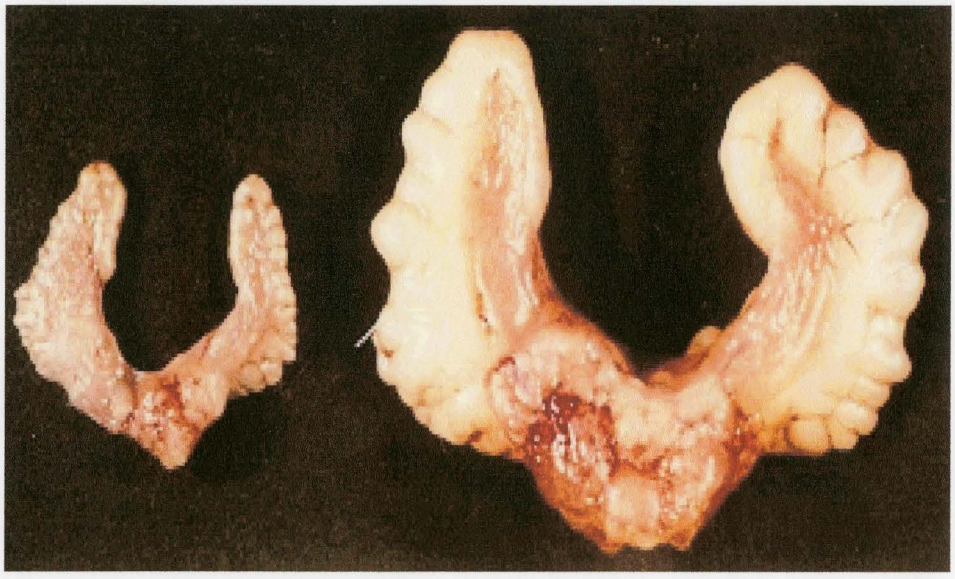
The Role of Androgens in the Bruce Effect

The Bruce effect is dependent upon androgen concentration in novel males. Juvenile males and castrated males can not disrupt pregnancy. When castrates are administered exogenous testosterone, their ability to disrupt pregnancy is restored (deCatanzaro et al., 1995; deCatanzaro & Storey, 1989; and Dominic, 1965).

The role of male accessory glands in the context of the Bruce effect has been well documented (deCatanzaro et al., 1996; deCatanzaro et al., 2000). The structural and function of the male accessory glands are dependent upon testicular androgens (University of Wyoming, 2003) (Figure 2) . The preputial glands are subcutaneous glands that are situated between the skin and the body wall anterior to the external genitalia (Figure 3). These degree of preputial gland activity in males is thought to be related to social experience and specifically to aggression (Hucklebridge et al., 1972). It has been suggested that these glands affect fighting behaviour through the release of an aggression-promoting pheromone (McKinney & Christian, 1970; Mugford & Nowell, 1970; Jones & Nowell, 1973). These glands are also thought to regulate anogenital licking (Brouette-Lahou et al., 1991) and in mice and other rodents, secretions from these glands are thought to be attractive to animals of the opposite sex (Bronson & Caroom, 1971). Despite the role of the preputial glands in such behaviours, the removal of these glands in novel males placed in the context of inseminated females does not inhibit the Bruce effect (deCatanzaro et al., 1996). DeCatanzaro et al. (1996) compared preputialectomized novel males to sham treated and controls in a direct exposure

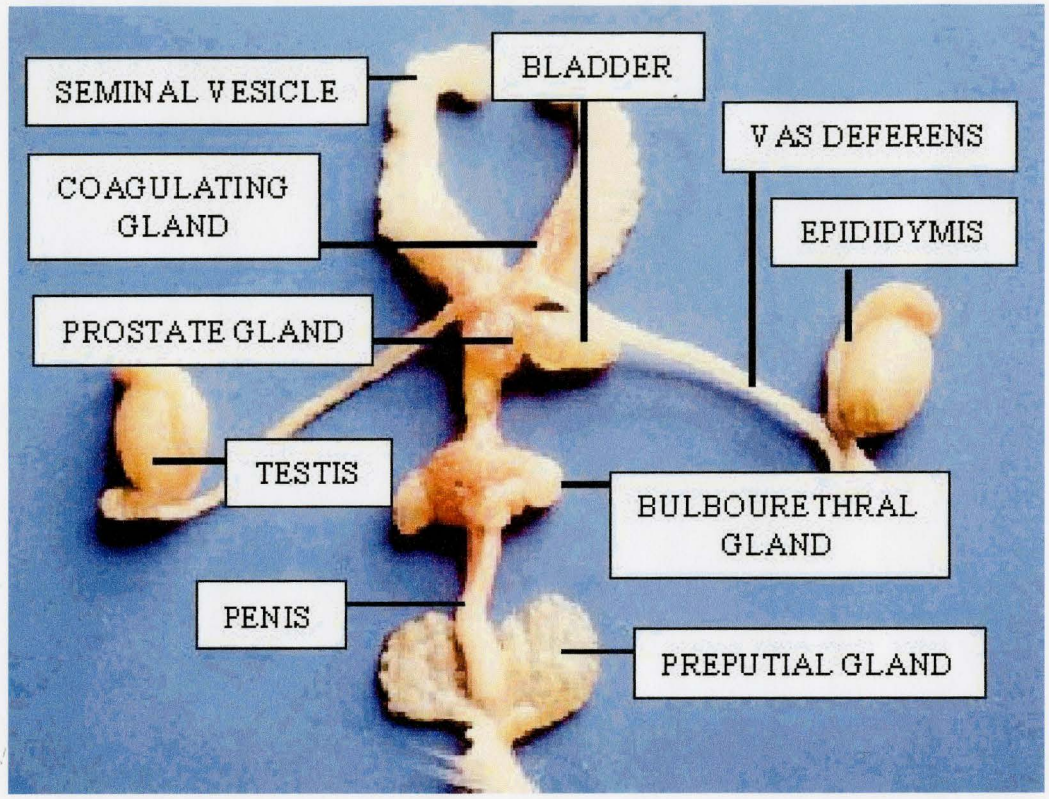
paradigm and found that males did not differ across conditions in their ability to induce pregnancy block. Thus the preputial glands are not necessary for the Bruce effect.

Figure 2: Seminal vesicles of castrated and sham-operated male rats



The effects of castration (left) on rat seminal vesicles. Glands for sham-operated control shown on right.

Figure 3: Reproductive Tract of the Male Mouse



In similar studies, the possible role of the vesicular-coagulating glands was examined. The vesicular-coagulating gland complexes are the largest androgen-dependent male accessory glands in mice and are located bilaterally on the urethral tract anterior to the testes. The vesicular glands contain the milky white substance that produces the copulatory plug inserted into the vagina immediately following insemination (Cukeirski et al., 1991; Hart & Greenstein, 1968). The size of these glands increases linearly under the influence of testosterone (Mann, 1947; 1950). A modulating role for pheromonal activity of these glands has also been suggested for intermale aggression (Jones and Nowell, 1973). DeCatanzaro et al. (2000) examined the possible role of these glands alone and in conjunction with the perputial glands in the context of the Bruce effect. They found that bilateral ablation of the vesicular coagulating glands, like that of the preputial glands does not interfere with the capacity for novel males to induce implantation failure in newly inseminated females. Furthermore, an either/or interaction of both of these glands is also not responsible for the Bruce effect. Removal of all six glands (bilateral excision of preputial, vesicular, and coagulating glands) disrupted pregnancy to the same degree as that found using sham-treated and control males. Castrated males however, with all six accessory glands removed did not significantly disrupt pregnancy where as those with chronic subcutaneous testosterone implants did induce a pregnancy block. This latter finding provides further support that the Bruce effect is largely dependent on androgens and or its metabolites. It may be that either another androgen-dependent excretory mechanism or a more direct action of these hormones is responsible for the Bruce effect.

Sexual satiety has also been examined in the context of the Bruce effect and it has been found to diminish the capacity of novel males to disrupt early pregnancy in females (Spironello & deCatanzaro, 1999). It is thought that this may be due to both pheromonal and behavioural changes in sexually sated males. These males may exhibit decreased testosterone levels in their excretions and may be less likely to engage in sexually motivated behaviour following sexual activity.

The nature of Pregnancy Outcome in the Context of the Bruce Effect

In all Bruce effect studies, the effect has been found to be both probabalistic and all or none. Some novel male-exposed females are almost always parturient. Furthermore, females are either parturient or not parturient; the number of pups per litter does not vary as a function of condition (deCatanzaro et al., 1996; Spironello Vella & deCatanzaro, 2001).

Hormonal Dynamics and the Bruce effect

Various hypotheses have been offered in the attempt to understand what actually causes implantation failure in females exposed to novel males. It has been debated whether novel males are similar to or differ from other stressors and whether implantation failure as a result of proximity to novel males is induced through the same physiological mechanisms as pregnancy disruption resulting from stress. For example, it has been argued that since novel males are likely to kill pups that they have not sired, it may be in the female's best reproductive interest to terminate pregnancy and return to

estrus (deCatanzaro, 1985). Conversely, others have suggested that this pregnancy block in only one example of a larger phenomenon in which females terminate pregnancy in the presence of various adverse stimuli including human handling (Runner, 1959), chronic physical restraint (deCatanzaro et al., 1994; Weibold et al., 1986), high ambient temperatures (Hsu, 1948), and predation (deCatanzaro, 1988).

Psychologically and physiologically demanding stimuli can elicit the stereotyped stress reaction in which the hypothalamic-pituitary-adrenal axis is activated. Traditionally, it had been believed that adrenal steroids released under stressful conditions are responsible for stress-induced pregnancy blocks. Adrenocorticotrophic hormone (ACTH) acts on the adrenal cortex to cause the release of corticosteriods. Administration of ACTH during pregnancy has been found to disrupt implantation of fertilized ova, reduce litter size, cause spontaneous abortion, and lead to fetal resorption (Robson & Sharaf, 1952; Veladro, 1957; Yang et al., 1969; Kittenger et al., 1980; deCatanzaro et al., 1986). Administering glucocorticoids to females has also been found to inhibit uterine growth and preparation for implantation (Szego, 1952; Velardo et al., 1956; Bitman & Cecil, 1967). It is not exactly clear whether major adrenal glucocorticoids such as cortisol and corticosterone directly inhibit pregnancy or whether adrenal activity directly mediates pregnancy block that is induced by psychological stress. Investigations in this area have produced varying opposing results (Syndar & Target, 1967; Sahu & Dominic, 1981).

In at attempt to better understand the physiological mediation of stress-induced pregnancy block as well as to understand the Bruce effect in relation to stress-induced

pregnancy block, various studies have been conducted that examine adrenal and ovarian steroids in relation to pregnancy. Most of these investigations have been founded on the fact that exogenous ACTH can result in an increase in serum estrogen levels (Arai et al., 1972; Strott et al., 1975) and that endogenous estrogen may rise during pregnancy in response to stressful conditions (MacNiven & deCatanzaro, 1990).

One study has compared the influences of corticosterone, androstenedione, dehydroepiandosterone, and estradiol on the course of pregnancy after insemination (deCatanzaro et al., 1991). The results from this experiment showed that 17β-estradiol had the most adverse effect on pregnancy, followed by dehydroepiandosterone, and then by androstenedione. A comparatively small amount of 17β-estradiol (0.333 μg injected once daily from days 1-6 of pregnancy) produced a complete pregnancy block in all inseminated females. Dehydroepiandosterone was second in potency, although only some and not all females experienced implantation failure. In order to get a significant block in pregnancy with androstenedione, a much higher dose had to be administered. Estradiol produced a pregnancy block at one thousandth of the lowest dose of dehydroepiandosterone and at one five-thousandth of the minimal effective dose of androstenedione. In contrast, corticosterone did not produce a significant pregnancy block when compared to vehicle-treated controls.

Assay studies on inseminated female rats with and without chronic restraint stress have examined endogenous corticosterone, progesterone, and 17β-estradiol levels. These studies showed that concentrations of all three steroids were significantly higher than normal in restraint stressed animals around the time of intrauterine implantation of

fertilized ova (MacNiven & deCatanzaro, 1990). Concurrent injection of 17β-estradiol antibodies has been found to partially prevent implantation failure in restraint stressed rats and mice (MacNiven et al., 1990; deCatanzaro et al., 1993). This reinstatement of pregnancy with the administration of estrogen antibodies may not be complete because endogenous progesterone levels can also be effected by stress. Although the estrogen antibodies serve to decrease the amount of available estrogen, they can not restore the critical ratio of estrogen and progesterone necessary for implantation (deCatanzaro et al., 1993).

Taken together, these results suggest that estrogens may be involved in mediating stress-induced pregnancy block. It may be that an increase in androgens and estrogens (which are metabolites of androgens) from either or both the adrenals and the ovaries provide the physiological mechanism through which pregnancy is disrupted in stress-induced pregnancy conditions (deCatanzaro et al., 1993). Harper (1967a; 1967b; 1969) found that dehydroepiandrosterone and androstenedione have deleterious effects on pregnancy. He suggested (1969) that the conversion of these steroids to estrogens may be responsible for their pregnancy-blocking effects. This suggestion would be consistent with our findings since only a fraction of dehydroepiandrosterone and androstenedione would need to be converted to estrogens to account for the pregnancy disruptions seen in these studies. Since small doses of estrogens have dramatic adverse effects on pregnancy, it is possible that only a small increase in natural estrogen levels by psychological and physiological stressors could account for the negative effects of these stimuli on early pregnancy (deCatanzaro et al., 1991).

Various important studies have been conducted examining the physiological mechanisms related to pregnancy disruption in the Bruce effect specifically. DeCatanzaro et al. (1995) administered various dosages of estrogen antibodies to newly inseminated females that were exposed to novel males through an indirect exposure paradigm. The results of their experiment showed that male-exposed females that were given a daily injection of 1 ml of the antibody had pregnancy outcomes comparable to controls. They showed that the administration of estrogen antibodies to inseminated females in the context of the Bruce effect was at least partially effective in protecting against implantation failure. These results are similar to those discussed earlier in which restraint stressed females were given estrogen antibodies (deCatanzaro et al., 1993; MacNiven et al., 1990).

Hormone profiles of the urine and feces of novel males and of inseminated females housed in proximity to these males have been collected using enzyme linked immunosorbent assay (ELISA) (Muir et al., 2001). An elevation of urinary testosterone was seen in females that failed to be parturient following exposure to novel males. An elevation of 17β-estradiol was seen in the urine of novel males.

In another related study, the time course of males' ability to induce pregnancy block following castration was examined and this was related to the concentration of urinary excretion of testosterone and 17β-estradiol (Vella & deCatanzaro, 2001). As was expected, more male-exposed females remained parturient with increasing time since novel male castration prior to exposure to the females. Following castration, novel males lose their ability to induce a pregnancy block over a six week period. This finding is

consistent with the gradual loss of sexually motivated androgen-dependent functions in castrated male rodents (Beach and Holz-Tucker, 1949; Whalen et al., 1961). 17β-estradiol levels were also found to decrease gradually following castration. Together, these results are especially interesting since they indicate that novel males show elevated estradiol levels and that estradiol levels are higher in male that are able to disrupt pregnancy than in males that are unable to do so.

Since it has been clearly established that pheromonal communication is involved in the complex of necessary stimuli that induce the Bruce effect, it has been hypothesized that estradiol, known for its pregnancy blocking effects in stressed females, may in fact be a component of the pheromonal stimuli involved in novel male-induced pregnancy block. In Bruce effect studies, it has been noted that while females tend to urinate in single puddles, males episodically spray small quantities of urine (Muir et al., 2001). Males actively target and spray their urine onto inseminated females (deCatanzaro et al., 1996; Drickamer, 1995; Reynolds, 1971; Vella & deCatanzaro, 2001) .

Based on this finding, a study was conducted to examine whether testosterone and estradiol painted directly on the nose of inseminated females could cause a sufficient pregnancy block (deCatanzaro et al., 2001). Nasal applications of minute quantities of 17β-estradiol during the first five days post-insemination reliably terminated pregnancy in inseminated females. In comparison, relatively high doses of testosterone applied to the nasal area had no adverse effects on pregnancy. When administered via subcutaneous injection, both estrogens and androgens cause implantation failure although estradiol does so at much smaller doses.

It may be that small doses of estrogens found in novel male urine are transmitted to females and that this contributes to the disruption of intrauterine implantation of fertilized ova. As described earlier, increased plasma estradiol levels are known to have deleterious effects on implantation including decreased rate of travel of the fertilized ova down the fallopian tubes and lysis of the corpus luteam (Whitney & Burdick, 1936; Burdick & Whitney, 1937; deCatanzaro et al., 1991). Together the data discussed here suggest that stress-induced pregnancy block as well as the Bruce effect may be mediated by elevated estrogen levels in inseminated females. In the case of the Bruce effect, it is possible that novel male urine may in part be a source of this additional estrogen. For example, it has been suggested that estrogens from novel male urine could potentially enter a female's circulation via the exposed vasculature of the nasal cavity (deCatanzaro et al., 2001).

There are competing hypotheses as to the exact nature of the Bruce effect and as to the physiological mechanism involved in the termination of pregnancy due to proximity of novel males. Another theory deals with olfactory memory and the prolactin levels in inseminated females.

The Olfactory Memory Hypothesis

The olfactory memory hypothesis argues that the female mouse creates an olfactory imprint of her coital partner and that this memory influences her bias response towards the stud male over novel males after insemination. It is thought that exposure to novel males after insemination suppresses the olfactory imprints of the sire (Lloyd-

Thomas & Keverne, 1982). Other studies have shown, however; that novel males may activate a different subset of olfactory neurons, triggering a cascade of neuroendocrine events in the female that result in implantation failure (Kaba et al., 1994). The Bruce effect has been examined to investigate the formation of olfactory memories and to study long term-potentiation (LTP) of the synapses between granule and mitral cells of the accessory olfactory bulb (Kaba et al., 1994; Brennan et al., 1990; Brennan et al., 1995). Ablation studies in which the olfactory bulbs (Bruce & Parrot, 1960), or vomeronasal organs (Bellringer et al., 1980; Lloyd-Thomas & Keverne, 1982), or olfactory tract (Rajendren & Dominic, 1986) of inseminated females are lesioned, lead to inhibition of the Bruce effect. These studies have been interpreted as evidence that if an olfactory imprint of the sire is not formed, the Bruce effect can not be induced.

Pheromones activate olfactory receptor neurons at the vomeronasal epithelium which converge in a specific pattern of glomeruli. Glomerular outputs diverge to the mitral cells of the accessory olfactory bulb which project sub-cortically to the amygdala and the hypothalamus (Dominic, 1996; Breer et al., 1994; Shepard, 1994). Dopamine is released here causing an inhibitory effect on prolactin production. Dominic (1996) has hypothesized that implantation failure occurs due to this decrease in circulating prolactin. Prolactin has luteotrophic effects and when the concentration of this hormone decreases, the corpus luteum fails to develop and function normally. Prolactin also promotes progesterone production in the corpus luteum which is vital to pregnancy maintenance. While progesterone levels are influenced by prolactin; estrogen levels influence the concentration of prolactin. High levels of circulating estrogen cause an increase in

dopamine which acts to decrease prolactin concentrations. Conversely, low serum estrogen levels are known to stimulate prolactin release (Marieb, 1989).

In summary, this competing hypothesis on the Bruce effect is based on the formation of an olfactory memory of the sire and the subsequent exposure to olfactory cues that differ from the ones originally used to form the imprint. This disparity between the olfactory cues emitted by the sire versus the novel male is thought to lead to a cascade of physiological events that ultimately result in the decrease in prolactin levels in the female and the subsequent failure of implantation.

Those who do not favour this hypothesis have argued that mice are too primitive to have sophisticated olfactory recognition abilities that could allow them to recognize other males as individuals with unique odours. The olfactory memory hypothesis requires that female mice be able to identify the difference between sire and non-sire odours. The competing hypothesis in which novel males provide a stimulus which signals potential danger and causes the female to experience stress does not require such a sophisticated olfactory pathway.

The Role of the Sire in the Bruce Effect: *The Sire Protection Effect*

Most studies on the Bruce effect have focussed on understanding the role of novel males in pregnancy disruption and on identifying the novel-male originating pheromone. Few studies have focussed on the role of the sire in the effect. When the Bruce effect is studied in a laboratory setting, the female is usually removed from the sire upon detection of a sperm plug (indicating successful insemination) and then placed in the context of

novel males in various conditions. The sire is needed for insemination only and is not used again in the experiment.

The role of the sire in the context of the Bruce effect was first examined by Parkes and Bruce (1961). They reported that the presence of the stud male with the female during exposure to novel males largely eliminated pregnancy disruption. In this condition, they reported that only eight of 40 females returned to estrus. They also found that when females were isolated upon sperm plug detection and then reintroduced to sire males 24 hours later, pregnancies were not disrupted. Parkes and Bruce interpreted their findings to suggest that females are able to "recognize" stud male odours for up to 24 hours post coitum and differentiate between stud odours and those of novel males. These studies did not include behavioural observations and focussed only on pregnancy outcome to determine the role of the sires, novel males, and females in the Bruce Effect (Parkes & Bruce, 1961).

In the 1980's, Dominic and Thomas began to revisit the role of the sire in pregnancy maintenance and specifically in the Bruce effect. They were interested in identifying a stud-originating stimulus that could afford protection to an inseminated female's pregnancy. To achieve this, they designed an experiment in which inseminated females in different experimental conditions could be differentially exposed to various stud-originating stimuli (Thomas and Dominic, 1987) (Figure 4). In the first condition, the inseminated female was housed in a cage with the free stud male. A novel male was confined above the two animals in a wire-mesh corral. In this condition the stud offered the female body contact, audio-visual signals, and non-volatile and volatile pheromones.

In the second condition, the stud and novel males were individually housed in wire-mesh corrals both placed above the inseminated female's cage. In this condition the female was exposed to the stud's audio-visual signals and non-volatile pheromones only. In the third group, the female was placed in the lower compartment of the cage (as in the previous groups) although in this condition, the cage was divided into smaller and larger chambers and the female was confined to the smaller one. The novel male was housed in a wire-mesh corral above this small chamber. The sire was placed in the large chamber of the lower compartment of the cage, adjacent to the female and separated from her by a wire-mesh partition. In this condition, the inseminated female was exposed to the stud's audio-visual signals and volatile pheromones only. The fourth condition served as a control condition and consisted only of the inseminated female with the novel male housed above her in a wire-mesh corral. This group served as the standard Bruce effect group and in this condition, the female was not exposed to any stimuli originating from the sire. Finally, the fifth group consisted of an isolated inseminated female only and served as a double-control in which a spontaneous abortion rate could be determined.

The first group in which the stud was able to have body contact with the female exhibited a severely diminished pregnancy disruption rate. In this group, 81.6% of the females remained pregnant despite the presence of the novel male. In the second group, 61.1% remained pregnant (indicating a weak Bruce effect). In the third group, and fourth groups respectively, 24.0% and 22.9% of the females remained pregnant. In the fifth group (control group with female only), 90.0% of females remained pregnant. Based on these results, Thomas and Dominic (1987) concluded that bodily contact between the

stud male and the inseminated female is the stud male-originating cue that affords protection to the inseminated female's pregnancy during the implantation phase of her pregnancy. They further argued that bodily contact and stud male pheromonal stimulation (through the presence of non-volatile pheromones present in the stud's excretions) act synergistically to inhibit the Bruce effect.

Figure 4:

Data Collected from Thomas and Dominic (1987):
Effect of the presence of the stud male on the Bruce effect

Group	Stimuli presented by the stud male	Proportion of females returning to estrus (%)	Proportion of females remaining pregnant (%)
1	Body contact, audio-visual signals, non-volatile and volatile pheromones	12.2	81.6
2	Audio-visual signals and non volatile and volatile pheromones	38.8	61.1
3	Audio-visual signals and volatile pheromones	72.0	24.0
4	No stimuli from stud	77.1	22.9
5	Untreated controls	7.5	90.0

Thomas and Dominic (1989) argue that a female makes an olfactory imprint of the odour of the stud male at the time of mating which leads to her differential response to any subsequent male exposure. The fact that inseminated females show an affinity to bedding soiled by the sire as opposed to by novel males during the critical days surrounding implantation has been interpreted as evidence of the female's behavioural tendency to procure protection against pregnancy disruption (Dickamer, 1989; Thomas & Dominic, 1987). In order to determine if mating is necessary for such an olfactory imprint to be established, a study was designed in which another male, coined the *familiar male,* was housed in a wire-mesh corral within the cage with the female and her stud. This familiar male was present during the pericopulatory phase but was not the female's coital partner. Upon successful insemination, the female was removed and placed in a cage with either the free familiar or stud male and a novel male confined in a wire-mesh corral. The results of this study indicated that the familiar male was able to afford some protection to the female's pregnancy. Under these experimental conditions, 53% of females remained pregnant when housed with a free familiar male, while 62% of females housed with free stud males remained pregnant. These findings were interpreted to suggest that the female not only creates an olfactory memory of her coital partner, but rather that she creates an olfactory imprint of *all* odours present during the pericopulatory phase. The presence of these odours in conjunction with the bodily contact of the animal from which the odours originated offer her protection against novel male induced pregnancy block (Kumar & Dominic, 1993). Furthermore, this finding is seen as support for the idea that implantation failure is caused by a suppression of prolactin release and subsequent luteal

failure. The study's results have been interpreted to mean that the presence of the stud male stimulates a luteo-trophic memory in the female that inhibits the luteal failure that would have otherwise led to a disruption in pregnancy.

The Role of the Stud Male in Preventing Stress-Induced Implantation Failure

The role of the stud male in preventing nutritional stress-induced implantation failure in inseminated females has also been examined. It is well-documented that food deprivation has deleterious effects on reproductive function (Leathem, 1966). Reproduction in female mammals is very costly and is closely regulated by the availability of metabolic fuels. When food is scarce reproduction is often interrupted and in many animals the estrous cycle is disrupted (Jones & Lubbers, 2001). For example, when Syrian hamsters are food-deprived for 48 hours on days 1 and 2 of estrus, ovulation and estrous behaviour are inhibited (Morin, 1986). Similarly, female mice that have been nutritionally stressed exhibit abnormal estrous cycles (Gangrade & Dominic, 1985). It has been suggested that food deprivation causes a depression in hypophysial (hypothalamic-pituitary) gonadotropin secretion and results in ovarian atrophy (Leathem, 1961). Inseminated female mice that are deprived of food for 48 hours during the pre-implantation period exhibit implantation failure (McClure, 1959, 1962; Bruce, 1963; Sahu & Dominic, 1985). Gangrade and Dominic (1985) showed that the irregularities in the female estrous cycle that can be induced by nutritional stress can be prevented by the presence of conspecific males.

In an isolated study, the presence of the stud male has been found to prevent implantation failure in nutritionally stressed newly inseminated females. Archunan and Dominic (1989) designed a study in which 11.8 % of females that were food deprived and isolated post-implantation remained pregnant, while 80.5% of the females that were food deprived but housed with a confined stud male during the fasting period remained pregnant. Interestingly, when females were housed with a confined strange conspecific male (not the stud), only 7.9% remained pregnant. One hypothesis is that this implantation failure is primarily caused by the depression of hypophysial prolactin release resulting in luteal failure and that the presence of the stud male may activate a luteotrophic memory in the inseminated female, maintaining normal luteal function (Sahu & Dominic, 1985; Dominic, 1966; Archunan & Dominic, 1989).

Together, Dominic's studies have been interpreted by some as support for the idea that olfactory memory of the sire can aid in the prevention of pregnancy block under Bruce effect conditions and stressful conditions (such as nutritional deprivation) during the implantation phase of pregnancy.

The Role of the Stud Male *after* Novel Male-Induced Implantation Failure

Thomas and Dominic have also investigated the role of the sire *after* novel male-induced pregnancy block (Thomas & Dominic, 1987). In their study, inseminated females were housed with corral-confined novel males to induce pregnancy block, and were then removed and re-introduced to corral-confined stud males. Implantation failure was determined by the existence of cornified vaginal cells found in vaginal smears taken

daily. The results indicated that a majority of pregnancy-blocked females who were re-exposed to stud males on days three to five post-insemination exhibited pseudo-pregnancy like cycles. Pseudo-pregnancy was confirmed though a positive deciduoma reaction after uterine traumatization in these females. In contrast, females who were not re-introduced to stud males and remained with novel males, as well as females who were isolated, did not show the same incidence of pseudo-pregnancy.

The incidence of new corpora lutea that formed in females following novel male-induced pregnancy block (and the resulting estrous cycle) were thought to become functional once again upon re-exposure to the stud male. Furthermore, since the stud males were corral-confined, Thomas and Dominic suggested that pheromonal stimulation originating from stud male excretions was responsible for the induction of pseudo-pregnancy, and that pseudo-pregnancy resulted from the re-commencement of hypophysial prolactin secretion, ultimately leading to the functional development of the corpora lutea. They argued that the same pheromonal cues that influence the female to become pseudo-pregnant following novel male-induced pregnancy block are also responsible for the ability of the sire to inhibit implantation failure in the context of novel males, also referred to as the *sire protection effect.*

It should be noted here that Thomas and Dominic were confident that stud male pheromones were responsible for the females' ensuing pseudo-pregnancy since all males in the experiment were corral-confined and thus could not vaginally stimulate the females (Thomas & Dominic, 1987). This reasoning can however be contested because it has also been reported that the gathering of daily vaginal smears can in fact *cause* pseudo-

pregnancy through vaginal stimulation during the process of taking the smear. This being the case, pseudo-pregnancy found through a smear can not be conclusively attributed to the presence of stud males (deCatanzaro & Storey, 1989; Komisaruk & Steinman, 1986).

The Present Study

Research investigating the role of the sire in the Bruce effect has been primarily based on the olfactory memory hypothesis. Furthermore, previous experimental work on the sire protection effect has not focussed on the behaviour of the sire. Although Thomas and Dominic sought to identify the stud-originating stimulus thought to protect the female against pregnancy block, their experimental design did not include any behavioural observations of the sire. Their results did indeed indicate that a free sire housed with the inseminated female produced a much larger sire protection effect (decreased Bruce effect) than did a confined sire. This finding was interpreted to mean that bodily contact between the sire and the inseminated female was necessary for the sire protection. Although this conclusion was drawn, behavioural observations of either a qualitative or quantitative nature of this bodily contact were not made.

The behaviour of an inseminated *female* in the context of the Bruce effect has been examined in order to determine whether females differentially approach or avoid novel males that can disrupt pregnancy (deCatanzaro & Murji, submitted). Inseminated females were placed in a four arm radial maze and at the end of each arm (behind a wire-mesh grid) were a novel alien male, a novel strange male (conspecific male), the sire, and no stimulus animal. The female was observed to spend the least amount of time

investigating the empty arm and the most amount of time investigating the arm containing he novel alien male that could disrupt her pregnancy. When the number of contacts with each animal in each arm was examined, similar results were found. The female made the least number of contacts with the wire-mesh at the end of the empty arm and the most number of contacts with the wire-mesh between her and the novel alien male.

In another experiment, in order to investigate female behaviour over a longer period of time, deCatanzaro and Murji placed inseminated females in the lower compartment of a cage above which were four separate compartments each housing a novel alien male, a novel strange male, the sire, and no stimulus animal respectively. The upper and lower compartments were separated by a wire-mesh grid. In this spatial configuration, female contacts with each of the four upper compartments as well as preferred nesting site were observed. No significant differences were found between the amounts of time females spent interacting with each of the four quadrants above, nor when comparing nesting sites below the quadrants.

DeCatanzaro and Murji's findings were unexpected based on Drickamer's (1989) previous findings that inseminated females prefer the soiled bedding of sires over that of novel males during the early stages of pregnancy. Thomas and Dominic (1987) had hypothesized that females prefer bedding soiled by studs over novel males as a means of gaining protection from pregnancy block. DeCatanzaro and Murji's findings indicate that females actually seek out interactions with novel alien males rather than seek protection from alien male-induced pregnancy block.

The present study is designed to examine stud behaviour in the context of the Bruce effect. Since it has been established that females interact with rather that avoid novel males, this study sought to examine whether the sire protection effect is actually driven by stud male behaviour, and specifically by stud male intervention between novel male and female interaction. This hypothesis does not rest on the notion of differential female olfactory recognition, but rather suggests that the pregnancy protection observed through the sire protection effect is driven directly by stud male behaviour. The Bruce effect is thought to be caused by a complex of stimuli including both pheromones and behaviour. If stud males are able to intervene between novel males and females they may in effect be reducing contact between these animals, and thus reducing a stimulus that is necessary for the Bruce effect.

In Experiments 1 and 2, stud male behaviour was examined using an indirect exposure paradigm, while in Experiment 3, this behaviour was examined using a direct exposure method. The indirect methods allowed us to examine behavioural interactions between the animals across a wire-mesh grid and to measure pregnancy outcome in various experimental conditions. The indirect method was used in the final experiment as a means of investigating sire aggression in the context of the Bruce effect. From an evolutionary perspective, we can expect to see increased aggression from sires in this context since they would be highly motivated to protect their reproductive investment from novel males with the capacity to block pregnancy.

EXPERIMENT 1

This experiment was designed to establish a sire protection effect in this laboratory. Inseminated females were exposed to novel males under two experimental conditions; *sire-present* and *sire-absent* conditions. We hypothesized that females in the sire-present condition would remain pregnant, while females in the sire-absent condition would exhibit implantation failure due to the Bruce effect. In order to examine both sire and female behaviour, we observed each animal's behaviour for a 15 minute interval between days 3-6 of the inseminated females pregnancy. We hypothesized that in the *sire-present* condition, the sire would intervene between the novel males and inseminated female. We further hypothesized that in this condition, the number of interactions between the females and the novel males would decrease when compared to the *sire-absent* condition.

METHODS

Subjects

HS (heterozygous strain) males, bred in our laboratory were used as novel males in this experiment. Thirty-five CF-1 males, obtained from Charles River Breeding Farms, La Prarie, Quebec, Canada, or bred from such stock in our laboratory were used as inseminating males. Thirty-five CF-1 virgin females between the ages of 70-100 days old, obtained from Charles River Breeding Farms were used. All animals were isolated

for at least one week prior to the experiment. Animals were housed in standard propylene cages measuring 28 x 16 x 11 (height) cm, filled with approximately 0.5 L of *carefresh* bedding material, with standard straight-wire tops. Animals had continuous access to food and water. The colony room was maintained under a reversed 14:10 hour light cycle at 21°C.

Preparation of Novel Males

HS males were isolated for a period of at least 14 days after which they were each paired with a single HS female to allow males to become sexually experienced. After a period of 4 days, females were removed and males remained isolated for a period of 7 days. This period was considered the recovery phase during which the sexually sated males could recover from their encounter with the females.

Subsequent to the recovery phase, HS males were moved into the double-decker cage system (Figure 1). This apparatus was constructed from clear Plexiglas measuring 30 x 21 x 27 (height) cm and was further divided into upper and lower compartments measuring 30 x 21 x 13 (height) cm each. These compartments were separated by a wire-mesh grid with square openings measuring 0.5 cm^2. The lower compartment was filled with approximately 0.5 L of clean bedding and had an independent and continuous supply of food and water. The upper compartment was divided into two equal portions by an opaque Plexiglas barrier at the lowest point of the cage lid (a standard straight-wire lid). This barrier served to provide two separate housing areas, each with an independent and continuous supply of food and water. An HS male was placed in each of these upper

chambers so that each double-decker system contained a total of two HS males. The double-decker cage system is designed so that the novel male excretions fall below (through the wire-mesh floor of the upper chambers) into the lower compartment of the cage.

Insemination

Each CF-1 female was paired with one CF-1 male in a standard cage, at the commencement of the dark phase of the light cycle. The hindquarters of each female were inspected three times daily during the dark phase of the light cycle for the presence of a sperm plug. All females with a sperm plug were marked as subjects and the day of detection was designated day 0 of pregnancy. Female subjects remained in their cages for 24 hours, after which they were each randomly assigned to one of three experimental conditions.

Experimental Conditions

Females in the *sire-absent condition* were removed from the stud males (after the 24 hour period described above) and were placed in the bottom portion of the double-decker apparatus. Females in the *sire-present condition* were each also placed in the lower compartment of the double-decker cage although with the stud male. Finally, females assigned to the control condition remained in the standard housing cage. The animals in the *sire-present* and *sire-absent conditions* remained in the double-decker apparatus for the entire duration of the female's implantation phase of pregnancy. On day

7 of pregnancy, each female was removed and isolated in a clean standard cage for the duration of gestation. Beginning on day 18 and until Day 25 of pregnancy, females were checked three times daily for parturition. Pregnancy outcome was measured by the presence or absence of live births.

Behavioural Recordings

A single 15 minute behavioural recording of the animals in the lower compartment of each double-decker cage was completed between days 3-6 of pregnancy. The following data were obtained: The number of contacts made by females with novel males above, the number of contacts made by the sire with the novel males above, and the nature of the sire's interactions with both the novel males and females. A single *contact* was defined by the animal either climbing up onto the wire-mesh above or by reaching up and making contact with the nose on the mesh above. (See Figure 5) for the chart format used for all recordings.

Figure 5:

Experiment 1: ***Behavioural Observations Chart***

Subject ID	**Condition** *(sire-present OR sire-absent)*	**Day of Pregnancy**	**# of Contacts Made by Females with Novel males**	**# of Contacts Made by Sires with Novel males**	**Number of times Sire bites Novel Males Above**

RESULTS

Behavioural Recordings

The mean number of contacts made by females in the *sire-present condition* with the novel males was compared to the mean number of contacts made by females in the *sire-absent condition* using an independent samples T-test: $t = -3.476$, $p = 0.005$. These results suggest that females spend significantly less time interacting with novel males when the sire is present.

The number of contacts made by the sire with the novel males above was recorded in the *sire-present condition.* The mean number of contacts made by the sires during the 15 minute recording was 44.5. This result indicates that sires do not avoid but rather make contact with novel males.

The nature of the sire's behaviour with respect to both the novel males and the female was also observed during the interval. We observed that the sire actively bites the novel males above through the wire-mesh grid. The mean number of times the sires bit the novel males above during the 15 minute interval was 15.83. The aggression we witnessed was significant. Novel males in the *sire-present condition* were found to have wounds on their paws, and many of them were missing toes or entire paws. Furthermore, a single HS novel male was found wounded and dead four days after entering the *sire-present condition.* The nature of the sire's behaviour towards the female was also qualitatively examined. It was noted that the female spent most of her time in the corner of the cage (as opposed to investigating the novel males above as described earlier

through the examination of the number of contacts made). The sire seemed to approach her and climb over her repeatedly, in between every few contacts that he made with the novel males above.

Pregnancy Outcome

The data for this experiment are summarized in Table 1, Figure 6 . In the *sire-present* condition, eight of nine females remained pregnant; in the *sire-absent* condition, two of eight females remained pregnant, and in the control condition, all 12 females maintained their pregnancies. A chi square test of association, relating occurrence of parturition to conditions, showed significant differences among conditions, χ^2 (2, N = 29) = 15.95, $p < 0.001$. A test of association reached significance when comparing control and *sire-absent* groups, χ^2 (1, N = 20) = 12.86, $p < 0.001$; and *sire-absent* and *sire-present* groups, χ^2 (1, N = 17) = 7.14, $p < 0.01$. Tests comparing control and *sire-present* groups did not reach the conventional level of significance.

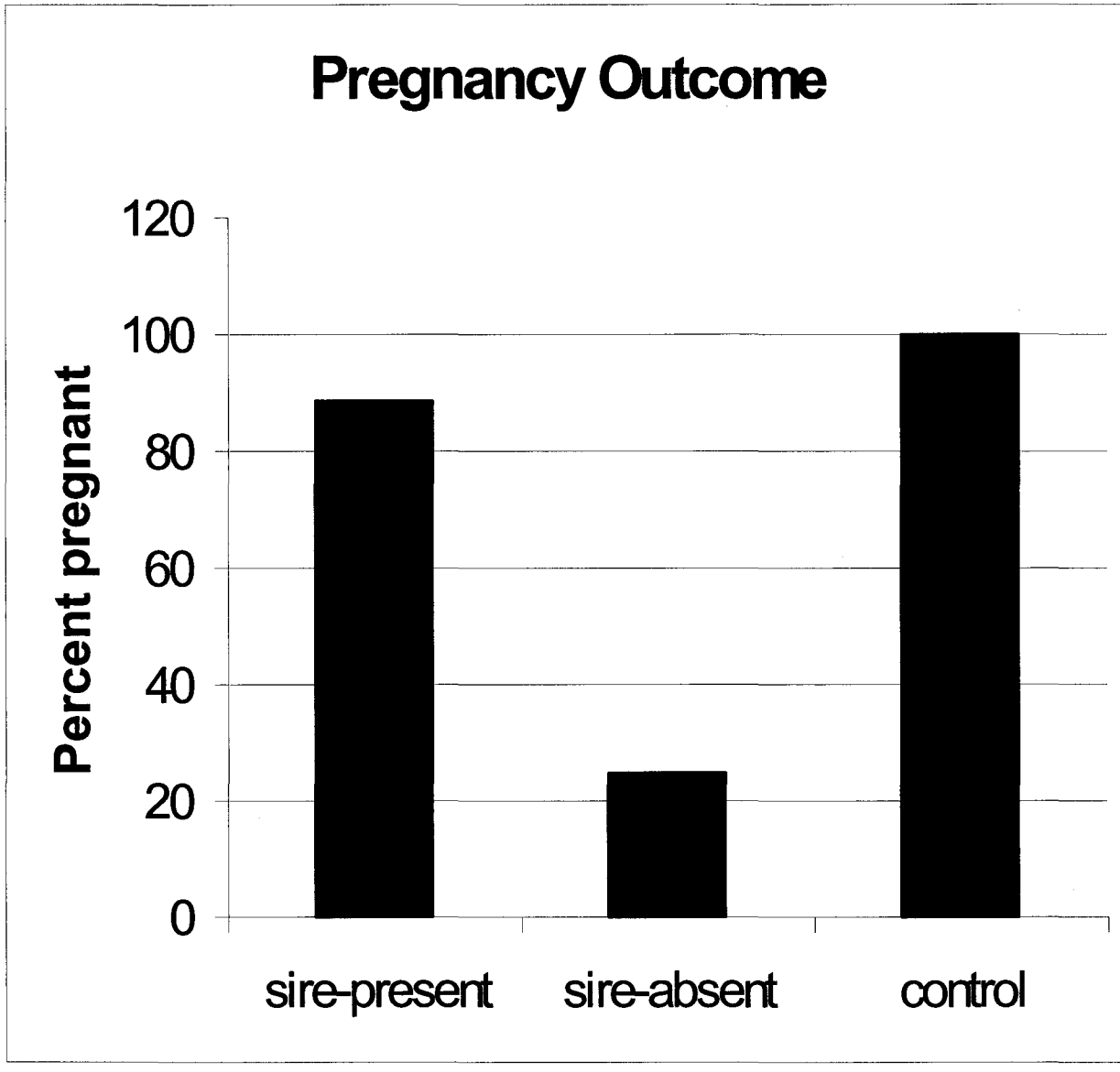
Table 1:

Experiment 1: *Mean (± SE) Number of Pups Born and Percent Parturient in Inseminated CF-1 Females Exposed to Sire-Present, Sire-Absent, and Control Conditions*

Outcome	Sire-Present Condition	Sire-Absent Condition	Control Condition
Number of pups born (all females)	10.75 ± 1.68	3.5 ± 2.29	13.67 ± 0.75
Number of pups born (pregnant only)	12.29 ± 0.78	14 ± 0.0	13.67 ± 0.75
Percent Parturient	89	25	100

Figure 6:

Experiment 1: *Pregnancy Outcome*



DISCUSSION

We were able to successfully establish a sire protection effect in our laboratory. The results indicate that females actively investigate the novel males above but that sires actively intervene between novel males and females. Sires are highly motivated not only to intervene between novel males and the inseminated female but also to behave aggressively towards novel males through the wire-mesh grid. This latter finding was unexpected, but can be understood in the context of evolutionary psychology. The sire is motivated to protect his future offspring and can be expected to engage in aggressive interactions with males that can place this offspring in danger.

Although the exact nature of the Bruce effect is still not entirely understood, it is likely that the effect is mediated by a complex of different variables including both pheromonal and behavioural cues (deCatanzaro et al., 1995). The presence of the sire at the time of implantation may have served to diminish the amount of novel male tactile stimulation received by the female and this in turn may have served to diminish the degree of risk towards successful implantation. Furthermore, by intervening between the novel males and female, the sire may decrease the amount of pheromone (present in the novel male urine) that is sprayed directly on the inseminated female.

It may also be the case that due to the extreme aggression exhibited by the sires towards novel males, the concentration of androgens in novel male urine decreased. Evidence suggest that following inter-male aggression, the subordinate male exhibits a decreased in pituitary-gonadal activity while the dominant male exhibits the opposite (Eleftheriou & Church, 1967). Defeat could depress the novel males' ability to induce

pregnancy block since androgens have been established as necessary for the Bruce effect (deCatanzaro et al., 1995; deCatanzaro & Storey, 1989; and Dominic, 1965). An increase in androgens emitted in the sires' urine is unlikely to affect the inseminated female since the sure does not target and spray his urine his urine at the female as the novel male does (Muir et al., 2001; deCatanzaro, 1996; Drickamer, 1995; Reynolds, 1971; Vella & deCatanzaro, 2001) .

EXPERIMENT 2

Based on our findings in Experiment 1, we hypothesized that sires act to inhibit the Bruce effect by actively intervening between the female and novel males. The present study was designed to investigate the role of sire behaviour in the Bruce effect. Inseminated females were exposed to novel males under two experimental conditions; *free-sire* and *corral-confined-sire* conditions. The *free-sire* condition is equivalent to the *sire present* condition as described in Experiment 1. We hypothesized that females in the *free sire* condition would remain pregnant, as found in Experiment 1. We further hypothesized that females in the *corral-confined-sire* condition would exhibit implantation failure due to the inability of the sire to intervene between contacts occurring between novel males and the female. As in Experiment 1, we examined both sire and female behaviour using the same behavioural measures. Each animal was observed for a 15 minute interval between days two and four of the inseminated females pregnancy. We used a tighter window of time in Experiment 2 in order to narrow in on behaviour occurring at the time of implantation. We did not run animals in a *sire-absent* or Bruce condition since we established a clear Bruce effect in Experiment 1. Furthermore, we have run this condition several times in our lab and have been repeatedly successful at producing a clear Bruce effect. For these reasons, re-running this condition would have been redundant and unnecessary.

METHODS

Subjects

Forty-four HS (heterozygous strain) males, as described in Experiment 1 were used as novel males. Twenty-nine CF-1 males were used inseminating males. Twenty-nine CF-1 virgin females, between the ages of 70-100 days old were used. All animals were isolated for at least one week prior to the commencement of the experiment. Animals were housed in standard propylene cages and had continuous access to food and water. The colony room was maintained at under a reversed 14:10 hour light cycle at 21°C.

Preparation of Novel Males

HS males were isolated for a period of at least 14 days after which they were each paired with a single HS female to allow males to become sexually experienced. After a period of 4 days, females were removed and males remained isolated for a 7 day recovery period. Subsequent to the recovery phase, HS males were moved into the double-decker cages

Insemination

Each CF-1 female was paired with one CF-1 male at the commencement of the dark phase of the light cycle. The hindquarters of each female were inspected as described in Experiment 1. All females with a sperm plug were marked as subjects and the day of detection was designated day 0 of pregnancy.

Experimental Conditions

Two HS males were placed in the upper compartment of each double-decker cage as described in Experiment 1. Twenty-four hours following detection of a copulatory plug, each inseminated female was randomly assigned to one of three conditions. Females in the *free-sire condition* were each removed from the stud males and placed in the bottom portion of the double-decker apparatuses. In order to properly follow AUP guidelines and to prevent the aggression that occurred in Experiment 1, an additional wire-mesh was inserted between the upper and lower compartments of the double-decker cages used in this condition. The additional wire-mesh was made of the same material and had the same measurements as the wire-mesh already present in the apparatus. There was a space of approximately 0.5 cm between the two pieces of mesh so that the animals in the lower and upper compartments could no longer make direct contact with each other. This design disallowed any direct contact between the sires and the novel males *and* between females and novel males.

Females in the *corral-confined-sire* condition were each placed in the lower compartment of modified double-decker cages. These cages included a wire-mesh corral measuring 12.7x10.2x7.6 cm (height) that was composed entirely of stainless steel wire-mesh, with square openings measuring 0.5x0.5 cm. The corral was secured to the cage using a stainless steel rod that was attached to the top of the corral and to the bottom of the wire-mesh partition that separated the upper and lower compartments of the double-decker cage (Figure 7). The stud males were each placed in the corrals. These males had

their own continuous supply of food and water and could only interact with the females through the grid, and were not able to interact with the novel males.

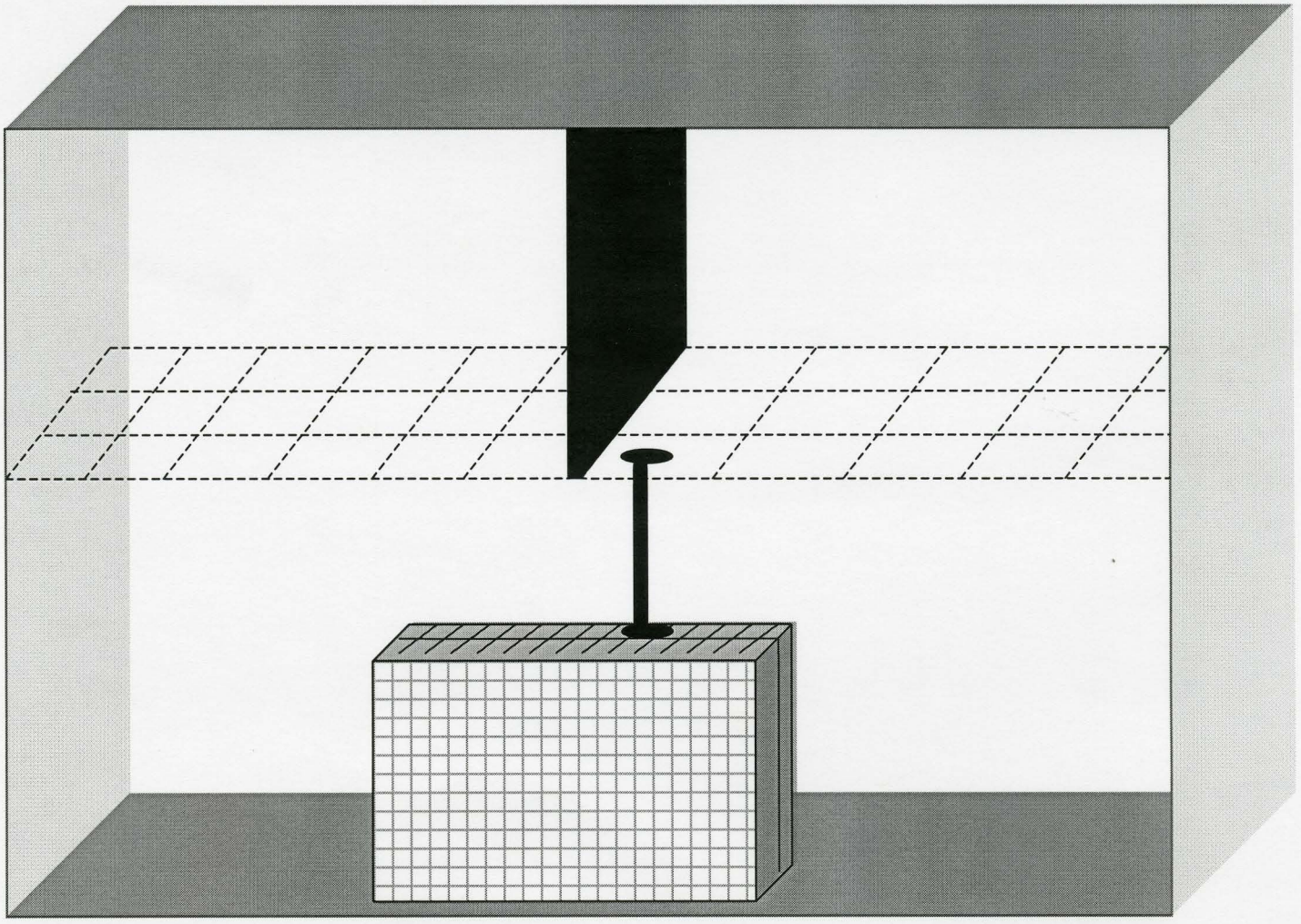
Finally, females assigned to the control condition remained in the standard housing cage. Animals in the *free-sire,* and *corral-confined sire* conditions remained in the double-decker apparatus for the entire duration of the female's implantation phase of pregnancy. On day 7 of pregnancy, each female was removed and isolated in a clean standard cage for the duration of gestation. Beginning on day 18 and until Day 25 of pregnancy, females were checked three times daily for parturition. Pregnancy outcome was measured by the presence or absence of live births.

Behavioural Recordings

A single 15 minute behavioural recording of the animals in the lower compartment of each double-decker cage was completed between days 2-4 of pregnancy. The number of contacts made by females with novel males above, and the number of contacts made by the sire with novel males above were measured. A contact was defined as described in Experiment 1. See Figure 5 for the chart format used to for all recordings.

Figure 7:

Experiment 2 : *Experimental Apparatus*



Note: drawing not to scale

RESULTS

Behavioural Recordings

An independent samples T- test (one-tailed), comparing the number of female contacts with the novel males in the *corral-confined-sire* condition with the *free-sire* conditions reached significance ($t = 2.02$, $df = 14$) at $p < 0.05$. Females in the *corral-confined-sire* condition spent significantly less time making contact with the novel males above (mean = 23.25 contacts in 15 minute interval) than did females in the *free-sire* condition (mean = 42.75 contacts in 15 minute interval). In the *free-sire* condition the mean number of contacts made by the sire with the novel males above was 73.25.

Pregnancy Outcome

The data for this experiment are summarized in Table 2 and Figure 8. In the *free-sire* condition, eight of nine females remained pregnant. In the *corral-confined-sire* condition, 10 of 13 females remained pregnant. A Chi square test of association which related occurrence of parturition to condition in all three conditions (*free-sire, corral-confined sire,* and control) did not reach conventional levels of significance ($\chi^2 = 2.117$, $df = 2$). A Chi square test of association which related occurrence of parturition to condition in the *free sire* and *corral-confined sire* conditions did not reach significance ($\chi^2 = 0.512$, $df = 1$).

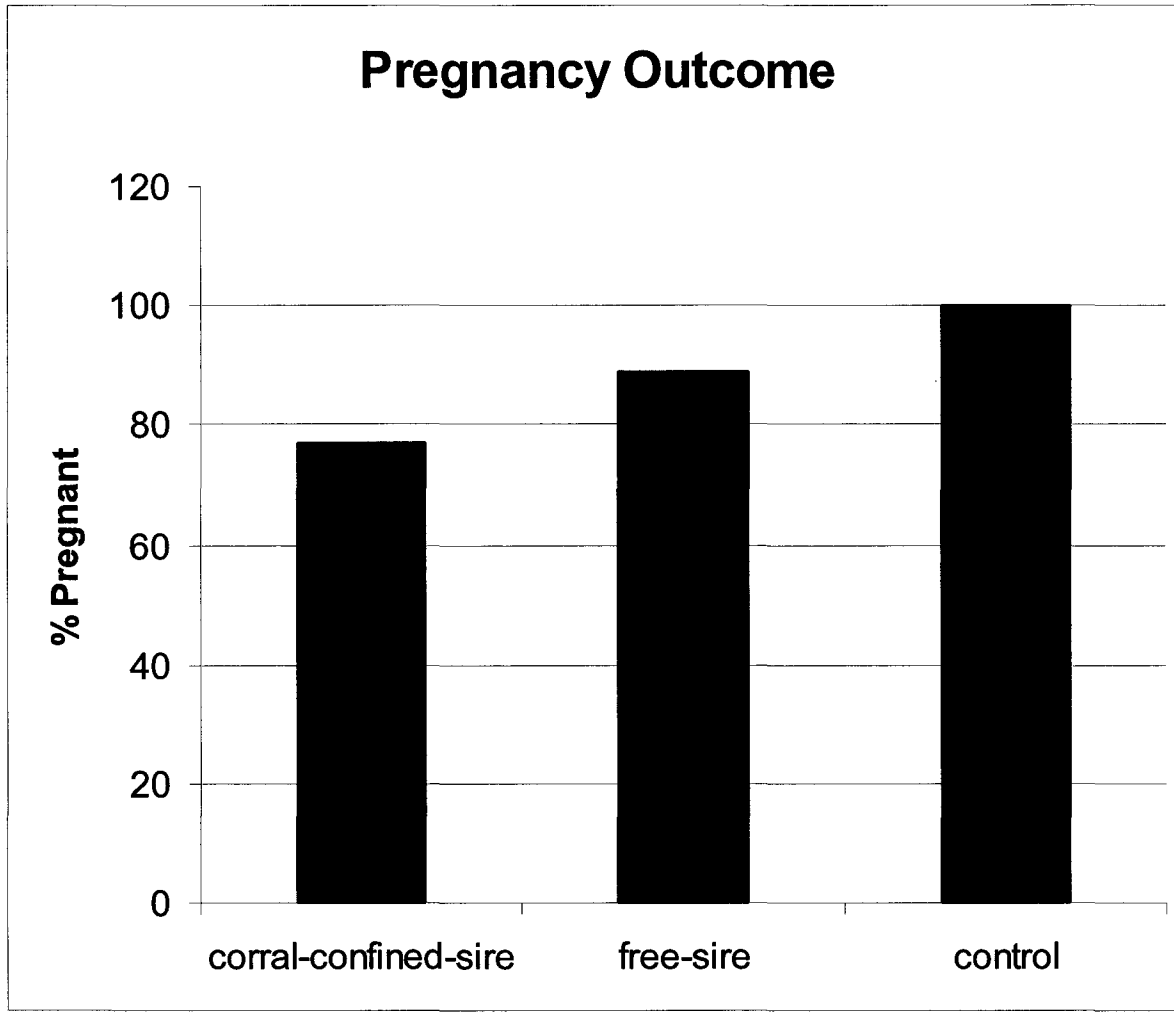
Table 2:

Experiment 2: *Mean (± SE) Number of Pups Born and Percent Parturient in Inseminated CF-1 Females Exposed to Free-Sire, Corral-Confined-Sire, and Control Conditions*

Outcome	Free-Sire Condition	Corral-Confined-Sire Condition	Control Condition
Number of pups born (all females)	10.14 ± 1.79	10.0 ± 2.50	12.57 ± 0.611
Number of pups born (pregnant only)	11.83 ± 0.703	15.0 ± 0.258	12.57 ± 0.611
Percent Parturient	89	77	100

Figure 8:

Experiment 2: *Pregnancy Outcome*



DISCUSSION

The interval sampling component of this Experiment yielded interesting results. In this experiment females in the *free-sire* condition made an average of 42.75 contacts with the novel males above. This result is unexpectedly high when compared to the results from the same condition in Experiment 1 where the average number of contacts was 26.33. An independent samples t-test comparing female contacts in the *free-sire* condition across the two experiments reached significance ($t = 1.59$, $df = 12$) @ $p < 0.05$.

This increase in the number of contacts in the *free-sire* condition of Experiment 2 may be due to either one or both of the design changes implemented in this experiment . The behavioural observations were conducted between days 2-4 of pregnancy while in Experiment 1, the recordings were taken between days 3-6 of pregnancy. The window of time during which behavioural observations were taken was decreased in an attempt to further narrow in on behavioural activity occurring during the implantation phase of pregnancy. It is unlikely that this acted to increase the number of contacts made by females with novel males because both time periods surround implantation and are very close to one another. No situational or physiological factors would change significantly between the time periods so as to affect the female's motivation to interact with the novel males.

Secondly, in this experiment, the double-decker apparatus in the *free-sire* condition was slightly altered. An additional piece of wire-mesh, was inserted between the upper and lower compartments of the apparatus so as to inhibit the animals in the lower compartment from making direct contact with animals in the upper compartment.

This additional wire-mesh was added in order to meet *animal use protocol* (AUP) guidelines and decrease the amount of physical injury incurred by novel males housed above due to the sire aggression witnessed in Experiment 1.

The additional wire-mesh piece not only reduced the amount of direct contact between the sire and novel males, but it also served to remove direct contact between the female and novel males. This lack of direct contact between the animals may have acted to decrease the sire's motivation to intervene between female and novel male interactions and might explain the increased number of female contacts with novel males seen in this condition (with the double mesh present) when compared to that of Experiment 1 (single mesh only).

As discussed earlier, the Bruce effect is thought to be mediated by a complex of both pheromonal and behavioural cues and so the removal of direct contact between the females and the novel males through the use of the additional wire-mesh grid could have influenced the nature of the effect (deCatanzaro et al., 1995; deCatanzaro et al., 1996). It may be useful to run all conditions with a double wire-mesh partition so that comparisons across conditions may be made more accurately.

In this experiment, an independent samples t-test comparing the mean number of female contacts in the *free-sire* condition and in the *corral-confined-sire* condition reached significance ($t = 2.02$, $df = 14$) at $p < 0.05$. Females in the *corral-confined-sire* condition made significantly fewer contacts with the novel males than did females in the *free-sire* condition. Based on our hypothesis we expected the opposite. We hypothesized that the female would make a greater number of contacts with the novel males above

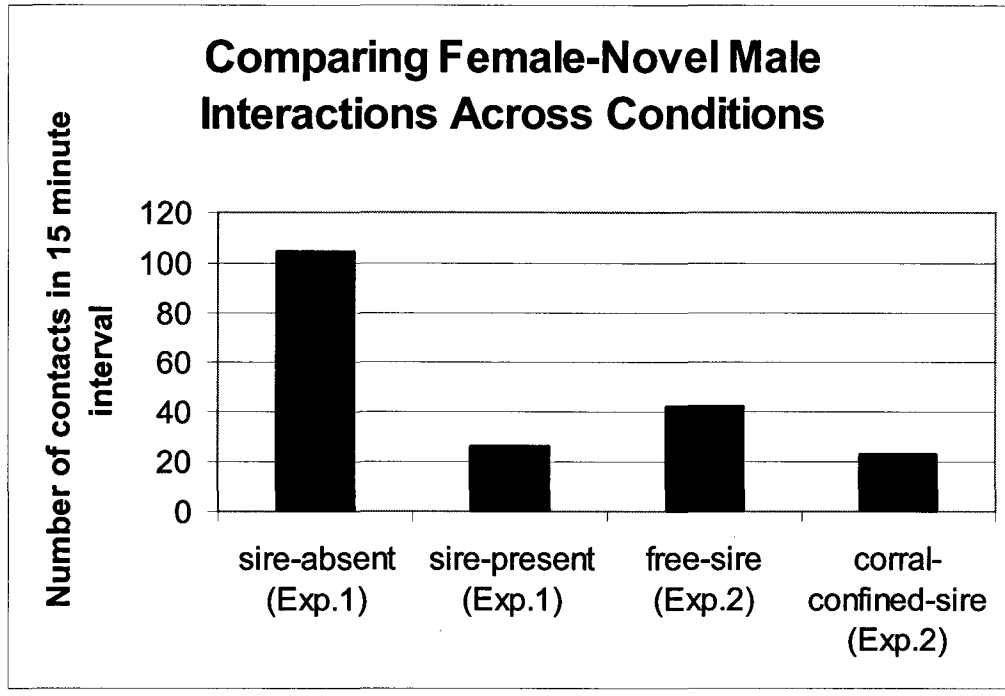
when the sire was confined and unable to intervene between her and the novel males housed above her.

It is useful to compare the mean number of female contacts with novel males in the *corral-confined-sire* condition of this experiment with the number of contacts in the experimental conditions of Experiment 1. In Experiment 1, a double mesh was not present and so a comparison between these conditions may yield more valuable results. Females in the *corral-confined-sire* condition made an average of 23.25 contacts with novel males, while females in the *free-sire* condition of Experiment 1 made an average 26.33 contacts. An independent samples t-test comparing the number of contacts across these two conditions does not reach conventional levels of significance ($t = 0.31$, $df = 12$). Females in the *sire-absent* condition of Experiment 1 made and average of 104.75 contacts. An independent samples t-test comparing the average number of female contacts in the *corral-confined-sire* condition to the *sire-absent* condition of Experiment reached conventional levels of significance ($t = 4.12$, $df = 12$) @ $p < 0.05$.

Together these results suggest that the number of female contacts with the novel males are similar when the sire is present and when he is present but confined in a wire-mesh corral. The number of contacts that the female makes with the novel males above are significantly higher when the sire is not present in the cage with the female. Thus the presence of the sire (free or confined) acts to influence the female to spend less time interacting with novel males that can disrupt her pregnancy. See Figure 9.

Figure 9:

A Comparison of the Number of Contacts Made Between Females and Novel Males Across Conditions



During our observations of the animals in the *corral-confined-sire* condition subjective observational accounts indicated that the female tended to spend a significant amount of time on top of the corral, interacting with the sire through the wire-mesh grid. It is possible that the corral served as a distraction and thus the female averted her attention towards this stimulus as opposed to towards the novel males.

The number of contacts made by the sire with the novel males above in Experiment 1 (in which the double-decker had a single mesh only) was 44.5 and the mean number of contacts made by the sire in Experiment 2 (double mesh) was 73.25. The results from these two experiments did not differ significantly ($t = 1.32$, $df = 10$) @ $p < 0.05$. This indicates that sire behaviour was consistent across the two experiments is the *free-sire* condition.

The pregnancy outcome of the *free-sire* condition of this experiment compared to that of the *sire-absent* condition (standard Bruce effect condition) from Experiment 1, is significantly different ($\chi^2 = 7.14$, $df = 2$) @ $p < 0.05$. In the *free-sire* condition, eight of nine females remained pregnant, while in the *sire-absent* condition of Experiment 1, only two of eight females remained pregnant. Thus a sire protection effect was established in this experiment. When the *corral-confined-sire* condition is compared to the *free-sire* condition, there is no significant difference ($\chi^2 = 0.512$, $df = 1$). There is a significant difference between the *corral-confined-sire* condition and the *sire-absent* condition of Experiment 1 ($\chi^2 = 5.45$, $df = 1$, $p < 0.05$).

Together these results indicate that females from both the *free-sire* and *corral-confined-sire* conditions maintained their pregnancies. We had originally hypothesized

that confining the sire to a wire-mesh corral and therefore inhibiting his ability to behave aggressively towards novel males and to intervene between novel males and females would serve to inhibit the sire's ability to protect the female's pregnancy. Our results however do not support this hypothesis. Despite the confinement of the sires in the *corral-confined-sire* condition, they were still able to inhibit the Bruce effect.

There were some complications that developed over the course of the experiment due to the use of newly constructed double-decker cages. These cages were built using the same basic design as the cages used in Experiment 1, however; the opaque border separating the two HS males in the upper compartment was not high enough to make direct contact with the cage tops. There was a small (0.5cm) space between the top of this divider and the cage top. During the course of the experiment we found that the some HS males were able to squeeze through this space so that these males had access to one another. In a situation such as this, where a receptive female is proximal and there is more than one male present, male mice will typically fight aggressively, often until one is found dead. It is due to this reason, the we normally separate the two HS males in the upper compartment of the cages. In the current experiment, we found that HS males had access to each other, and were fighting aggressively. In some cases, one or both of the animals were found dead following three or more days in the experimental apparatus. Four HS males from the *corral-confined-sire* condition, and two from the *sire-absent* condition were found dead. It is possible that this may have affected our results. The males were found dead towards the end of the seven day exposure period however and

since this is after the narrow window of implantation we are confident that this did not effect pregnancy outcome.

EXPERIMENT 3

This experiment was designed to examine inter-male aggression between stud and novel males. The purpose of the study was to examine if stud male aggression towards novel males increases in the presence of the inseminated female. Stud males were grouped with novel males in one of two experimental conditions: *female-present* and *female-absent* conditions. The *female-absent* condition was intended to serve as a control condition and was meant to examine territorial aggression, while the *female-present* condition was meant to examine aggression related to sire protection. We hypothesized that the stud male would initiate and dominate more aggressive encounters with novel males in the presence of the inseminated female.

METHODS

Subjects

HS (heterozygous strain) males, bred in our laboratory were used as novel males in this experiment. Thirty-five CF-1 males, were used as inseminating males, and thirty-five CF-1 virgin females, between the ages of 70-100 days old, were used. All animals were isolated for at least one week prior to the commencement of the experiment. Animals were housed in standard propylene cages and had continuous access to food and water. The colony room was maintained at under a reversed 14:10 hour light cycle at 21°C.

Insemination

Each CF-1 female was paired with one CF-1 male at the commencement of the dark phase of the light cycle in the standard cage. The hindquarters of each female were inspected as described in Experiment 1 for the presence of a sperm plug. All females with a sperm plug were marked as subjects and the day of detection was designated day 0 of pregnancy. Females remained housed with the stud males.

Experimental Conditions

Between days three and five of each female's pregnancy, stud males were placed in one of two experimental conditions: *female-present* and *female-absent.* In the *female-present* condition, the sire and pregnant female remained in their cage and a single novel HS male was introduced into the cage. The point of introduction was designated *time = 0.* Behavioural recordings were taken (Figure 10). The novel male was removed from the cage when the aggressive encounter between the males came to an end and a clear dominant and subordinate positions were established, or when the aggressive encounter between the two males became significantly aggressive and either one or two of the mice suffered from more than three bleeding wounds. The latter was done so that basic *Animal Use Protocol* (AUP) guidelines could be respected.

In the *female-absent* condition, the female was removed from her home cage and placed temporarily in a clear standard cage. The stud male remained in the cage and a novel male was introduced to the cage. The point of introduction was designated *time = 0.* The same behavioural recordings were taken using the table in Figure 10. The novel

male was removed as in the *female-present* condition. Upon removal of the novel male the female was returned to the cage containing the stud male.

Behavioural Recordings

Behavioural recordings and observations were taken during the aggressive encounters between the stud males and the novel males The amount of time that elapsed before initiation of aggressive contact between the two males and the identity of the initiator (sire or novel male) were recorded. The identity of the more aggressive male was also noted. Degree of aggression was measured by frequency of aggressive behaviours including tail rattling, biting, wrestling, and vocalizations. In situations where a clear winner and loser of the conflict were established (determined by dominant and subordinate postures), the identity of each was also noted.

Figure 10:

Experiment 3: *Interval Sampling - Aggression Data*

DATE:	
Time:	
Condition:	
Day of Female's Pregnancy:	
Initial Behaviour:	
Time of First Aggressive Behaviour:	
Identity of Initiator:	
Description of Aggressive Behaviours Exhibited by Each Animal:	
How Long Before Animals Are Separated?	

RESULTS

Behavioural Recordings

In all cases in both conditions, the sire initiated the conflict. An independent samples T-test comparing the mean time of conflict initiation between the *female-absent* and the *female-present* conditions reached significance, $t = 2.529$, $p < 0.05$. The *female-present* condition had a much longer time elapse before initiation of a conflict (175.89 s) than the *female-absent* condition (74.38 s) (Figure 11). In the *female-present* condition, the sire won six of seven aggressive encounters and in the *female-absent* condition, the sire won four of five aggressive encounters. A chi square tests of association which related identity of winner to conditions, marginally escaped conventional significance, $\chi^2 (1, n = 12) = 0.069$, $p > 0.05$ See Figure 12.

Other important qualitative observations were subjectively recorded. The observers noted that the sire spent most of his time in between the female and the novel male in the *female-present* condition. If the novel male was adjacent to the female at any time, the sire immediately forced his way in between the two animals. Finally, the sire was noted to be more aggressive when the novel male was proximal to the female.

Figure 11:

Experiment 3: *Conflict Initiation Time*

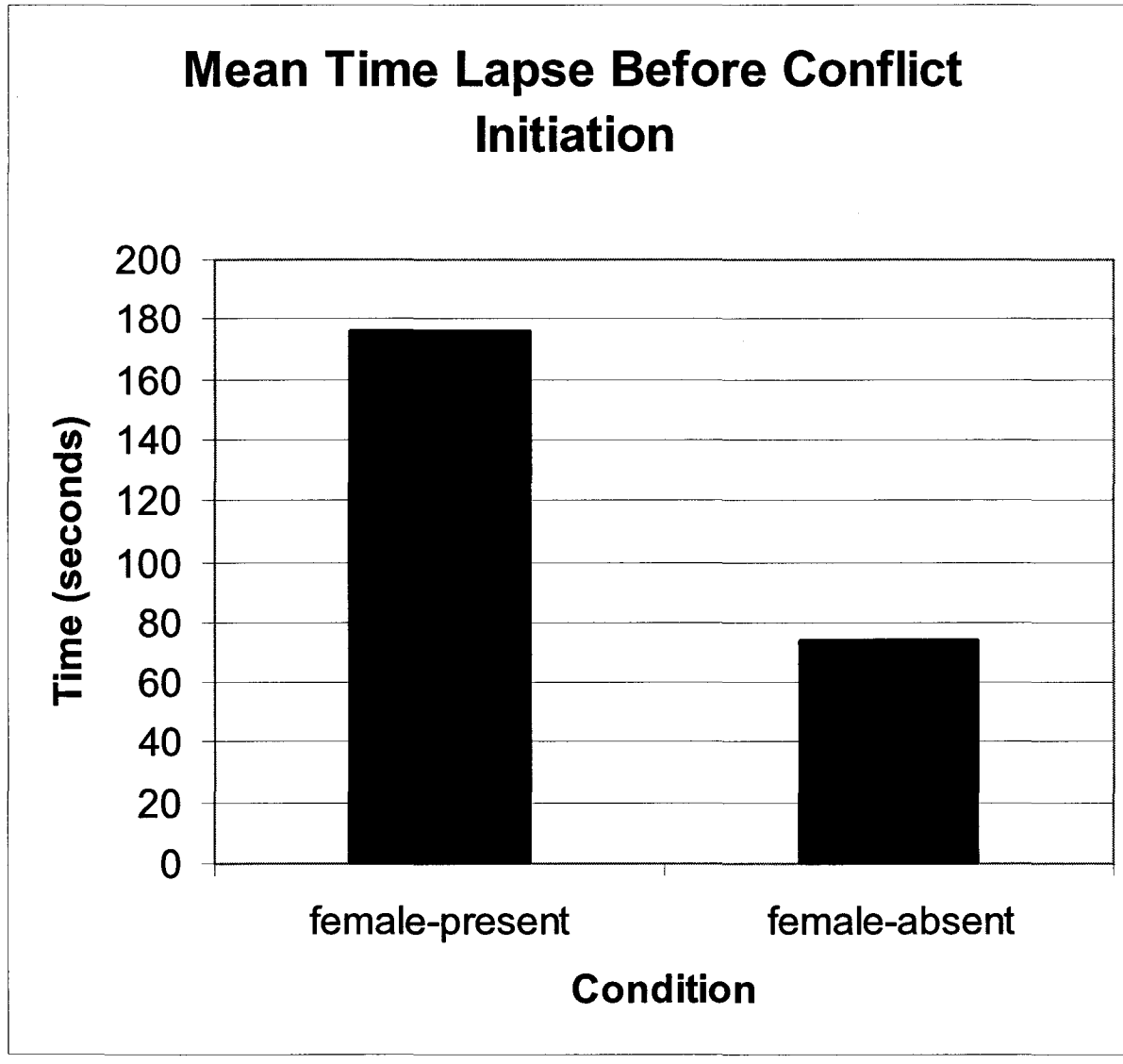


Figure 12:

Initiation of Conflict

Condition	%age of times the sire initiates
Experimental	100%
Control	100%

DISCUSSION

The time difference between the two conditions before initiation of conflict may be due to the fact that the addition of a third animal in the cage acts as a distracter stimulus. All animals investigated one another prior to the commencement of any aggressive conflicts.

The results of this study indicate that the sire won all conflicts, independent of the experimental condition. This can be interpreted in an evolutionary context as the reflection of his motivation to protect his reproductive investment. Although the female was removed in the *female-absent*, no time lapsed before her removal from the cage and the introduction of the novel male. For this reason, it is likely that the sire is still acting out aggression that is motivated by sire protection.

Suggestions for further research in this area include adding another condition in which the stud male is removed from the female and isolated after the female is inseminated. After a designated time period of isolation, both the stud male and novel male can be removed from their cages and placed in a clean cage. In such a condition, the sire may no longer exhibit intense aggression motivated by sire protection since he has been removed from the female for some time. Furthermore, since the cage would be fresh, neither animal would have an established territory. Such a condition may yield results that are closer to chance levels and could serve as a control condition in the study of sire aggression.

The qualitative observations made during the recordings also indicate that the sire is highly motivated to intervene between interactions occurring between the novel male

and inseminated female. These results can be compared to those of Experiment 1 in which the number of female contacts with novel males was significantly reduced in the presence of the sire.

GENERAL DISCUSSION

In Experiment 1, it was found that in the presence of a free sire, a significant proportion of females maintained their pregnancies despite the presence of novel males, when compared to females exposed to novel males in the absence of sires. Behavioural observations indicated that the sire behaves aggressively towards novel males through a wire-mesh grid and that he is highly motivated to engage in conflict with novel males. Females in this condition exhibited a reduced number of contacts with novel males suggesting that the presence of the sire serves to reduce behavioural interaction between females and novel males. Together, these data provide evidence that sires do in fact afford protection to inseminated females' pregnancies. This protection effect involves intervention between novel males and females as well as extreme aggression from the sire aimed at novel males. In previous studies, it was thought that sire protection was mostly due to tactile stimulation between the sire and the inseminated female (Thomas & Dominic, 1987). Sire behaviour was not observed during these previous studies. The present study indicates that sire behaviour may in fact be an important component of the sire protection effect and that the sire actively intervenes between novel male and female contact as well as behaves aggressively towards novel males.

In Experiment 2, sire behaviour was partially limited as a stimulus offering protection to the inseminated female by confining sires to a wire-mesh corral. In this study,

females that were exposed to confined sires also maintained their pregnancies. This result was unexpected since the sires were unable to behave aggressively towards novel males and were unable to intervene in female-novel male interactions. In addition to this finding, the number of average female contacts with novel males were similar in both *free-sire* and *corral-confined-sire* conditions. In Experiment 1, the average number of female contacts with novel males in the absence of the sire was 104.75. This average is significantly higher than that seen in the *free-sire* condition (mean = 26.33) and in the *corral-confined-sire* condition (mean = 23.25). Based on these results it seems that presence of the sire, irrespective of whether he is free or confined serves to decrease the number of contacts the female makes with the novel males. It may be that the inclusion of an additional animal serves as an additional stimulus and distracts the female from the novel male. The female may be dividing her time between the animals and thus the total number of contacts with the novel males decreases.

DeCatanzaro and Murji (submitted) conducted an experiment in which an inseminated female was placed in a four arm radial maze and at the end of each arm (behind a wire-mesh grid) were a novel alien male, a novel strange male (conspecific male), the sire, and no stimulus animal. The female was observed to spend the least amount of time investigating the empty arm and the most amount of time investigating the arm containing he novel alien male that could disrupt her pregnancy. Time spent in the empty arm differed significantly from time spent in all the other arms. When the number of contacts with each animal in each arm was examined, similar results were found. The female made the least number of contacts with the wire-mesh at the end of

the empty arm and the most number of contacts with the wire-mesh between her and the novel alien male. Again, the number of contacts made in the empty arm differed significantly from the number of contacts in all other conditions. These results indicate that the female does not avoid novel males that can block her pregnancy. Furthermore, the time spent and number of contacts made with each of the three types of males did not differ significantly. The results of deCatanzaro and Murji's study may help to explain the results obtained in Experiment 1 and 2. When the sire was present, the female spent time investigating both the sire and the novel males. Although the decrease in female contacts made with novel males in the *free-sire* condition was initially interpreted to be a result of the sire's intervention, it may have been a combination of his intervention and the female's own motivation to explore the sire as well as the novel males (thus decreasing the total time she spent interacting with the novel males).

In Experiment 2, females in both the *free-sire* and *corral-confined-sire* conditions remained pregnant. This result was contrary to our hypothesis that a confined sire would be unable to intervene in female-novel male contacts and would be unable to engage in aggressive behaviours towards novel males (thus failing to produce a sire protection effect). In contrast, Experiment 2 showed that the presence of the sire, whether he is free or confined leads to the protection of a female's pregnancy.

It is useful to place these results in the context of Thomas and Dominic's (1987) studies (Figure 4). They concluded that the presence of a free sire allows for body contact between females and sires and that this stimulus is responsible for the sire protection effect. In their experiment, 81.6 % of females housed with a free sire and exposed to a

novel male house above remained pregnant. They also found that if females were housed alone below a novel male and a sire (separated by a wire-mesh gird between them), 61.1 % of them remained pregnant. If sires and females were housed in the same compartment but separated by a wire-mesh grid and females were exposed to novel males housed above them, 24.0% of females remained pregnant.

The design used for the second condition in Thomas and Dominic's (1987) study in which female were housed with both a sire and a novel male can be criticized because females could have spent their time differentially under each of the two males and this could have affected the pregnancy outcome. The group in which the sire and female are separated by a wire-mesh grid is different from the design used in the *corral-confined-sire* condition of Experiment 2 because in this condition *both* the sire and the female were housed directly below the novel males.

The results from Thomas and Dominic's (1987) study indicate that when the female and sire were separated by a wire-mesh grid, the sire protection effect was lost, and only 24% of females remained pregnant. These results are contrary to the results found in Experiment 2. When the sire was confined to a wire-mesh corral, 77% of females remained pregnant. It is possible that this difference in pregnancy outcome across these two conditions in the two experiments is partly due to the fact that in Thomas and Dominic's study, only the females were housed beneath the novel males, and the sires were not. It may be that the sire emits pheromonal cues that differ when he is exposed to novel male pheromones (as the sires were in the *corral-confined-sire* condition of Experiment 2) and that this change in pheromonal cues result in increased

pregnancy protection. It is plausible that the sire's pheromones (when the sire is housed in the context of novel males) alter the female's physiology in such a way as to make implantation failure less likely in the context of novel males.

The findings in Experiment 2 suggest that the aggression initiated by the sire towards novel males and the active intervention of the sire between the inseminated female and novel males may only be one component of the stimuli offered by the stud male that act to prevent implantation failure in the presence of novel males. Stud male pheromones and interaction between stud males and females may also be important in producing the sire protection effect.

Due to *animal use protocal* (AUP) guidelines we were unable to keep the *sire-present* and *free-sire* conditions consistent across experiments. In Experiment 1 we witnessed an extreme amount of aggression between the sire and the novel males. Sires engaged in aggressive behaviours including biting the toes and feet of the novel males through the wire-mesh grid and a single incidence of death (caused by severe wounding) of a novel male was seen. In order to eliminate such aggression in the laboratory, we added an additional wire-mesh grid to the cages used in the same condition in Experiment 2. Although this additional barrier served to remove direct contact between the animals and thus removed any aggressive conflicts, it may have served to alter our results (as noted earlier). It also created a weakness in the design of Experiment 2 because comparisons across conditions within this experiment were now affected by this factor. It is for this reason that we introduced comparisons between experiments 1 and 2. All CF-1 mice used in both experiments were bred from the same stock and were of the same age

and condition. Furthermore, the HS males (novel males) used in Experiment 1 and 2 were the same. This enabled us to make comparisons across the two studies without having to be concerned about any confounding variables that may affect our results.

In Experiment 3, sire aggression was examined and it was found that sires initiated and won all conflicts regardless of whether the female was present or was just removed from the cage. As was seen in Experiment 1, sires were found to be highly motivated to behave aggressively towards novel males.

These combined observations of sire behaviour can all be understood from an evolutionary context. The sire is motivated to protect his reproductive investment from novel males with the ability to induce pregnancy block and therefore behave in such a way as to limit novel male contact with inseminated females.

These data also help to confirm the importance of contact cues in the Bruce effect. When contact between novel males and females is reduced due to sire intervention, significantly more females maintained their pregnancies. DeCatanzaro et al. (1995) showed that contact cues are a necessary component of the complex of stimuli that cause the Bruce effect. When inseminated females were exposed to novel male urine alone in their bedding, a Bruce effect could not be established. Furthermore, when novel male urine was painted on the noses of inseminated females, the a pregnancy block could not be established. The importance of contact has also been seen in studies by deCatanzaro and Storey (1989) in which females housed directly with novel males experienced a much larger pregnancy block when there was a wire-mesh grid between them. When

novel males are housed directly with females they either re-inseminate the females or block their existing pregnancies.

It is useful to examine the results of the present study in the context of Archunan and Dominic's (1989) study of sire protection under the conditions of nutritional stress. In their study, they reported that females that were deprived of food and isolated after insemination exhibited pregnancy disruption, while females that were similarly food-deprived but housed with a confined stud male during the fasting period remained pregnant. It may be, for example, that nutritional stress affects the normal hormonal dynamics necessary for successful implantation and that the stud male offers some biologically active hormones in his excretions that serve to return abnormal hormone levels in the female to levels that are conducive to successful implantation. We must then address why the stud and novel conspecific males differed on the degrees of protection they each afforded to the female's pregnancy. Although this is not clear, it could be hypothesized that the sire's excretions differ from those of the novel conspecific male based simply on the fact that the sire is sexually sated. We know that androgen levels decrease in sexually sated males and that sexually primed males show increased androgens in the context of a receptive female (Spironello & deCatanzaro, 1999).

Alternatively, it may be that the presence of other healthy animals in proximity to the nutritionally stressed female influence the female's physiology in such a way as to support pregnancy maintenance. Again, it should be noted that Archunan and Dominic's methods included the use of daily vaginal smears as an indication of state of pregnancy.

As discussed earlier, this method is not as accurate as is the direct observation of parturition (deCatanzaro & Storey, 1989; Komisaruk & Steinman, 1986).

Suggestions for future studies include the examination of sire behaviour after the implantation phase of pregnancy. We know that the Bruce effect only affects females during the implantation phase of pregnancy. It can be argued that sires may intervene between female and novel male interactions less frequently after implantation since novel males are unable to block pregnancy after this time. Conversely, it can be argued that sires should continue to be motivated to behave in aggressively towards novel males since they pose a risk to the sire's offspring after parturition. Novel males are likely to kill pups that they have not sired (deCatanzaro, 1985).

Ideally, a paradigm in which sire, novel male, and female urine could all be collected in the context of the Bruce effect, could be used to examine and compare hormone content in each of the three groups of animals. Such a study may bolster our understanding of the hormonal dynamics of both the Bruce and sire protection effect.

It may also be interesting to examine the Bruce effect under conditions where the female is not housed with the sire, but is housed in bedding soaked in sire excretions. As seen in the study conducted by Parkes and Bruce (1962) and deCatanzaro et al. (1995), the bedding would have to be sufficiently soiled and renewed often so as to maximize the amount of sire pheromone available to the female. Such a study might might help us to gain a better understanding of the role of sire-originating pheromonal cues in the sire protection effect.

The present study provides new and important findings on the nature of sire and female behaviour in the context of the Bruce effect and the sire protection effect. This data is useful in understanding the complex of cues that cause an inseminated female to lose her pregnancy in the context of novel males and to maintain her pregnancy in the presence of her sire.

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