MALE INDUCED IMPLANTATION FAILURE (THE BRUCE EFFECT) IN MICE: ROLE OF LEARNING AND MEMORY

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Thesis submitted in partial fulfillment of the requirements for the Degree of
Doctor of Philosophy in Zoology
under the faculty of science of the
University of Calicut

Under the supervision of

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DECLARATION

I, Preeji K P, hereby declare that the work embodied in the thesis "**Male induced implantation failure (the Bruce effect) in mice: role of learning and memory"** submitted to the University of Calicut in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Zoology is a bonafide record of the research work carried out by me under the supervision of Dr. John K. Thomas, Associate Professor (Rtd), and Dr. Leyon Varghese, Assistant Professor, Department of Zoology, Christ College, in the Research and Post-graduate Department of Zoology, Christ College, Irinjalakuda, University of Calicut and no part of the thesis has formed the basis for the award of any degree, diploma or other similar titles of any university.

IRINJALAKUDA **PREEJI K P**

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KERALA, INDIA

CERTIFICATE

This is to certify that the thesis "**Male induced implantation failure (the Bruce effect) in mice: role of learning and memory"** submitted to the University of Calicut in partial fulfillment of the requirements for the award of the degree of Doctor of Philosophy in Zoology is an authentic record of the work carried out by Ms. Preeji K P, under our supervision in the Department of Zoology, Christ College, Irinjalakuda, University of Calicut and no part of the thesis has formed the basis for the award of any degree, diploma or other similar titles of any university.

Dr.JOHN K. THOMAS (Guide)

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CERTIFICATE

This is to certify that Ms. Preeji K P has completed the research work for the full period prescribed under the Ph. D. ordinance of the University of Calicut. This thesis "**Male induced implantation failure (the Bruce effect) in mice: role of learning and memory"** embodies the results of her investigations conducted during the period at which she worked as a research scholar. I recommend the thesis to be submitted for evaluation for the award of the degree of Doctor of Philosophy in Zoology of the University of Calicut.

IRINJALAKUDA **PRINCIPAL**

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Dedicated to

My Husband

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PREFACE

During my short tenure as a guest faculty of the Department of Zoology, Christ College, Irinjalakuda, I assisted a few of my B.Sc. students in their dissertation work on 'Human olfactory discrimination'. They used odorants from leaf extracts of local plants and commonly used fruit extracts to test the ability of humans to discriminate and remember the odors that they were allowed to smell for a short period. The methodology was very simple but the results were very interesting. When I approached Dr. John K Thomas, the Asst. Prof. of the Department of Zoology, Christ College and expressed my interest in doing research on olfactory memory, he gave me a few papers on the Bruce effect and suggested me to work on pheromone and its influence on reproduction in mice.

The thesis is the report of the work I have done on the context dependent learning and the formation of olfactory memory in the newly inseminated female mice during the process of mating. Imprinting of the nonpheromonal cues in newly inseminated mice and the luteotrophic effect of imprinted nonpheromonal cues are discussed. Olfactory preference of estrus females to the urine of dominant vs. subordinate male and the ability of females to learn the nonpheromonal cues paired with the urine of male mice is also reported in this thesis. The protective effect of the imprinted nonpheromonal cues against male rat-induced implantation block and starvation-induced implantation block in inseminated females is also discussed. Post-mating free female choice of stud vs. alien male is discussed in the last chapter.

Preeji K.P.

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General Introduction

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Karlson and Butenandt (1959) identified a chemical substance (Bombykol) which silkworm moths use for intraspecific communication and named it 'pheromone' (Karlson and Luscher 1959). Influence of chemical substances on the reproductive processes of mammals was reported as early as in 1955. Van der Lee and Boot (1955, 1956) reported mutual disruption of estrus cycle in unisexually grouped female mice. Induction of the estrus in group-housed females was reported by Whitten in 1956. However, the male-induced implantation failure reported by Hilda M. Bruce in laboratory mice (the Bruce effect) is one of the best investigated pheromonal effects in mammals (Bruce 1959). When a newly inseminated female mouse is separated from her mating partner and exposed to another male (alien male) the fertilized ova fail to implant in the uterus and the female returns to estrus as if mating had not been occurred (Bruce 1959, 1960a). Direct physical contact with the alien male is not necessary for pregnancy failure to occur in the female. Exposure of the inseminated female to the soiled bedding of alien male (Parkes and Bruce, 1962) or to its urine also results in the implantation failure (Dominic 1964, 1966a). These observations lead to the conclusion that Bruce effect is a pheromone-mediated phenomenon.

Pheromones are secreted outside the body of an animal and bring out specific response when perceived by another individual of the same species. Functionally pheromones are classified into 'releaser', 'primer' and 'imprinting' pheromones based on the responses elicited in the recipients. The pheromones involved in the Bruce effect are placed under the category of primer pheromones (Parkes and Bruce 1961; Dominic 1987). One of the characteristics of the primer pheromones is that they require prolonged stimulation and the responses are often mediated through the neuroendocrine pathways (Bruce 1966, 1967, 1970; Dominic, 1983, 1987a).

There seems to be variation in the effectiveness of pheromone present in the urine of males in inducing implantation failure in newly inseminated females. A male belonging to a different strain from that of the stud male (alien male) is significantly more effective than a different male from the same strain of the stud male (strange male). This is indicated by the fact that exposure of the inseminated female to a male belonging to the wild strain (alien male) or to its urine results in about more than 80% of implantation failure, whereas exposure to a different male belonging to the same strain of the stud male (strange male) or to its urine results in 30–35% of pregnancy failure (Parkes and Bruce 1961; Thomas 1989).

The Bruce effect has been observed in several strains of laboratory mice (e.g. CBA, BALB/c, and C3H). However, it is not observed in highly inbred *Mus* strains (Bruce 1968; Kakihana et al. 1974). Several other rodent species also exhibit the Bruce effect. For example, deer mouse *Microtus agrestis* (Bronson and Eleftheriou 1963), Meadow vole *Microtus pensilvanicus* (Clulow and Langerford 1971), Prairie vole *Microtus ochrogaster* (Stehn and Richmond 1975) and in Norway rats *Rattus norvigicus* (Marashi and Rulicke 2012). A recent field study reported for the first time that the Bruce effect occurs in a wild primate, the gelada (*Theropithecus gelada*) and the authors concluded that their data support the hypothesis that the Bruce effect can be an adaptive strategy for females (Roberts et al. 2012). Bruce effect is not observed in Mongolian gerbil *Meriones unguiculatus* (Norris and Adams 1979).

Investigations carried out during the past five decades have unequivocally demonstrated that the urine of intact male mouse is the source of pheromone involved in the Bruce effect. The methodology of investigations seems to interfere with the effectiveness of male urine in inducing implantation failure

in inseminated females. Exposure of inseminated Parkes albino female mice to urine-soiled bedding of strange males in boxes failed to induce implantation block (Bruce 1960a). On the other hand, housing females in tall glass jars with reduced air circulation containing bedding sprinkled with urine from strange males was effective in inducing pregnancy block (Bruce 1960b). In another study, Parkes and Bruce (1962) reported in Parkes strain of mice that twice-daily renewal of male bedding is more effective in inducing Bruce effect than once daily renewal of soiled bedding. The authors suggested that the pheromone involved in the Bruce effect is volatile and ephemeral.

The role of urinary androgens in male-induced implantation failure is demonstrated in several studies. An inbred strain of SJL males does not normally secrete pregnancy blocking pheromones in their urine. When SJL females and males were administered depo-testosterone cypionate, the secretion of pregnancy-blocking pheromone was stimulated (Hoppe 1975). Rajendren and Dominic (1988) treated intact alien males with steroidal antiandrogen cyproterone acetate and found that the pheromone involved in the Bruce effect may be a by-product of androgen metabolism. deCatanzaro et al. (2006) demonstrated that the urine of male mice contains unconjugated estradiol and testosterone. Male mice treated with anastrozole (aromatase inhibitor) and a diet containing less phyto-estrogen exhibited reduced ability to induce implantation failure. The Bruce effect is mimicked in inseminated females treated with small quantities of exogenous estrogen (deCatanzaro et al. 2001, 2006). Adult males are shown to develop polyuria on exposure to inseminated or developing females and they direct the urine to the nasal area of these females (Reynolds 1971; Arakawa et al. 2007; deCatanzaro et al. 2009). Guzzo et al. (2010) implanted osmotic pumps containing tritium-labeled E_2 (^{3}H-E_2) in males and the radioactivity was detected in the urine of these males. These authors were able to detect ^{3}H-E_2

in inseminated females after intranasal irrigation of tritium-labeled male urine and concluded that the presence of an excess quantity of estrogen in male urine may disrupt implantation in inseminated females.

A newly inseminated female is susceptible to pregnancy block for 48 h post-mating, after which exposure to an alien male is ineffective (Bruce 1961). Intermittent exposure to an alien male during the post- mating period had been shown to result in pregnancy block in certain strains of mice. For example, Chipman et al. (1966) reported that females of outbred Swiss mice exhibited implantation failure when exposed to alien males for 15 minutes for four days. Increasing the number of alien males exposed to inseminated females failed to affect the rate of pregnancy block (Bronson et al. 1964; Bruce 1963; Chipman and Fox 1966).

However, the exact definition and role of pheromones in chemical communication in mammals are still a controversial subject (Stowers and Marton 2005; Wyatt 2010). It is interesting to note that normal odors when coupled with experience may produce stereotyped behaviors similar to what is observed in the case of response to a pheromone (Stowers and Kuo 2015). The olfactory process in mammals is currently classified as associative olfaction and specialized olfaction. In associative olfaction the meaning of the odor is decoded depending on the experience of the individual and may vary among individuals; whereas, specialized odors activate specific neuronal circuitry bringing out pre-set behaviors irrespective of the experience of the individual (Wyatt 2010).

Due to the complexity of the mammalian pheromones and the contextual variations in the behavioral responses exhibited by mammals to different chemical substances, Wyatt (2003, 2005, 2010) introduced the term ‘signature mixture’ to designate chemical substances that exhibit individual variation in their composition and which requires learning from the part of

the recipient in exhibiting a specific response. According to him, signature mixtures are 'the subsets of variable molecules from the chemical profile that is learned by other conspecifics and used to recognize an organism as an individual or as a member of a particular social group'. On the other hand, 'pheromones are evolved signals which exhibit specific reaction, for example, a stereotyped behavior and/or a developmental process in a conspecific' (Wyatt 2010).

Even after years of hefty research on pheromones only a few pheromones have been identified so far. Pheromones are distinguished from other odorants on the basis that it can elicit a stereotyped, innate social and reproductive behavior even in inexperienced recipients (Stowers and Marton 2005; Wyatt 2010). They are said to be special odors that regulate hormone secretion and the perception and decoding of the meaning of pheromones are often determined by developmentally predetermined subsets of neural circuits that have a high likelihood of eliciting pre-programmed behaviors (Liberles 2014; Stowers and Kuo 2015). However, recent evidences suggest that responses to pheromones may vary depending on the gender, internal state, past experiences, genetic profile and concomitant environmental stimuli (Stowers and Liberles 2016). Furthermore, it has been demonstrated that chemical cues that are emitted by individuals can be interpreted in a combinatorial manner based on their relative ratios and may elicit innate and learned behavioral patterns (Kaur et al. 2014). In mice a nonvolatile major urinary protein darcin is said to be attractive to females. Initially darcin activates the vomeronasal organ (VNO) and then only the female exhibits attractive behavior to the volatile pheromones associated with the same individual (Roberts et al. 2010). However, this learned attraction to volatile pheromones of the male urine may vary depending on the health status of the male. Lanuza et al. (2014) demonstrated that female mice are not attracted to

the urine collected from males infected by a nematode *Aspiculuris tetrapetra*. This indicates that the behavioral response of mice to a pheromone is highly flexible and learning plays a pivotal role in pheromone communication.

The role the stud male in the Bruce Effect

One of the interesting features of the Bruce effect is that even though the pheromone that induces implantation failure is present in the urine of all adult males, re-exposure of the newly inseminated female to the stud male after separation for 24 hours does not result in implantation failure (Parkes and Bruce 1961). It is often suggested that the female is able to identify the individual odor of its mating partner and it is this ability that prevents the female from exhibiting implantation failure on exposure to the stud male (Thomas and Dominic 1989a). Yamazaki et al. (1980) demonstrated that MHC genes are responsible for the production of individual odor in mice. It is suggested that the female mouse gets imprinted with the individual odor of the stud male during the critical period of mating. It has been shown that a short period of 15 minutes stay with the stud male during the pericopulatory period is enough for the female to get imprinted with the olfactory memory of the stud male (Thomas 1989) and the memory thus imprinted in the female lasts for several days after mating (Brennan et al. 1989). *In vivo* studies have shown that AOB neurons are capable of encoding the sexual and genetic status of the donor individual based on the olfactory signals relayed through the VNO (Ben-shaul et al. 2010; Tolokh et al. 2013). Mating alters the function of the neural circuitry and disrupts the implantation blocking effect of the pheromones of the stud male (Brennan and Keverne 1997).

Neural mechanisms of olfactory imprinting

Brennan et al. (1990) have shown that imprinting of the olfactory memory of the stud male takes place in the AOB following mating- induced activation of

norepinephrine. Vaginal stimulation is essential for the formation of the olfactory memory of the stud male in the AOB neurons of mated females. Once this olfactory memory is formed in the female, it no longer responds to the pheromones of the stud male which would otherwise induce implantation failure. The neural mechanism involved in the formation of pheromonal memory of the mating partner in the female mouse is elucidated in several studies. It has been shown that vaginocervical stimulation during mating decreases the dendrodendritic feedback inhibition of mitral/tufted (MT) cells in the accessory olfactory bulb (AOB) (Otsuka et al. 2001; Ichikawa 2003; Gao et al. 2017), leading to GABA-mediated inhibition of MT cells. Otsuka et al. (2001) have shown that artificial vaginocervical stimulation often considerably reduces dentrodendritic feedback inhibition of mitral cells and enhances their activity. This self-inhibition ultimately disrupts the pheromone signals of the stud male reaching the central brain, thus preventing the ability of his pheromone to induce implantation failure in his coital partner. Choi et al. (2011) reported that the olfactory system of the mouse has a subset of projections that are responsible for decoding pheromones resulting in certain stereotyped behaviors.

Luteotrophic effect of stud male pheromone

Several investigators have recognized the crucial role played by the stud male in the Bruce effect (Parkes and Bruce 1961; Dominic 1969; Lott and Hopwood 1972; Milligan 1980). It is suggested that the female mouse gets 'imprinted' with the odor of the olfactory cues emanating from the stud male during mating and this forms the basis for their differential response to strange male exposure after mating (Milligan 1980; Keverne and de la Riva 1982). Female field voles *Microtus agrestis*, which were allowed to stay with a male for one hour during pericopulatory period, do not exhibit implantation failure when allowed to mate with the same male or re-exposed to it after separation

for 48 h (Milligan et al. 1979). These authors concluded that prolonged stay with the stud male is not necessary to develop the ability to recognize the coital partner in this species.

The presence of the stud male can prevent implantation failure in newly inseminated female exposed to alien male (Parkes and Bruce 1961). Thomas and Dominic (1987a) demonstrated that physical contact with the stud male or its urine is essential for preventing alien male-induced implantation block (Thomas and Dominic 1987b). The primary endocrine response of the inseminated females exposed to alien male is shown to be the inhibition of hypophysial prolactin secretion and the failure of development of corpora lutea (Dominic 1966b, 1970). It is suggested that presence of the stud male or its urine exerts luteotrophic effect in the females in protecting the female from the Bruce effect (Thomas and Dominic 1987b; Archunan 2014). Adult males exposed to the females before mating (familiar males) failed to protect newly inseminated females against the Bruce effect (Thomas and Dominic 1989a). This shows that the protective luteotrophic effect of the stud male is not due to the familiarity that develops during pre-mating interaction with the female.

The luteotrophic effect of stud male or its urine is further emphasized in the studies of Kumar and Dominic (1996) which demonstrated that the presence of the stud male significantly reduces implantation failure in newly inseminated females exposed to male rat. Starvation or food deprivation during the first two days of pregnancy significantly affects implantation ratio in laboratory mice (Bruce 1963; McClure 1963; Sahu and Dominic 1985). But a significant reduction in implantation failure was found in food-deprived female mice when they were housed with the stud male (Archunan and Dominic 1989). These studies provide strong evidences for the luteotrophic influence of the stud male.

Additional evidence for the luteotrophic influence of the stud male or its urine comes from the studies of Thomas and Dominic (1987b). These authors demonstrated that when the pregnancy of newly inseminated female is terminated by exposure to alien male or by injection of bromocriptine, a dopamine agonist, the majority of females exhibited implantation failure and they returned to estrus. When the pregnancy- blocked females were re-exposed to the stud males on the third day *post coitum*, a vast majority of experimental females showed pseudopregnancy. However, exposure to alien male or to a different male that belongs to the same strain of the stud male failed to induce pseudopregnancy in pregnancy-blocked females. It is suggested that the new crop of corpora lutea formed during the first estrus after the pregnancy block became active on re-exposure to the stud male or its urine. In rodent species like rats and mice, vaginal stimulation is essential for the development of luteal function (Everett 1967). In the above-mentioned studies, the stud males remained confined during the experimental period, ruling out the possibility of any mounting or vaginal stimulation. Moreover, exposure to the urine of the stud male is equally effective in the induction of pseudopregnancy in the implantation-blocked females (Thomas and Dominic 1987c). These authors argued that the imprinted memory of the odor of the stud male acts as a luteotrophic 'mnemonic' and re-activation of this memory by the stud male or its urine stimulates the release of prolactin and the subsequent reactivation of the corpora lutea in the females.

Chemical nature of pheromones

The discovery of pheromones as chemical substances employed for intraspecific communication was mainly based on the observed role they play in altering the physiology and behavior of animals. The exact chemical nature of pheromones and the mechanism by which they produce physiological or behavioral changes in the recipients were largely unknown until recently

(Doty 2010). Pheromones are traditionally classified based on their function as releaser (signaling), primer and imprinting pheromones (Wilson and Bossert 1963; Bruce 1970; Dominic 1983). However, the original source of several mammalian pheromones, their molecular structure and the means of their detection has been largely tentative. One of the principal reasons for the delay in molecular characterization is that the pheromones are exceptionally rare and often remain as complex mixtures and set off behaviors that are not seen under standard laboratory conditions. It is believed that most pheromones are non-volatile and require physical contact for inducing pheromone-mediated behaviors (O'Connell and Meredith 1984). In mammals, considerable number of diverse chemical substances acts as pheromones. Metabolic products of steroid molecules, peptides, high molecular-weight protein-ligand complexes, and low-molecular-weight volatile substances are shown to induce pheromonal effects (Liberles 2014). 2-sec-Butyl-4-5-dihydrothiazole identified from the urine of male mice is said to be one of the candidates for chemically characterized volatile pheromones. 2, 3-Dehydro-exo-brevicomin is said to be involved in estrus induction, inter- male aggression and female attraction (Dulac and Torello 2003). MHC class I peptides (Methylthio) methanethiol is another volatile pheromone that acts as an agent that promotes olfactory memory and female attraction (Lin et al. 2005). Leinders et al. (2004) reported that MHC class I peptides function as ligands for individual identification and determine the response of the female to the pheromones of a strange/alien male in the context of the Bruce effect. Recent studies carried out in several laboratories were successful in identifying the molecular structure and mechanism of transduction of some of these chemical signals (Stowers and Kuo 2015).

Sense organs for pheromone detection

The majority of the vertebrate pheromones are detected through the olfactory

pathways. The olfactory system of vertebrates consists of the olfactory epithelium proper, the Jacobson's organ or the VNO, the trigeminal nerve and terminal endings of the nervus terminalis and the septal organ of Rodolfo Masera (Meredith 1983). Unlike the case of other vertebrates, in the mammals, olfactory sense is very complex and learning and memory have a significant influence on the behavioral outcome of pheromone detection. In mammals, there are two anatomically distinct systems involved in olfaction viz., the main olfactory system and the accessory olfactory (vomeronasal) system (Scalia and Winans 1975; Wysocki 1979; Meredith 1980, 1983). Whitten (1963) suggested for the first time the involvement of VNO in pheromone perception. Pheromone receptors of the main olfactory system are present in the olfactory epithelium and the VNO or the Jacobson's organ is shown to be specialized for the detection of pheromones through the accessory olfactory system. The nerve projections of the main olfactory system are connected to the anterior cortical nucleus, olfactory cortex, olfactory tubercles, anterolateral cortical amygdaloid nucleus and lateral entorhinal cortex of the brain. On the other hand, the nerve terminals from the VNO are connected to the bed nucleus of the accessory olfactory tract, the medial nucleus of amygdale and bed nucleus of stria terminalis (Scalia and Winans 1975; Wysocki 1979). VNO is the primary sense organ specialized in the detection of non-volatile odorants and the main olfactory system is said to detect the airborne or volatile substances (Parsons 1970; Beauchamp et al. 1982; Halpern 1987). Powers and Winans (1975) reported that in male hamsters, removal of VNO produced a significant deficiency in their mating behavior, providing first experimental evidence for the involvement of VNO in pheromone detection. Surgical ablation of VNO is shown to eliminate the male-induced acceleration of puberty (Vandenberg effect) in impubertal female mice (Kaneko et al. 1980; Lomas and Keverne 1982). Traditionally it is believed that pheromones are nonvolatile molecules which are perceived

through the VNO and control innate social behaviors. Recent evidences indicate that this concept is highly limiting and differing to several observed response in animals. Pheromones are now considered as volatile or nonvolatile and activate the neurons connected to the main olfactory epithelium (MOE) or to the neurons projecting from the VNO and connected to the AOB. Moreover, contrary to the general belief, the behavioral responses to pheromones are not always innate and may vary depending on the context and experience of the recipient (Stowers and Marton 2005).

Recently, it has been shown that VNO is involved in the inherent attraction of females to male scents and learning the identity of individual males; subsequently, females can identify the individual male through the volatile pheromones alone (Roberts et al. 2010). Keller et al. (2006) demonstrated that female mice employ volatile cues detected through the main olfactory system in the context of mate recognition. However, signals from the VNO is essential for promoting physical contact with the male and expression of lordosis behavior for further progress of mating. Social status of the male is one of the determinant factors in the context of female mate choice. Several studies have shown that preference to male pheromone largely depends on the reproductive status of the female mice and the dominant status of the male. Veyrac et al. (2011) investigated the involvement of main and accessory olfactory system in the detection of the social status of the male. It has been shown that accessory olfactory pathways of the female are strongly activated when the urine of dominant male is presented, whereas urine from the subordinate male is detected by the main olfactory system and activated piriform cortex. This indicates that both main and accessory olfactory systems are involved in the identification of the social status of the male.

Bruce and Parrott (1960) demonstrated that any damage to the olfactory bulb

of the female mouse abolish the male-induced implantation failure, the Bruce effect (Reynolds and Keverne 1979; Bellringer et al. 1980; Lloyd-Thomas and Keverne 1982; Rajendren and Dominic 1985). The evidence for the involvement of VNO in the Bruce effect comes from the studies of Bellringer et al. (1980) and Rajendran and Dominic (1984b). However, Thomas and Dominic (1988) reported that the luteotrophic effect of stud male on pregnancy-blocked females had not abolished by VNO removal; but intranasal irrigation with $ZnSO_4$ completely prevented stud male-induced pseudopregnancy in pregnancy-blocked females.

Endocrine response in the Bruce effect

There are sufficient evidence that the primary endocrine reaction of the inseminated females exposed to alien male is the inhibition of hypophysial prolactin secretion and the failure of development of corpora lutea (Parkes 1961; Dominic 1966c, 1970). Administration of exogenous prolactin or the presence of ectopic pituitary graft (Dominic 1967a, b) has been shown to inhibit the influence of alien male on newly inseminated females. Treatments with prolactin-releasing agents are reported to prevent the Bruce effect. Dominic (1966d) reported that reserpine; a potent prolactin-releasing agent prevents implantation failure in inseminated females exposed to alien males. Subsequently, several other prolactin-releasing agents were also tested for their effectiveness in preventing the Bruce effect. Chloropromazine (Sahu and Dominic 1980a), quipazine (Sahu and Dominic, 1980b), α-methyl dopa (Sahu and Dominic 1983a), tryptophan (Sahu and Dominic 1983b) and haloperidol (Rajendran and Datta 1988) were also shown effective in avoiding implantation failure in females exposed to alien males.

It has been shown that male-induced implantation failure occurs on activation of tuberoinfundibular dopamine system in the hypothalamus. Dopamine released from the median eminence inhibits prolactin release from the

pituitary, resulting in implantation failure in inseminated females (Brennan 2009). Boehm et al. (2005) using the genetic transneuronal tracer technique has shown that GnRH neurons obtain pheromone signals from both odor and pheromone relays in the brain. It is suggested that feedback loops are apparent in GnRH neurons, indicating the possible influence of both odor and pheromone processing in mice. These findings strongly support the involvement of prolactin in the Bruce effect.

Nonpheromonal odors and innate behavior in mammals

Olfactory experience during early life may modify social behavior in several mammals. Mainardi et al. (1965) found that spraying artificial perfumes (prima violet) on parents during the suckling period in mice may show deficiency in sexual selection when they become adult. Similarly, in rats, pups reared from birth to one month of age in an atmosphere scented with artificial odor exhibited a lack of discrimination in the context of mate choice (Marr and Gardner Jr. 1965).

The role of learning in pheromone communication

Traditionally pheromones are distinguished from other odorants based on two major characteristics: (1) they are externally voided chemical substances from the body of animals for intraspecific communication and (2) they produce innate stereotyped behaviors in the recipients with little role for learning and experience. For example, Martin (1980) defined pheromones as 'isolated chemicals shown to be relatively species specific which elicit a clear and obvious behavioral or endocrinological function and which produce effects involving a large degree of genetic programming, influenced little by experience'.

Another proposed distinction of pheromones from other odorants is regarding the sensory pathways for pheromone detection. It is often suggested that VNO is specialized for the perception of pheromones and chemical signals that

activate VNO are considered as pheromones (Keverne 1983). Electrophysiological studies have shown that chemicals other than 'pheromones' also activate VNO (Hatanaka 1992). Sam et al. (2001) demonstrated that neurons of VNO can detect pheromones as well as other odorants. Bronson (1976) suggested that the role of experience in mammalian pheromone communication is relatively less investigated. The results of a few attempts to examine the function of learning and memory in mammalian pheromone communication have shown that experience plays an important role in the expression of pheromone- mediated behaviors. It has been shown that social odors experienced during the early life of a mammal influence species identification and later sexual selection in mammals. Carter and Marr (1970) have shown that the ability of guinea pigs for species identification based on pheromones can be altered using artificial odors presented during early life.

Muller-Schwarze (1977) was well aware of the role of learning and memory in chemical communication in mammals. He suggested the term 'informer pheromone' to designate the chemical signals which are 'stored in the memory and can be recalled later in a variety of contexts'. The importance given by him for learning is a major deviation from the traditional concept of pheromone. Doty (2003) suggested that learning is involved in establishing the meaning of chemicals perceived by mammals. Even though a neonate is attracted to the odor of its mother, learning and memory are involved in distinguishing the mother's odors from the smell of other adult females of the colony. The remarkable ability of rats for odor discrimination is well established in several studies (Jennings and Keffer 1969; Slotnick et al. 2000). Mammalian olfactory system is capable of providing information on the physical environment; the meaning of such information is often deciphered through contextual learning and experience (Doty 2003). Brennan

(2010) also had expressed that mammalian pheromone communication largely relies on context and learning as compared to insects. Marshall et al. (1981) reported the flexibility and sensitivity of the mammalian olfactory system for detecting synthetic novel odorants.

Olfactory learning may occur during different stages of the life history of a mammal. The olfactory system of mammals is functional even during the early stages of development and intrauterine learning are often manifested during the postpartum period. For example, Pedersen and Blass (1982) demonstrated that rat pups treated in utero with citral (a mixture of two aldehydes that have the same molecular formula but different structures) prefer to attach to washed nipples scented with citral and not to unwashed nipples. Smotherman (1982) injected an odorant to the amniotic fluid of pregnant rats on day 20 of gestation and later the animals were made sick by treatment with lithium chloride (LiCl). On day 10 of the post-natal period the pups were trained to approach anesthetized dam in a runway. It is reported that when the odor cue experienced by the pups during the intrauterine period was introduced into the experimental arena the running speed of the pups decreased and several of them failed to reach their mother. This indicates that the pups were able to learn in uteri an odor signal associated with the sickness of their mother and exhibit aversive behavior after birth.

Odor learning in mammals takes place during the early periods of development. Recognition of odors associated with the mother is often learned during the suckling period. It has been shown that in rat pups pairing an artificial odor with tactile stimulation results in conditioned olfactory preference (Dominguez et al. 1999) indicating neonatal learning in rats. Cross fostering studies in several mammalian species also indicate that odor preference during infancy and subsequent mate preference is a learned response and need not be a genetically determined behavior. Mainardi (1965)

demonstrated that female pups of *Mus musculus domesticus* reared in the presence of male and female parents preferred male odors of their own subspecies when they are adults to that of another subspecies, *Mus musculus bactrianus*. However, female pups reared only by their mother in the absence of the male parent do not exhibit any such discrepancy in their mate preference between these two subspecies. Female pups of *Mus musculus domesticus* cross fostered to pigmy mice (*Baiomys taylori*), when tested for their male odor preference during adulthood preferred the odor of *Baiomys taylori* to that of *Mus musculus domesticus* (Quadagno and Banks 1970). Several studies demonstrate the involvement of MHC genes in determining the individual odors in mice (Yamazaki et al. 1976; Beauchamp et al. 1985). Mice reared in semi-natural conditions (Potts et al. 1991) as well as those bred and fostered under laboratory conditions (Yamazaki et al. 1976; Egid and Brown 1989) preferred to mate with opposite sex having unrelated MHC genes. Later studies indicate that this genetically determined preference is often superseded by cross-fostering and alteration in the diet of parents. Penn and Potts (1998) showed that female mice avoid mating with males bearing MHC genes of their foster family. The authors suggested that their data support the familial imprinting hypothesis in the context of mate selection in mice. Several other rodent species also exhibit learned response to a number of artificial odorants perceived during their pre- weaning period (Janus 1993).

Learning and memory are shown to guide odor-mediated behavioral responses of several mammals during their adulthood. Adult sexual experience plays a major role in developing odor preference or augment pre-existing faint preference for odors of females in estrus to the odor of diestrus females (Doty and Dunbar 1974; Lydell and Doty 1972). Kippin and Pfaus (2001) have shown that in rats adult male that had sexual experience with females

scented with artificial odors preferred to mate with females anointed with the same odor. However, it should be noted that this type of olfactory conditioning with artificial odors requires the presence of the female during the post-ejaculatory period (Kippin et al. 2001). Mammals possess a fabulous capacity for acquiring and maintaining memory of several odor types and to recall the context in which it is perceived. For example, a female mouse in estrus prefer dominant male odor to subordinate male odor or to odor of juveniles (Mossman and Drickamer 1996).

The significance of the Bruce effect

The significance of the Bruce effect remains a controversial issue since its discovery in 1959 by Bruce. Bruce and Parrot (1960) viewed male- induced implantation failure as a strategy for exogamy. Several authors have suggested Bruce effect to be a selection process that operates at the level of the individual in the context of male-male competition (Bronson 1968). Rogers and Beauchamp (1976) compared male-induced implantation failure with infanticidal behavior of male langurs and chimpanzees. Infanticide evolves in certain mammalian social groups with rigid hierarchy and where mating is controlled by a limited number of dominant males (Lukas and Huchard 2014). The Bruce effect is generally considered to be a counter-strategy of females towards expected infanticide. Eccard et al. (2017) studied bank voles (*Myodes glareolus*) in large open-air enclosures and found that the Bruce effect may be adaptive for female rodents living in social groups with seasonally fluctuating populations. As the viability of the progeny is in danger in presence of a strange male, the female avoids investment in reproduction through implantation failure and the male strategy for increasing its chances for mating with females returning to estrus soon after the failure of implantation (Hrdy 1977). It can also be a female strategy for selecting better genes of a male competitor who can offer parental care (Dawkins 1976).

Delayed pregnancies after displacement of a resident male by a new male is observed in free living gelada baboons (*Theropithecus gelada*) is believed to be a sign of the Bruce effect (Roberts et al. 2012). Similar phenomenon is observed in rodents like marmots *Marmota marmota* (Hacklander and Arnold 1999) and odd-toed ungulates *Equus caballus* Berger 1983). The possibility of male-induced implantation failure as a laboratory artifact can be ruled out in the light of studies in free living populations of mammals. The genetic basis of individual recognition which is pivotal in the Bruce effect is elucidated in several studies. Yamazaki et al. (1983) suggested that the adaptive significance of the Bruce effect is that it may increase genetic compatibility.

Unlike the pheromones in insects which trigger innate, stereotyped behaviors in recipients, the Bruce effect involves a learning process which enables the female to distinguish a strange male or its odor from that of the stud male. The formation of the memory of the stud male odor in the AOB of the newly inseminated female is well documented (Brennan and Keverne 1989) and it has been suggested that the strange/alien male obliterates the luteotrophic effect of the imprinted memory of the stud male resulting in implantation failure (Thomas and Dominic1987b, c). Recently, it has been demonstrated that females can be imprinted with a nonpheromonal cue which it perceives during the pericopulatory period and this imprinted nonpheromonal cue acts analogous to the stud male odor in protecting the female from the Bruce effect, emphasizing the role of learning in the phenomenon (Thomas et al. 2018).

General Methodology

General methodology

Animals

The females, stud males and the strange males employed in the present investigation belonged to the BALB/c strain. The alien males used for the induction of implantation failure in newly inseminated mice belonged to the wild strain *Mus musculus domesticus* and the Swiss mice. Wild mice were collected from houses in and around Thrissur and they were housed in a separate room. The rats used for the study were of Wistar strain. All mice and rats were purchased from Kerala Veterinary and Animal Sciences University, Mannuthy, Thrissur, Kerala.

All mice were housed individually in polypropylene cages of size 29 ×22 × 14 cm and rats were in cages of size 42 × 21 × 20 cm with rice-husk bedding. The temperature (23°C) and reverse light-dark cycle of 12:12 (lights on at 18:00 h) were kept constant. Animals were fed on a standard diet purchased from Small Animal Breeding Station of Kerala Veterinary and Animal Sciences University and water was provided *ad libitum.*

Study of the estrous cycle by examination of the vaginal smear

Stages of the estrous cycle of the mice are reflected in the appearance and composition of the vaginal cells and these stages can be determined by examining the cells under a compound microscope. Vaginal smear was obtained by gentle scraping of the dorsal wall of the vagina with a steel spatula with smooth edges. The female was held in a head-down position and the tip of the spatula moistened with water was introduced into the vagina and the vaginal wall was gently scraped to obtain the smear. This technique will not cause any injury to the vagina or cervix. A small sample of the cells from the vaginal epithelium was spread on a clean glass slide, dried and examined without staining under a microscope. The approximate proportion of

each cell type in the smear was recorded (McLean et al. 2012).

There are four stages in the estrous cycle of mice: proestrus, estrus, metestrus and diestrus. When the female is in proestrus, mostly nucleated and some cornified epithelial cells are present. Some leukocytes may be present if the female is in early proestrus. As the stage of the cycle advances to estrus, mostly cornified epithelial cells are present. If the cycle is not interrupted by pregnancy, pseudopregnancy, or other phenomena, metestrus will begin. Metestrus is a brief stage when the corpora lutea form but fail to fully luteinize due to the lack of progesterone. The uterine lining will begin to slough and evidence of this is seen in the form of cornified epithelial cells and polymorphonuclear leukocytes present in vaginal swabs. Some nucleated epithelial cells will also be present in late metestrus. Diestrus is the longest of the stages lasting more than 2 days. Vaginal swabs during diestrus show primarily polymorphonuclear leukocytes and a few epithelial cells during late diestrus. Leukocytes remain the predominant cell type having removed cellular debris. The cycle then repeats (Byers 2012).

Mating and exposure to alien male

Virgin BALB/c females (10-20 weeks old and weighing 25-30 g) were monogamously paired with experienced stud males and examined daily to confirm mating. Upon finding the vaginal plug, the females were removed from the stud males and individually housed in fresh cages (42 × 21 × 20 cm) with fresh bedding. The day on which the vaginal plug was detected was designated as day 0 *post coitum* (Bruce 1960; Dominic 1965). In most of the experiments reported in the Thesis, the females were individually exposed to a confined alien male 24 h after finding the vaginal plug (day 1 *post coitum*). The alien male was confined in a wire mesh corral, 16 × 13 × 10 cm, and placed inside the cage housing the newly inseminated female on day 1 *post coitum*. The females generally remained with the alien males for two

days (48 h) and had some access to both urine and excreta of the alien males. After 48 h the females were separated from the alien males and kept in a fresh cage with fresh bedding. All animals had free access to food and drinking water during experimentation.

Confirmation of implantation failure

Vaginal smear was examined daily from all the females to assess their reproductive status. Presence of abundant irregular shaped, nonnucleated, cornified squamous epithelial cells in the smear on day 4 *post coitum* was taken as the indication of pregnancy block and return to estrus (Thomas and Dominic 1987a; McLean et al. 2012). Vaginal smear with abundant leucocytes and mucus was taken as an indication of pregnancy/pseudopregnancy.

Urine collection

The male mouse was taken out from its cage and placed on a glass plate. Holding the tail of the animal in the left hand, the abdominal region of the male was gently pressed. The expelled urine was then collected using a clean dropper and 100 µL was used as pheromonal cue.

Nonpheromonal cues

Two drops of food-grade groundnut (*Arachis hypogaea*) oil (PRO PRIMIO Refined Groundnut Oil, R.R. Oomerbhoy Pvt. Ltd.) and gingelly oil/sesame (*Sesamum indicum*) oil (Agmark Swarnam Gingilly Oil, United Oil Industries) were used as nonpheromonal cues. A cotton ball of 150 mg weight smeared with two drops (100 11L) of oil was given to the experimental animals as nonpheromonal cue.

Chapter 1

Effect of exposure to alien male, strange male, stud male and nonpheromonal cues on the pregnancy of newly inseminated female mice

Chapter 1

Effect of exposure to alien male, strange male, stud male and nonpheromonal cues on the pregnancy of newly inseminated female mice

Introduction

Olfactory cues play a significant role in the behavior and reproduction of mice. In the natural environment, some olfactory cues may be attractive and some other cues produce a repulsive response from individual mice. While the smell of food materials predicts a rewarding experience to both sexes, olfactory cues associated with a receptive female foretell a pleasant experience to the male. The smell of food with pathogens and olfactory signals of a predator predicts imminent danger and produce an aversive response in both sexes of mice. Several behavioral responses of mice to environmental odors are learned responses. First, suckling of mother's milk once thought as an innate behavior is now demonstrated to be a response to the signature mixture of mother learned *in utero* and at the time of parturition (Logan et al. 2012). Pheromones, the chemicals employed by animals for intraspecific communication elicit mostly innate or unlearned responses in a wide range of animal species including mammals.

Bruce (1959, 1960a) reported that when a newly inseminated female mouse is separated from the stud male and exposed to another male, implantation fails and the female returns to estrus. A male of a different strain (alien male) is more effective in inducing pregnancy block in inseminated females than another male belonging to the same strain of the stud male (strange male). Exposure to the stud male after separation for 24 h does not result in pregnancy block (Bruce 1961). Bruce (1960b) and Dominic (1966b) demonstrated that the inseminated females are susceptible to pregnancy failure during the pre-implantation period and in fact this phenomenon is

essentially a case of failure of implantation.

Pheromone responsible for implantation block is present in the urine of adult males (Dominic 1965) and direct physical contact with the urine of the alien male is necessary for implantation block to occur. This implies that the pheromone involved in the Bruce effect is nonvolatile and acts through the VNO (Rajendran and Dominic 1984a, b, 1985). The primary hormonal response of a newly inseminated female to alien male/strange male urine is the inhibition of prolactin secretion and treatment with exogenous prolactin would prevent the occurrence of implantation block (Dominic 1967b).

All intact adult males are capable of producing pheromones involved in the Bruce effect. However, re-exposure of the mating partner of the female fails to induce implantation failure in inseminated females. It has been suggested that the female becomes imprinted with the individual odor of the stud male during mating and this learning process and the subsequent memory of the stud male is responsible for the failure of stud male to induce pregnancy block in females (Thomas and Dominic 1986, 1987a). Other studies indicate that the presence of the stud male during exposure to the alien male protects the female from implantation block (Thomas and Dominic 1987b). It is suggested that the pheromones of the stud male exerts a luteotrophic effect on the female and prevents the inhibition of prolactin in females exposed to the stud male (Thomas and Dominic 1987c; Archunan 2014). The luteotrophic influence of the stud male on the inseminated females is further demonstrated in the studies of Thomas and Dominic (1987b, c) where re-exposure of pregnancy- blocked females to the stud male or his urine results in pseudopregnancy in the majority of females tested.

Imprinting is a process of associative learning that takes place at certain unique developmental stage in the life of animals. There is a critical sensitive period in the female during which the animal is more susceptible to

imprinting. It is possible to imprint the animal with any object during the critical or sensitive period. Precocial birds like domestic fowl and duck are shown to be imprinted with any moving object exposed during their critical period and exhibit "following behavior" an instinctive behavior is usually shown by chicks towards their mother (Spalding 1872; Lorenz 1961).

These preliminary investigations are intended to provide background information on the Bruce effect. It is also intended to test whether two edible oils (groundnut oil and gingelly oil) extracted from ground nut and sesame seeds (gingelly), respectively, which form a part of the food supplied to the mice, have any influence on the pregnancy of newly inseminated females.

Methodology

40 adult virgin females, 10 adult males (10–20 weeks; BALB/c) and 6 adult Swiss males were purchased from Kerala Veterinary and Animal Sciences University. Two drops of food-grade groundnut oil and gingelly oil were used as nonpheromonal cues. The estrus cycle of the females was assessed using standard vaginal smear technique (see general methodology).

Out of the 40 females, 36 females which exhibited regular estrous cycle were selected and they were divided into six groups, each group consisting of 6 females. They were allowed to mate with adult males. Vaginal smear was taken daily to examine the reproductive status of the female. On finding the vaginal plug, females were separated from their stud male (day 0 *post coitum*) and housed in cages with fresh bedding. After 24 h (day 1 *post coitum*) females of each group was treated as follows:

Group I: exposed to an alien male

Group II: exposed to a strange male

Group III: exposed to the stud male

Group IV: exposed to groundnut oil

Group V: exposed to gingelly oil

Group VI: left undisturbed

Alien male, strange male and stud male were confined in a wire net cage during exposure to inseminated females. However, the female mice had access to the urine and excreta of the alien male, strange male and the stud male. Two drops groundnut oil or gingelly oil were smeared on cotton balls and presented to the females allowing direct contact with the olfactory cues.

After 48 h (day 3 *post coitum*) females of Groups I, II, III, IV, and V were separated from the experimental cage to a fresh cage and observed until the termination of the experiment on day 20 *post coitum*. Females of Group VI was left undisturbed till day 20 *post coitum* (Untreated control). Vaginal smear was examined daily from all females. Presence of abundant irregular-shaped, nonnucleated, cornified squamous epithelial cells in the smear was taken as an indication of pregnancy block and return to estrus (Thomas and Dominic 1987a; McLean et al. 2012). Vaginal smear with abundant leucocytes and mucus was taken as an indication of pregnancy/pseudopregnancy. The number of females that were pregnant after showing estrous smear and the number of pups delivered by females that did not exhibit estrous smear were recorded.

Statistical analysis

The data were analyzed using Fisher's exact test.

Result

P value of pair-wise comparison of the incidence of pregnancy block using Fisher's exact test revealed that there was statistically significant

variation when Group IV was compared with Groups I and II; but Group IV showed no statistically significant variation when compared with Groups III, V and VI. Similarly, *P* value of pair-wise comparison of the incidence of pregnancy block using Fisher's exact test revealed that there was statistically significant variation when Group V was compared with Groups I and II; but Group V showed no statistically significant variation when compared with Groups III, IV and VI.

Table 1: Effect of exposure of different pheromonal and nonpheromonal cues on the pregnancy of inseminated females

Groups	Treatment	No. of females selected (*n*)	Results		
			Pregnant *n* (%)	Pregnancy block *n* (%)	No. of pups delivered (*n*)
Group I	Exposed to the confined alien male for 48 h	6	0 (0)	6 (100)	0
Group II	Exposed to the confined strange male for 48 h	6	1 (16.67)	5 (83.33)	2
Group III	Exposed to the confined stud male for 48 h	6	5 (83.33)	1 (16.67)	45
Group IV	Exposed to groundnut oil for 48 h	6	6 (100)	0 (0)	48
Group V	Exposed to gingelly oil for 48 h	6	6 (100)	0 (0)	47
Group VI	Left undisturbed until the termination of the experiment (control)	6	6 (100)	0 (0)	49

P value using Fisher's exact test of Group IV with Groups I, II, III, V and VI
Group IV vs. Group I=**0.0022**
Group IV vs. Group II=**0.0152**
Group IV vs. Group III=1.0000
Group IV vs. Group V=1.0000
Group IV vs. Group VI=1.0000

P value using Fisher's exact test of Group V with Groups I, II, III, IV and VI
Group V vs. Group I=**0.0022**
Group V vs. Group II=**0.0152**
Group V vs. Group III=1.0000
Group V vs. Group IV=1.0000
Group V vs. Group VI=1.0000

This study indicates that there is no significant variation in the pregnancy of females exposed to groundnut oil and gingelly oil. The pregnancy exhibited by females in these groups (IV and V) is not significantly different from the pregnancy exhibited by females exposed to confined stud males and females left undisturbed (Group VI) but the results significantly vary with the pregnancy observed in females exposed to confined alien and strange males.

The females that exhibited cornified epithelial smear did not show any sign of pregnancy whereas those females that failed to show estrous smear continued their pregnancy and delivered a varying number (7–10) of pups (Table 1).

Discussion

The present results confirm earlier reports (Bruce 1959; Dominic 1966a) that exposure to a male other than the stud male induces implantation block in inseminated females. Exposure to alien males resulted in a higher rate of pregnancy failure than that observed in females exposed to strange males. This is in conformity with earlier reports (Bruce and Parkes 1961; Thomas 1989). The present study corroborates earlier reports (Bruce 1959) that a male belonging to another strain from that of the stud male (alien male) is more efficient in inducing pregnancy block than a male belonging to the same strain of the stud male (strange male). Parkes and Bruce (1961) suggested that greater effectiveness of an alien male to induce pregnancy failure in newly inseminated females is due to the higher genetic dissimilarity between the alien male and the stud male.

Re-exposure to the stud male after 24 h of separation did not result in implantation failure in inseminated females. This indicates the ability of females to discriminate the coital partner from other males (alien or strange). It is suggested that the newly inseminated female is able to identify the stud male through the individual odors and this forms the basis of the

differential response of the female to the stud male and another male (Parkes and Bruce 1961; Dominic 1969). Bowers and Alexander (1967) reported that mice show exceptional ability to recognize and differentiate individuals based on olfactory cues. It has been suggested that the ability of female mice to distinguish individual male odor is based on the genetic variability at T locus (Lenington and Egid 1985). Yamasaki et al. (1980) reported that variability at the major histocompatibility complex (MHC) is the source of individual odors. Evidences demonstrate that mating forms a recognition memory of the pheromones of the stud male in the female and thus they lose their capacity to induce implantation failure in its mating partner (Keverne and de la Riva 1982; Thomas and Dominic 1987a; Brennan and Keverne 1997). Recent studies indicate that pheromonal memory is encoded in the AOB and this memory selectively inhibits firing of the mitral cells (MC) resulting in the suppression of neuroendocrine responses that induce implantation failure (Brennan et al. 1990; Ichikawa 2003; Gao et al. 2017).

The female exhibits implantation failure only if there is some 'strangeness' in the odor of the second male (Rogers and Beauchamp 1976). It has been shown that males familiar to the females are less effective in inducing pregnancy block in females (Thomas and Dominic 1989a). Sahu and Dominic (1985) housed females with confined alien males a few days before mating. When they were re-exposed to the female after mating the percentage of pregnancy block was less as compared with females exposed to unfamiliar alien males. Thomas and Dominic (1989b) demonstrated that immediate post-copulatory exposure to alien males depressed the effectiveness of alien males to induce the Bruce effect in the majority of the females tested. However, exposure of inseminated females after 2 hours resulted in 43% of pregnancy block and exposure after 6 hours induced more than 60% of implantation failure. The authors argued that the ability

of females to differentiate the odors of the stud male from alien male develops slowly after mating and any interference of alien male odor during the sensitive period (critical period) depressed the implantation blocking effect of alien males. All these evidences indicate that inseminated females get imprinted with the odor of the stud male and the females have the ability to differentiate the odors of other males comparing the imprinted odor of the stud male.

Pericopulatory exposure of inseminated females to alien male odors was shown to depress the Bruce effect in other rodent species like prairie vole *Microtus ochrogaster* (Gray et al. 1974; Dewsbury and Baumgardener 1981). However, there are other rodent species that exhibit implantation block on exposure to strange males during the pericopulatory period. Newly inseminated females of deer mouse *Peromyscus maniculatus* (Dewsbury 1982) and Djungarian hamsters *Phodopus campbelli* (Wynne-Edwards and Lisk 1984) showed pregnancy failure on exposure to strange males during pericopulatory period. The plasticity in the formation of the olfactory memory of the stud male in inseminated females may account this differential response.

Exposure of inseminated females to groundnut oil or gingelly oil did not influence the pregnancy of the newly inseminated females. All females exposed to groundnut oil or gingelly oil completed the term of pregnancy and delivered healthy pups, indicating that there was no adverse impact on the pregnancy of females exposed to oils.

Summary

Incidence of the Bruce effect in BALB/c females was investigated in this study. Implantation failure was observed in a large proportion of inseminated females of BALB/c on exposure to alien male or to the strange male.

Effectiveness of strange males in inducing implantation block was lesser than that of alien males. Here also re-exposure to the stud male after separation for 24 h did not result in the implantation block. Exposure to groundnut oil or gingelly oil did not affect implantation in BALB/c females. All females exposed to these oils delivered healthy pups on completion of their term of pregnancy.

Chapter 2

Exposure of a nonpheromonal cue during mating and its effect on inseminated females exposed to an alien male

Chapter 2

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Introduction

Pheromones are substances that are secreted externally by an individual and elicit a specific response when perceived by another individual of the same species (Karlson and Butenandt 1959). Alien male-induced implantation failure is a well-studied pheromonal effect in mice (Bruce 1959; Dominic 1987; Archunan 2014). Exposing a newly inseminated female to alien male results in implantation failure and revert the female to estrus stage (Bruce 1959). Re-exposure to the stud male 24 h after mating did not induce implantation failure (Parkes and Bruce 1961). Pregnancy failure was not observed when the newly inseminated female was exposed to alien male in the presence of the stud male or its urine (Thomas and Dominic 1987a). It is suggested that inseminated mouse gets imprinted with the odor of the stud male and so responds differentially to a strange male than the stud male (Parkes and Bruce 1961; Thomas and Dominic 1987a). This implies that each male has its signature odor to be imprinted on females.

The efficacy of the odor of the stud male in protecting pregnancy in newly inseminated female mice is well documented in several other contexts. For example, Kumar and Dominic (1996) demonstrated that the presence of the stud male significantly reduces implantation failure in newly inseminated females exposed to the male rat. Nutritional stress or food deprivation during the first 2 days of pregnancy significantly affects implantation ratio in laboratory mice (Bruce 1963; McClure 1963; Sahu and Dominic 1985). But a significant reduction in implantation failure is found in female mice when they were housed with stud male, even when the females were food deprived of food for 48 h (Archunan and Dominic 1989).

The present study addressed the question of whether it is possible to imprint the female mouse with a nonpheromonal cue during the pericopulatory sensitive period. If imprinting is possible, re-exposure to the imprinted nonpheromonal cue will protect pregnancy on exposure to an alien male, in the absence of the stud male. Finally, the critical time point and the role of direct contact with nonpheromonal cue for imprinting were also evaluated.

Methodology

100 adult virgin females and 14 adult males (10–20 weeks; BALB/c) were purchased from Kerala Veterinary and Animal Sciences University. Fourteen adult wild strains of male mice were collected from houses in and around Thrissur. Different stages of the estrous cycle were monitored daily using a standard vaginal smear technique. Two drops of food-grade groundnut oil smeared cotton ball was used to provide the nonpheromonal cue.

There were 3 experiments. In each experiment, 30 adult virgin females with regular estrus cycle were divided into 5 groups comprising 6 females in each group (Groups I–V). Their behavior was observed using an infrared camera connected to a monitor kept in the adjacent room.

Urine collection and exposure

Two drops of urine from the stud male were smeared on a fresh cotton ball and placed on the bedding of the cage, allowing the female to have direct contact with it.

Experiment 1: Effect of a nonpheromonal cue on alien male-induced implantation failure

All females were monogamously paired with adult males and allowed to mate (Groups I–V). In Group III, a cotton ball smeared with groundnut oil was provided at the time of pairing. In Group IV, the mating pair was exposed a fresh cotton ball without groundnut oil. Females with vaginal plug (day 0

post coitum) were separated from the stud male and housed individually in a new cage with fresh bedding. After 24 h (day 1 *post coitum*), they were subjected to the following treatments:

Group I: exposed to a confined alien male in the presence of confined stud male, allowing contact with urine and excreta of both males.

Group II: exposed to a confined alien male, allowing contact with its urine and excreta and exposed to a cotton ball smeared with the urine of the stud male.

Group III: exposed to a confined alien male allowing contact with its urine and excreta and exposed to a cotton ball smeared with groundnut oil.

Group IV: exposed to a confined alien male allowing contact with its urine and excreta, and exposed to a cotton ball without groundnut oil.

Group V: Female was left undisturbed (untreated control).

After 48 h, all females were returned to their cages and housed individually till the termination of the experiments (day 5 *post coitum*).

Experiment 2: Critical period for imprinting of a nonpheromonal cue

Group I: The mating pair was exposed to a cotton ball smeared with 2 drops of groundnut oil during free interaction (pre-mating exposure). On initiation of copulation, the male and the female were transferred to a new cage and allowed to mate. They were observed until the formation of the vaginal plug and after 1 h, the female was transferred to a new cage with fresh bedding.

Group II: One hour after vaginal plug formation was confirmed in female, the pair was exposed to a cotton ball smeared with groundnut oil (post- mating exposure). After 2 h, the female was transferred to a new cage with fresh bedding.

Group III: Female was exposed to a cotton ball smeared with groundnut oil on the initiation of copulation (pericopulatory exposure). The formation of the vaginal plug was confirmed and after 1 h of the post-mating period, the female was transferred to a new cage with fresh bedding.

Group IV: Female was exposed to a cotton ball without groundnut oil on initiation of copulation (pericopulatory exposure). The formation of the vaginal plug was confirmed and after 1 h of the post-mating period, the female was transferred to a new cage with fresh bedding.

Group V: After mating and formation of the vaginal plug, female was separated from the stud male and kept undisturbed until the termination of experiment (untreated control).

After 24 h (day 1 *post coitum*), the newly inseminated females in all groups except those in Groups IV and V, were exposed to a confined alien male in presence of a fresh cotton ball smeared with groundnut oil for 48 h. Females in Group IV were exposed to alien male in the presence of a cotton ball without groundnut oil. Females in Group V were left undisturbed. Experiments were terminated on day 5 *post coitum.*

Experiment 3: The role of contact during imprinting of nonpheromonal cue

All females were monogamously paired with adult males and allowed to mate (Group I-V). In Group II and Group III, a cotton ball smeared with groundnut oil was provided at the time of pairing and females were allowed to have direct contact (non-volatile and volatile cues) with it during mating. In Group IV, a cotton ball smeared with groundnut oil was provided at the time of pairing on which the female had no direct contact with it. Females with vaginal plug (day 0 *post coitum*) were separated from the stud male and housed individually in new cages with fresh bedding. After 24 hours (day 1

post coitum) they were subjected to the following treatments:

Group I: Female was exposed to a confined alien male in the presence of confined stud male, allowing contact with the urine and excreta of both males.

Group II: Female was exposed to a confined alien male allowing contact with its urine and excreta and exposed to a cotton ball smeared with groundnut oil on which the female had no direct contact.

Group III: Female was exposed to a confined alien male allowing contact with its urine and excreta and exposed to a cotton ball smeared with groundnut oil on which the female had direct contact.

Group IV: Female was exposed to a confined alien male allowing contact with its urine and excreta and exposed to a cotton ball smeared with groundnut oil on which the female had no direct contact.

Group V: Female was left undisturbed (untreated control).

After 48 h, all females were returned to their cages and housed individually until the termination of the experiments (day 5 *post coitum*).

All females were housed separately in their home cages up to day 20 *post coitum* to ascertain their reproductive status. Vaginal smear was examined daily from all females of Experiments 1, 2 and 3. Presence of abundant irregular-shaped, nonnucleated, cornified squamous epithelial cells in the smear was taken as the indication of pregnancy block and return to estrus (Thomas and Dominic 1987a; McLean et al. 2012). Vaginal smear with abundant leucocytes and mucus was taken as the indication of pregnancy/pseudopregnancy. The number of females that were pregnant after showing estrus smear and the number of pups delivered by females that did not exhibit estrus smear were recorded.

Statistical analysis

Fisher's exact test was used for analyzing the data.

Result

Experiment 1: Effect of nonpheromonal cue on alien male-induced implantation failure

P value of pair-wise comparison of the incidence of pregnancy using Fisher's exact test revealed that there was no statistically significant variation when Group III was compared with Groups I, II, and V; but Group III showed a very statistically significant variation compared with Group IV.

Table 1: Protective effect of nonpheromonal cue on alien male-induced implantation failure.

Groups	Treatment	No. of females (*n*)	Results		
			Pregnancy *n* (%)	Pregnancy block *n*(%)	No. of pups delivered (*n*)
Group I	Exposed to alien male along with stud male	6	6 (100)	0 (0.0)	47
Group II	Exposed to alien male along with urine of stud male	6	5 (83.3)	1 (16.7)	38
Group III	Exposed to alien male along with a cotton ball smeared with groundnut oil	6	6 (100)	0 (0.0)	48
Group IV	Exposed to alien male along with a cotton ball without groundnut oil	6	0 (0.0)	6 (100)	0
Group V	Untreated control	6	6 (100)	0 (0.0)	49
P value using Fisher's exact test of Group III with other groups Group III with Group I=1.000 Group III with Group II=1.000 Group III with Group IV=**0.0022** Group III with Group V=1.000					

The nonpheromonal cue used in this study (groundnut oil) is capable of protecting the female from alien male-induced implantation failure (Group III; Table 1). This is not, however, significantly different from Group I (exposed to the alien male in the presence of the stud male), or Group II (exposed to the alien male in presence of the stud male's urine) or Group V that was left undisturbed during the post-mating period. The females in Group IV, which were exposed to alien male allowing free access to a cotton ball without nonpheromonal cue in the absence of the stud male or its urine showed a significantly higher rate of implantation failure.

Experiment 2: Critical period for imprinting of a nonpheromonal cue

P value of pair-wise comparison of the incidence of pregnancy using Fisher's exact test revealed that there was a very statistically significant variation when Group III compared with Groups I, II, and IV; but Group III showed no statistically significant variation compared with Group V.

Table 2: Critical period for imprinting of a nonpheromonal cue

Groups	Treatment	No. of females (*n*)	Results		
			Pregnancy *n* (%)	Pregnancy block *n* (%)	No. of pups delivered (*n*)
Group I	Pre-mating exposure to a cotton ball smeared with groundnut oil	6	1 (16.7)	5 (83.3)	7
Group II	Post mating exposure to a cotton ball smeared with groundnut oil	6	0 (0.0)	6 (100)	0
Group III	Pericopulatory exposure to a cotton ball smeared with groundnut oil	6	6 (100)	0 (0.0)	44
Group IV	Pericopulatory exposure to a cotton ball without groundnut oil	6	1 (16.7)	5 (83.3)	8
Group V	Untreated control	6	6 (100)	0 (0.0)	47
P value using Fisher's exact test of Group III with other groups Group III with Group I=**0.0152** Group III with Group II=**0.0022** Group III with Group IV=**0.0152** Group III with Group V=1.000					

The nonpheromonal cue exposed during copulation (initiated with lordosis behavior and ended in ejaculation and formation of the vaginal plug) protected the pregnancy in females on re-exposure to the same cue at the time of exposure to the alien male (Group III; Table 2). This is not statistically different from pregnancies observed in inseminated females which were left undisturbed after mating (Group V). Females exposed to the nonpheromonal cue during pre- (Group I) or post-copulatory periods (Group II) exhibited pregnancy failure when exposed to the alien males.

Most of the females exposed to the alien males in the presence of a cotton ball without the nonpheromonal cue (Group IV) returned to estrus stage, terminating their pregnancies.

Experiment 3: The role of contact during imprinting

P value of pair-wise comparison of the incidence of pregnancy using Fisher's exact test revealed that there was a very statistically significant variation when Group III compared with Groups II, and IV; but Group III showed no statistically significant variation compared with Group I and V.

Table 3: Protective effect of nonpheromonal cue on alien male induced implantation failure- Role of contact

Groups	Treatment	No. of females (n)	Results		
			Pregnancy *n* (%)	Pregnancy block *n* (%)	No. of pups delivered (*n*)
Group I	Exposed to alien male along with stud male	6	6 (100)	0 (0.0)	48
Group II	Exposed to alien male along with a cotton ball smeared with groundnut oil on which the female had no direct contact	6	1 (16.7)	5 (83.3)	3

Group III	Exposed to alien male along with a cotton ball smeared with groundnut oil on which the female had direct contact	6	6 (100)	0 (0.0)	50
Group IV	Exposed to alien male along with a cotton ball smeared with groundnut oil on which the female had no direct contact	6	0 (0.0)	6 (100)	0
Group V	Untreated control	6	6 (100)	0 (0.0)	51
P value using Fisher's exact test of Group III with other groups Group III with Group I=1.0000 Group III with Group II=**0.0152** Group III with Group IV=**0.0022** Group III with Group V=1.0000					

The nonpheromonal cue, the groundnut oil on which the female had a direct contact is capable of protecting the female from alien male-induced implantation failure (Group III; Table 3). This is not, however, significantly different from Group I (exposed to the alien male in the presence of the stud male), or Group V that was left undisturbed during post-mating period. The female of Group II which had a direct contact with the nonpheromonal cue during mating and no direct contact with it during exposure to alien male and females in Group IV, which were exposed to alien male allowing free access to a cotton ball without nonpheromonal cue in the absence of the stud male or its urine showed significantly higher rate of implantation failure.

In all experiments, the females that exhibited cornified epithelial smear did not show any sign of pregnancy whereas those females that failed to show estrous smear continued their pregnancy and delivered varying number (7–9) of pups (Tables 1, 2 and 3)

Discussion

Present results unequivocally demonstrate that the virgin females allowed to mate in the presence of a nonpheromonal cue, retained their pregnancy when

they were re-exposed to the nonpheromonal cue during exposure to the alien males in the absence of their stud males. The majority of these females retained their pregnancy, which is comparable with pregnancy observed in inseminated females exposed to alien males in the presence of their stud males or their urine. This indicates that a nonpheromonal cue exposed to females at the time of mating is imprinted in the females and re-exposure to the same cue protects the females from alien male-induced implantation failure. In contrast, females allowed to mate with males in the presence of a cotton ball without nonpheromonal cue exhibited a significantly higher rate of pregnancy failure when exposed to alien males. It should be remembered that this effect is not specific to groundnut oil. Exposure to gingelly (*Sesamum indicum*) oil during the mating period is also capable of protecting the pregnancy of the inseminated females exposed to alien males (unpublished data).

It is generally believed that a female is imprinted specifically with the individual odor of the stud male and the female has the ability to discriminate him at least for a few days after the original mating and respond differentially to the odor of the stud male and to the odor of other males (Parkes and Bruce 1961; Thomas and Dominic 1987c). It has been demonstrated that pheromones other than the stud male can interfere with the imprinting of the odor of the stud in the inseminated female. Thomas and Dominic (1989b) reported that the ability of alien males to induce implantation failure in inseminated females is significantly reduced when they were exposed to alien males immediately after insemination.

Presence of the stud male during exposure to the alien male considerably lowers the incidence of implantation failure in inseminated females (Thomas and Dominic 1986, 1987a). It is suggested that the stud male could protect the newly inseminated female from implantation failure because its

pheromones have a luteotrophic effect in inseminated females (Thomas and Dominic 1987b, 1987c; Archunan 2014). The luteotrophic effect of the cues associated with the stud male is further demonstrated in a study in which a majority of the pregnancy-blocked females when re- exposed to confined stud males exhibited pseudopregnancy (Thomas and Dominic 1987c), indicating that the olfactory memory of the stud male is retained in the females for a long time after the actual mating.

The protective effect of the nonpheromonal cue against the implantation blocking influence of the alien male shows that nonpheromonal cue can be imprinted in the memory of the inseminated female provided the female is exposed to it during the mating process. In other words, the female identifies the stud male and shows differential response to the stud male and another male, not because of the specificity of the individual odor of the stud male, but because of the associative learning that takes place during the mating time.

A critical period or sensitive period is one of the major characteristic features of imprinting and in most cases it takes place during certain unique experience of the individual. Precocial birds are shown to get imprinted with any moving object that they see first and exhibit 'following behavior', an instinctive behavior usually exhibited by the chicks to their mother (Spalding 1872; Lorenz 1961), indicating that animals can be imprinted with any stimulus with certain characteristic features, if they were exposed to it during the sensitive period. In precocial birds, movement is the characteristic feature to which the young pay attention to than the shape or the color of the stimulus.

In this study, the protective effect of the nonpheromonal cue against the pregnancy blocking influence of the alien male is observed only when the nonpheromonal cue is exposed to females during pericopulatory sensitive

period (Experiment 2; Group III). The majority of the females exposed to the nonpheromonal cue during the pre or post-copulatory interactions, (Experiment 2; Groups I and II), exhibited a higher rate of pregnancy failure on re-exposure to the same cue at the time of exposure to the alien males. It seems that vaginocervical stimulation is the key factor in imprinting the nonpheromonal cue in these females. In sheep, studies demonstrate that sudden rise in estrogen in females just before parturition and vaginocervical stimulation during the expulsion of the fetus plays a significant role in imprinting the odor of the neonate in the mother (Kendrick et al. 1991; Nowak et al. 2011). It was revealed that in mice the formation of pheromone-induced olfactory memory in AOB depends on vaginocervical stimulation during mating with the stud male (Otsuka et al. 2001; Ichikawa 2003).

This study on the protective effect of a nonpheromonal cue against implantation failure induced by an alien male indicates that the nonpheromonal cue the groundnut oil, which the females experience during pericopulatory sensitive period has the ability to act as a luteotrophic signal in inseminated mice. However, imprinting of the nonpheromonal cue is possible only if the female is allowed direct contact (the nonvolatile and volatile cues) with the nonpheromonal cue during pericopulatory sensitive period. In other words, exposure of the female, only to the volatile cues associated with the groundnut oil will not imprint the odor in the inseminated female. Similarly, contact with the nonvolatile cues of the nonpheromonal cue is essential for inducing protective effect against implantation block by alien male. To the best of our knowledge, this is the first report that demonstrates the effectiveness of an imprinted nonpheromonal cue in protecting pregnancy of a female against male-induced implantation failure (the Bruce effect). This study shows that ambient chemical cues or nongenetically determined odors that the female mouse

perceives at the time of mating may blend with the genetically determined pheromonal cues of the stud male in forming a chemical signature of the stud male (Wyatt 2010). The female learns or gets imprinted with this chemical signature, rather than only with the pheromones of the stud male. It is important to note that the nonpheromonal cue, once imprinted, acts analogous to the stud male pheromones, and may form a memory in the olfactory system offering protection against the Bruce effect. However, the mechanism involved in the protective effect of nonpheromonal cue is presently unknown.

Summary

Bruce effect or alien male-induced implantation failure is a well-studied phenomenon in mice. The presence of the stud male during exposure to an alien male protects the female from implantation failure. The pheromones of the stud male are imprinted in the female at the time of mating and act as a luteotrophic agent. The hypothesis whether a nonpheromonal cue exposed to the female during pericopulatory sensitive period could protect pregnancy in newly inseminated females exposed to alien males was tested. Virgin females were allowed to mate in the presence of a cotton ball smeared with groundnut oil as a nonpheromonal cue. When these females were exposed to alien males in presence of groundnut oil, the majority of the females retained their pregnancy. We evidenced that a nonpheromonal cue could possibly protect the female from alien male-induced implantation failure. The majority of the females exposed to the nonpheromonal cue during the pre- or post-copulatory interactions with the males exhibited a higher rate of pregnancy failure on re-exposure to the same cue at the time of exposure to alien males. The protective effect of the nonpheromonal cue is observed only when the female is exposed to it during the pericopulatory sensitive period. However, imprinting of the nonpheromonal cue is possible only if the

female is allowed direct contact (the nonvolatile and volatile cues) with the nonpheromonal cue during pericopulatory sensitive period. In other words, exposure of the female, only to the volatile cues associated with the groundnut oil will not imprint the odor in inseminated female. Similarly, contact with the nonvolatile cues of the nonpheromonal cue is essential for inducing the protective effect against implantation block by the alien male.

Chapter 3

Induction of pseudopregnancy in implantation blocked females on exposure to an imprinted nonpheromonal cue

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Introduction

Chemical communication is one of the major pathways through which animals share information with other organisms living around them. Karlson and Butenandt (1959) identified a chemical substance silkworm moth uses for intraspecific communication and named it "pheromone" (Karlson and Luscher 1959). Bruce in 1959 reported the existence of pheromones that induce implantation failure in female mice exposed to a male other than its original mating partner. It is suggested that mating forms a recognition memory of the pheromones of the stud male in the female and thus they lose their capacity to induce implantation failure in its mating partner (Thomas and Dominic 1987a; Brennan and Keverne 1997). Recent evidence indicate that pheromonal memory is encoded in the AOB and this memory selectively inhibits the firing of the mitral cells (MC), resulting in the suppression of the neuroendocrine responses that induce implantation failure (Brennan et al. 1990; Ichikawa 2003; Gao et al. 2017). It has been shown that implantation failure in inseminated females exposed to alien males is due to the depression of hypophysial prolactin secretion and the subsequent failure of corpora lutea (Dominic 1966b, 1970; Keverne 1983). However, the presence of the stud male or its urine during exposure to an alien male protects the female against implantation failure (Thomas and Dominic 1987a). It is suggested that the presence of the stud male or its urine has a luteotrophic effect in newly inseminated female mouse (Thomas and Dominic 1987b; Archunan 2014). This is further supported by the report that re-exposure of implantation-blocked females to the stud male or its urine results in pseudopregnancy in the majority of females (Thomas and Dominic 1987b).

This is true irrespective of whether the original pregnancy is terminated by exposure to an alien male or by a single injection of bromocriptine, a dopamine agonist (Thomas and Dominic 1987c).

It is demonstrated that a nonpheromonal cue (groundnut oil) exposed to the female during the pericopulatory period is capable of protecting the female from the alien male-induced implantation failure, the Bruce effect (Thomas et al. 2018). The authors suggested that the nonpheromonal cue exposed to the female during the pericopulatory sensitive period blend with the individual pheromone of the stud male forming its chemical signature. It is important to note that the nonpheromonal cue acts analogous to the stud male pheromones, offering protection against the Bruce effect (Thomas et al. 2018).

The present study is designed to evaluate whether the nonpheromonal cue imprinted in the female during pericopulatory period is capable of inducing pseudopregnancy in implantation-blocked females. We hypothesized that if the imprinted nonpheromonal cue acts analogous to the pheromones of the stud male, then exposure of implantation-blocked females to the nonpheromonal cue would induce pseudopregnancy, indicating its luteotrophic effect. This hypothesis was tested in female mice whose implantations were blocked by exposure to alien males or by treatment with bromocriptine.

Methodology

Sixty adult virgin females and 15 adult males (10–20 weeks; BALB/c) were purchased from Kerala Veterinary and Animal Sciences University. Fourteen

adult wild strain male mice were collected from houses in and around Thrissur.

All females with regular estrous cycles were then allowed to mate with adult males in the presence of a cotton ball smeared with 2 drops of food-grade groundnut oil as a nonpheromonal cue. Mated females, indicated by the presence of vaginal plug (day 0 *post coitum*), were divided into two categories (Table 1). On day 1 *post coitum* inseminated females of category 1 were individually exposed to confined alien males to induce implantation block and the females of category 2 were treated with bromocriptine for terminating their pregnancy.

Preparation of bromocriptine for injection

Bromocriptine mesylate (European Pharmacopoeia Reference Standard) was dissolved at a concentration of 5mg/mL consisting of 500µL of 70% alcohol and 500µL of sterile water. All injections were given subcutaneously at a dose of 16.7mg/kg of body weight.

Tables 1: **Induction of implantation block in inseminated females**

Category	Treatment	Total no. of females (*n*)	No of inseminated females (*n*)	No of implantation blocked females (*n*)
Category 1	Exposure to alien male	30	29	27
Category 2	Injection of bromocriptine	30	28	27

After 48 h (day 3 *post coitum*), 24 females from each category that exhibited implantation failure was selected and divided into 4 groups comprising 6 females in each group (Groups I-IV) for further treatments.

Experiment 1: Effect of re-exposure to an imprinted nonpheromonal cue in implantation blocked (induced by exposure to alien male) females

Group I: Re-exposed to the confined stud male

Group II: Re-exposed to a cotton ball smeared with 2 drops of groundnut oil

Group III: Re-exposed to the confined alien male

Group IV: Left undisturbed

Experiment 2: Effect of re-exposure to an imprinted nonpheromonal cue in implantation blocked (induced by injection of bromocriptine) females

Group I: Re-exposed to the confined stud male

Group II: Re-exposed to a cotton ball smeared with 2 drops of groundnut oil

Group III: Re-exposed to the confined alien male

Group IV: Left undisturbed

Different stages of estrous cycle were monitored daily using vaginal smear technique (McLean et al. 2012). Presence of abundant irregular shaped, non-nucleated, cornified squamous epithelial cells in the smear was taken as the indication of implantation block and return to estrus (Thomas and Dominic 1987a; McLean et al. 2012). Vaginal smear with abundant leucocytes and mucus was taken as the indication of pregnancy/pseudopregnancy.

On day 7 *post coitum*, animals were anesthetized (according to the guidelines of CPCSEA) by subcutaneous injection of atropine sulphate at 0.02mg/kg and after 15 min, a combination of xylaxine at 10mg/kg plus ketamine hydrochloride at 80mg/kg were administered intraperitonially. The right uterine horns of all females were traumatized by passing 3–4 transverse loops of thread across the lumen of the uterus. After 4 days, on day 11 *post coitum*, females were sacrificed and their uteri were examined for the presence of pseudopregnancy. Females with positive decidual cell reaction in traumatized right uterine horn were considered to be pseudopregnant. Pregnancy-blocked females, which were left undisturbed, were sacrificed on

day 11 *post coitum* and their uteri were examined to rule out pregnancy/pseudopregnancy (Thomas and Dominic 1987c).

Statistical analysis

Fisher's exact test was performed for analyzing the data.

Result

The majority of the implantation-blocked females (induced either by alien male exposure or by injection of bromocriptine) exhibited pseudopregnancy following re-exposure to the stud male or to the imprinted nonpheromonal cue (Group I and Group II of Tables 2 and 3, Plate I). By contrast, re-exposure of implantation-blocked (either by alien male or by bromocriptine) females to alien male or those females left undisturbed (Group III and Group IV of Tables 2 and 3, Plate II) failed to exhibit pseudopregnancy.

Table 2: Induction of pseudopregnancy on re-exposure of imprinted nonpheromonal cue in implantation-blocked (induced by alien male) females

Groups	Treatment	No. of females (*n*)	Results	
			Females with pseudopregnancy *n* (%)	Females without pseudopregnancy *n* (%)
Group I	Re-exposed to confined stud male	6	6 (100)	0 (0.0)
Group II	Re-exposed to cotton ball smeared with groundnut oil	6	5 (83.3)	1 (16.7)
Group III	Re-exposed to confined alien male	6	0 (0.0)	6 (100)
Group IV	Left undisturbed after implantation block	6	0 (0.0)	6 (100)
P value using Fisher's exact test of Group II with other groups Group II with Group I=1.000 Group II with Group III=**0.0152** Group II with Group IV=**0.0152**				

P value of pair-wise comparison of the incidence of pseudopregnancy using Fisher's exact test revealed that there was no statistically significant variation when Group II was compared with Group I; but Group II showed a statistically significant variation compared with Groups III and IV.

Re-exposure of the imprinted nonpheromonal cue (groundnut oil) is capable of inducing pseudopregnancy in female mice whose implantations were blocked by an alien male (Group II; Table 2). This is not significantly different from Group I (exposed to confined stud male). The females in Group III (exposed to confined alien male) failed to exhibit pseudopregnancy and showed statistically significant variation with Group II. No implanted embryos were found in the uterus of females left undisturbed after implantation block (Group IV)

Table 3: Induction of pseudopregnancy by re-exposure of imprinted nonpheromonal cue in implantation-blocked (induced by injection of bromocriptine) females

Groups	Treatment	No. of females (*n*)	Results	
			Females with pseudopregnancy *n* (%)	Females without pseudopregnancy *n* (%)
Group I	Re-exposed to confined stud male	6	5 (83.3)	1 (16.7)
Group II	Re-exposed to cotton ball smeared with groundnut oil	6	5 (83.3)	1 (16.7)
Group III	Re-exposed to confined alien male	6	0 (0.0)	6 (100)
Group IV	Left undisturbed after implantation block	6	0 (0.0)	6 (100)
P value using Fisher's exact test of Group II with other groups Group II with Group I=1.000 Group II with Group III=**0.0152** Group II with Group IV=**0.0152**				

P value of pair-wise comparison of the incidence of pseudopregnancy using

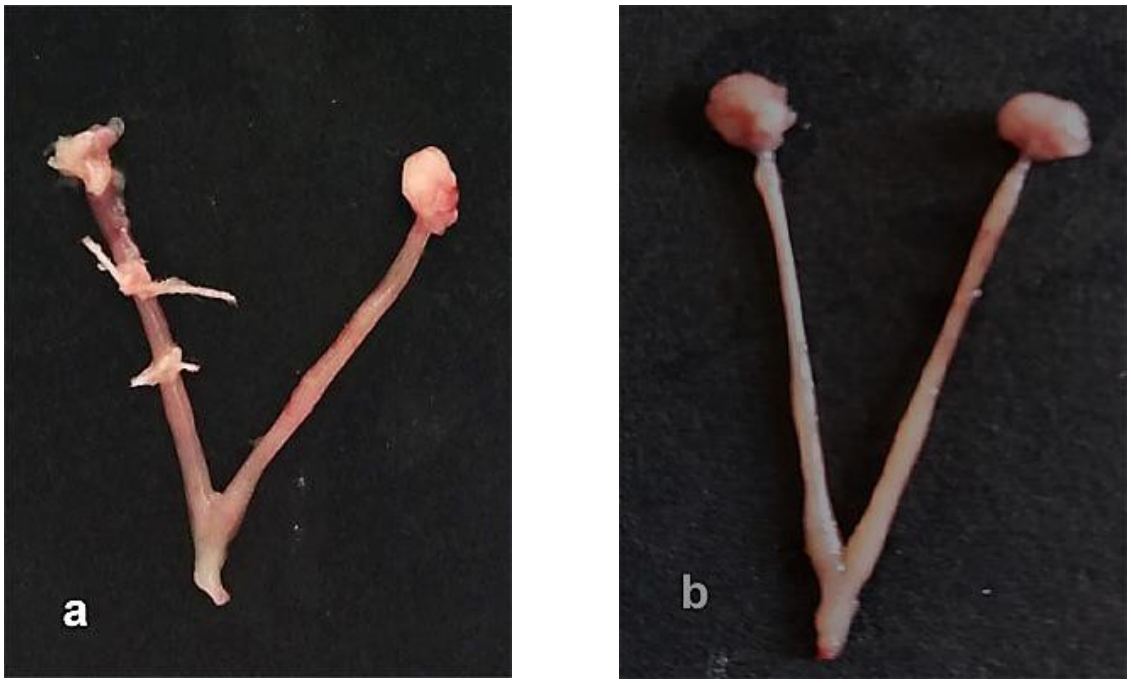
Fisher's exact test revealed that there was no statistically significant variation when Group II was compared with Group I; but Group II showed a statistically significant variation compared with Groups III and IV.

Re-exposure of the imprinted nonpheromonal cue (groundnut oil) is capable of inducing pseudopregnancy in female mice whose pregnancies were terminated by injection of bromocriptine (Group II; Table 3). This is not, however, significantly different from Group I (exposed to confined stud male). The females in Group III (exposed to confined alien male) failed to exhibit pseudopregnancy and showed statistically significant variation with Group II. No implanted embryos were found in the uterus of females left undisturbed after implantation block (Group IV).

PLATE – I

Uterus with pseudopregnancy. a) Decidual cell reaction in traumatized uterus of pregnancy blocked females on re-exposure to stud males. b) Decidual cell reaction in traumatized uterus of pregnancy blocked females on re-exposure to the nonpheromonal cue.

PLATE – II



Uterus without pseudopregnancy a) traumatized uterus (without decidual cell reaction) of pregnancy blocked females on re-exposure to alien males. b) Uterus without implanted embryos in pregnancy blocked females left undisturbed.

Discussion

This study indicates that a nonpheromonal cue (groundnut oil) presented to the female during pericopulatory period is capable of inducing pseudopregnancy on re-exposure to the same cue after implantation block. The rate of pseudopregnancy observed in implantation blocked females re-exposed to the imprinted nonpheromonal cue is not significantly different from pseudopregnancy observed in pregnancy blocked females re-exposed to their stud males. This indicates that the new batch of corpora lutea formed after implantation block became functional on re-exposure to the imprinted nonpheromonal cue. Thomas and Dominic (1987b) reported that re-exposure of pregnancy-blocked females to their stud males induced pseudopregnancy in the majority of females tested. Since the stud male remained confined during the re- exposure period the possibility of vaginal stimulation or any other direct body contact contributing to the luteal function can be ruled out. However in the above study, the female had free access to the pheromones of the stud male. It is evident that the imprinted pheromones of the stud male acted as a luteotrophic agent in pregnancy-blocked females (Thomas and Dominic 1987c; Archunan 2014).

Thomas et al. (2018) demonstrated that a nonpheromonal cue presented to the female during pericopulatory period is capable of protecting the inseminated females against alien male-induced implantation block. In this study, pseudopregnancy was also observed in newly inseminated females whose pregnancies were terminated by exposure to alien male or by treatment with bromocriptine, on re-exposure to the imprinted nonpheromonal cue (groundnut oil) or to the stud male. This indicates that the olfactory cues presented to females during pericopulatory period, irrespective of whether it is the pheromones or nonpheromonal cues, exert luteotrophic effect in inseminated female mice. The possibility of associative

learning between the pheromones of the stud male and the nonpheromonal cues perceived by the female during pericopulatory period, offering luteotrophic ability to the nonpheromonal cue, cannot be ruled out in this context. It is also possible that the olfactory cues the female perceive during mating get integrated with the pheromonal cues of the stud male forming its chemical signature (Wyatt 2010).

It is well documented that during mating the female mice are imprinted with pheromones of the stud male (Thomas and Dominic 1987a, b). Evidences indicate that the pheromonal memory is stored in the AOB of the female (Kaba et al. 1989; Kaba and Keverne 1992). The formation of the pheromonal memory depends on vaginocervical stimulation during mating and prolonged exposure to the stud male pheromones (Keverne 1983; Kaba and Nakanishi 1995). The protective effect of nonpheromonal cues against the Bruce effect (Thomas et al. 2018) and the induction of pseudopregnancy reported in this study shows that the female learns or gets imprinted with this chemical signature, rather than only with the pheromones of the stud male (Thomas et al. 2018).

The failure of familiar males to protect newly inseminated females from implantation-blocking effect of the alien male (Thomas and Dominic 1989a) is often suggested as an example of the ability of females to identify the stud male as an individual. Yamazaki et al. (1980) suggested that an individual odor is determined by MHC genes in mice. It is often suggested that the differential response of the newly inseminated female to the stud male and another male is due to the ability of the female to identify the individual pheromones of the stud male (Thomas and Dominic 1987c). The results of the present study corroborate earlier suggestion that the ambient olfactory cues the female perceive during pericopulatory period get integrated with the olfactory memory of the pheromones of the stud male. Reactivation

of the imprinted olfactory memory by re-exposure to the imprinted nonpheromonal cue stimulates luteotrophic effect in females. This luteotrophic effect is responsible for the protective effect of nonpheromonal cue against the alien-male- induced implantation failure in female mice (Thomas et al. 2018) and induction of pseudopregnancy in implantation-blocked females as observed in this study. The memory of the imprinted nonpheromonal cue remains active even after blocking the pregnancy for several days after mating. Thomas and Dominic (1987c) demonstrated that the re-exposure to the stud male on day 4 *post coitum* induces pseudopregnancy in females whose pregnancies were terminated by a single injection of bromocriptine. However, lower incidence of pseudopregnancy was observed when they were re-exposed to the stud male beginning on the day of the second estrus, indicating the obliteration of olfactory memory by the onset of second estrus (Thomas and Dominic 1989). This is in contrast with the observation of Kaba et al. (1989) in which they reported that the pheromonal memory lasts at least for 30 days after mating.

The primary endocrine response of newly inseminated females exposed to alien males is shown to be the depression of hypophysial prolactin secretion and the subsequent failure of corpora lutea, resulting in implantation failure (Dominic 1966b, 1970; Keverne 1983). It is possible that the incidence of pseudopregnancy in implantation-blocked females results from the reactivation of the olfactory memory of the stud male which exerts luteotrophic effect in these females (Thomas and Dominic 1987c; Archunan 2014).

The neural mechanism involved in the formation of pheromonal memory of the mating partner in female mice has been elucidated in several studies. It has been shown that vaginocervical stimulation during mating decreased the dendrodendritic feedback inhibition of mitral/tufted (MT) cells in the AOB (Otsuka et al. 2001; Ichikawa 2003; Gao et al. 2017), leading to GABA-mediated inhibition of MT cells. This self-inhibition ultimately disrupts the pheromone signals of the stud male reaching the central brain, thus preventing the ability of its pheromone to induce implantation failure in its coital partner. Whether this alteration of neural mechanisms involved in the release of pituitary prolactin on exposure to the imprinted cue leading to the induction of pseudopregnancy in pregnancy blocked females needs to be studied further.

Summary

A nonpheromonal cue exposed during pericopulatory period gets imprinted in the female mice and offers protection against alien male- induced implantation failure. In this study whether the nonpheromonal cue imprinted during mating has any luteotrophic property is investigated by exposing implantation blocked females to the imprinted nonpheromonal cue. Here the pregnancy of newly inseminated females was blocked by exposure to alien male or by treatment with bromocriptine a dopamine agonist. It is found that the majority of these females exhibited pseudopregnancy on re-exposure to the imprinted nonpheromonal cue irrespective of whether the original pregnancy is blocked by exposure to alien male or injection of bromocriptine. The rate of pseudopregnancy seen in these females is similar to pseudopregnancy observed in implantation blocked females exposed to the stud males. The results confirm that during mating the female mouse gets imprinted with a blend of nonpheromonal cues along with the individual pheromones of the stud male. The imprinted nonpheromonal cue acts analogous to the pheromonal cues of the stud male in inducing pseudopregnancy in implantation blocked females.

Chapter 4

Influence of imprinted nonpheromonal cues on inseminated females exposed to stressful stimuli

Chapter 4

Influence of imprinted nonpheromonal cues on inseminated females exposed to stressful stimuli

Introduction

The efficacy of the odor of stud male in protecting newly inseminated female mice against male induced implantation failure is well documented (Parkes and Bruce 1961; Thomas and Dominic 1987a, b). Thomas et al. (2018) reported that a nonpheromonal cue (groundnut oil) exposed to the female during pericopulatory period acts analogous to the pheromones of the stud male. It has been shown that the imprinted nonpheromonal cues exposed to inseminated females are capable of protecting the females from implantation blocking effect of alien males. The present study is designed to test whether a nonpheromonal cue exposed to female mice during pericopulatory period is capable of protecting inseminated females from implantation failure in the presence of two different stressful stimuli -exposure to male rat and starvation.

In the wild, rats are natural predators of mice. deCatanzaro (1988) reported that inseminated female C57 mice housed with rats for 7 days produced fewer litters as compared with the control. Similarly, female mice exposed to the urine of male or female rat produced very few litters indicating that rat urine, irrespective of whether collected from male or female affected the reproductive outcome of female mice. Newly inseminated female mice of Parkes strain (P mice) exposed to male rat or to rat urine exhibited a significantly high rate of pregnancy failure (Kumar and Dominic 1996). However, inseminated female mice exposed to a female rat or to urine of a female rat exhibited a significantly lower rate of pregnancy block.

Newly inseminated females that were allowed to remain with their stud male during exposure to male rat or to the soiled bedding of male rat showed significantly lower rate of pregnancy failure as compared to pregnancy failure observed in females exposed to male rat or male rat soiled bedding without the stud male (Kumar & Dominic 1996). These authors suggested that the lower rate of pregnancy failure observed in females housed with the stud male during exposure to male rat soiled bedding is due to the luteotrophic effect of the odor of the stud male.

Food deprivation during the initial days of implantation is a very stressful stimulus to newly inseminated female mice and in the majority of females implantation fails and the female returns to estrus. Short-term complete deprivation of food during mating is shown to induce implantation failure in mice (McClure 1958). In a detailed study, McClure (1963) further demonstrated that complete starvation for 48 h from day 4 *post coitum* resulted in pregnancy failure in the majority of females. It was suggested that starvation depressed gonadotropin secretion resulting in the degeneration of the decidua. Sahu and Dominic (1986) reported that administration of exogenous prolactin prevented starvation-induced implantation failure in inseminated females. Ectopic pituitary graft was also shown to mitigate the effect of starvation-induced pregnancy block (Archunan and Dominic 1991). Archunan and Dominic (1989) demonstrated that exposure of stud male during the period of starvation prevented implantation failure in the majority of the inseminated females. The authors suggested that the activation of imprinted luteotrophic olfactory memory of pheromones of the stud male is responsible for prevention of implantation failure in food-deprived females.

The present investigations are designed to evaluate whether a nonpheromonal cue exposed to the female during the pericopulatory period could be able to protect the pregnancy of inseminated female mouse during exposure to

male rat odor and during the period of food deprivation. The hypothesis tested in this study is that if the imprinted nonpheromonal cue is analogous to the imprinted olfactory memory of pheromones of the stud male, then the inseminated females would be protected from the stressful effect of exposure to male rat odor and 48 h of starvation during pre-implantation period.

Methodology

70 adult virgin females, 14 adult males (10–20 weeks; BALB/c) and 14 adult rats (Wistar) were purchased from Kerala Veterinary and Animal Sciences University. Different stages of the estrous cycle were monitored daily using a vaginal smear technique (see general methodology for details).

Two experiments were conducted and in each experiment, 30 adult virgin females with regular estrus cycle were divided into 5 groups comprising 6 females in each group (Groups I–V). Their behavior was observed using an infrared camera connected to a monitor kept in the adjacent room. A cotton ball smeared with 2 drops of food-grade groundnut oil was used to provide a nonpheromonal cue. Two drops of urine from the stud male were smeared on a fresh cotton ball and placed on the bedding of the cage, allowing the female to have direct contact with it.

Experiment 1: Effect of a nonpheromonal cue on male rat-induced implantation failure

All females were monogamously paired with adult males and allowed to mate (Groups I–V). In Group III, a cotton ball smeared with groundnut oil was placed in the cage at the time of pairing. In Group IV, the mating pair was exposed to a fresh cotton ball without groundnut oil. Females with vaginal plug (day 0 *post coitum*) were separated from the stud male and housed individually in a new cage with fresh bedding. After 24 h (day 1 *post coitum*) they were subjected to the following treatments:

Group I: Female was exposed to a confined male rat in the presence of a confined stud male, allowing contact with urine and excreta of both males.

Group II: Female was exposed to a confined male rat, allowing contact with its urine and excreta and exposed to a cotton ball smeared with urine of the stud male.

Group III: Female was exposed to a confined male rat, allowing contact with its urine and excreta and exposed to a cotton ball smeared with groundnut oil.

Group IV: Female was exposed to a confined male rat, allowing contact with its urine and excreta and exposed to a cotton ball without groundnut oil.

Group V: Female was left undisturbed (untreated control).

After 48 h, all females were returned to their cages and housed individually until the termination of the experiments (day 20 *post coitum*).

Experiment 2: Effect of a nonpheromonal cue on starvation-induced implantation failure

All females were monogamously paired with adult males and allowed to mate (Groups I–V). In Group III, a cotton ball smeared with groundnut oil was provided at the time of pairing. In Group IV, the mating pair was exposed to a fresh cotton ball without groundnut oil. Females with vaginal plug (day 0 *post coitum*) were separated from the stud male and housed individually in a new cage with fresh bedding. After 24 h (day 1 *post coitum*) they were subjected to the following treatments:

Group I: Female was deprived of food for 48 h and exposed to the confined stud male, allowing contact with its urine and excreta.

Group II: Female was deprived of food for 48 h and exposed to a cotton ball

smeared with the urine of the stud male.

Group III: Female was deprived of food for 48 h and exposed to a cotton ball smeared with groundnut oil.

Group IV: Female was deprived of food for 48 h and exposed to a cotton ball without groundnut oil.

Group V: Female was left undisturbed (untreated control). All

animals were provided with water *ad libitum.*

After 48 h, all females were returned to their home cages and housed individually till the termination of the experiments up to day 20 *post coitum* to ascertain their reproductive status.

Vaginal smear was examined daily from all the females of experiments 1 and 2. Presence of abundant irregular-shaped, nonnucleated, cornified squamous epithelial cells in the smear were taken as the indication of pregnancy block and return to estrus (Thomas and Dominic 1987a; McLean et al. 2012). Vaginal smear with abundant leucocytes and mucus was taken as an indication of pregnancy. The number of females that were pregnant after showing estrous smear and the number of pups delivered by females that did not exhibit estrous smear were recorded.

Statistical analysis

The data were analyzed using Fisher's exact test.

Result

Experiment 1: Effect of a nonpheromonal cue on male rat-induced implantation failure

P value of pair-wise comparison of the incidence of pregnancy using Fisher's exact test revealed that there was no statistically significant variation

when Group III was compared with Groups I, II and V, whereas Group III showed a very statistically significant variation when compared with Group IV.

Table 1: Response of females to imprinted nonpheromonal cue on male rat-induced implantation failure.

Groups	Treatment	No. of females (*n*)	Results		
			Pregnancy *n* (%)	Pregnancy block *n* (%)	No. of pups delivered (*n*)
Group I	Exposed to the male rat along with the stud male	6	6 (100)	0 (0.0)	48
Group II	Exposed to the male rat along with urine of the stud male	6	5 (83.3)	1 (16.7)	39
Group III	Exposed to the male rat along with a cotton ball smeared with groundnut oil	6	5 (83.3)	1 (16.7)	44
Group IV	Exposed to the male rat along with a cotton ball without groundnut oil	6	0 (0.0)	6 (100)	0
Group V	Untreated control	6	6 (100)	0 (0.0)	54
P value using Fisher's exact test of Group III with other groups Group III with Group I=1.000 Group III with Group II=1.000 Group III with Group IV=**0.0152** Group III with Group V=1.000					

The nonpheromonal cue, the groundnut oil is capable of protecting the female from male rat-induced implantation failure (Group III; Table 1). The protective effect of the imprinted nonpheromonal cue is not, however, significantly different from Group I (exposed to the male rat in the presence of the stud male) or Group II (exposed to the male rat in the presence of the stud male's urine) or Group V that were left undisturbed during the post-mating period. The females in Group IV, which were exposed to male rat

allowing free access to a cotton ball without nonpheromonal cue in the absence of the stud male or its urine showed significantly higher rate of implantation failure.

Experiment 2: Effect of a nonpheromonal cue on starvation-induced implantation failure

P value of pair-wise comparison of the incidence of pregnancy using Fisher's exact test revealed that there was no statistically significant variation when Group III was compared with Groups I, II and IV, whereas Group III showed a very statistically significant variation when compared with Group V.

Table 2: Response of food-deprived inseminated females to imprinted nonpheromonal cue

Groups	Treatment	No. of females (*n*)	Results		
			Pregnancy *n* (%)	Pregnancy block *n* (%)	No. of pups delivered (*n*)
Group I	Food-deprived females exposed to the confined stud male	6	1 (16.7)	5 (83.3)	7
Group II	Food-deprived females exposed to the urine of stud male	6	0 (0.0)	6 (100)	0
Group III	Food-deprived females exposed to a cotton ball smeared with groundnut oil	6	1 (83.3)	5 (16.7)	6
Group IV	Food-deprived females exposed to a cotton ball without groundnut oil	6	0 (0.0)	6 (100)	0
Group V	Females left undisturbed after mating (untreated control)	6	6 (100)	0 (0.0)	53
P value using Fisher's exact test of Group III with other groups Group III with Group I=1.000 Group III with Group II=1.000 Group III with Group IV=1.000 Group III with Group V=**0.0152**					

The imprinted nonpheromonal cue, the groundnut oil is not capable of protecting the female from starvation-induced implantation failure (Group III; Table 2). The number of pregnancy in females is not significantly different from Group I (food-deprived females exposed to the stud male) or Group II (food-deprived females exposed to the urine of stud male) or Group IV (food-deprived females exposed to a cotton ball without groundnut oil). The females of all groups except Group V (females which were left undisturbed during the post-mating period) exhibited a significantly higher rate of pregnancy failure. In conclusion, the presence of a confined stud male or its urine and the imprinted nonpheromonal cue failed to protect the pregnancy of food-deprived inseminated females.

In both experiments, the females that exhibited cornified epithelial smear did not show any sign of pregnancy, whereas those females that failed to show estrous smear continued their pregnancy and delivered varying number (7–9) of pups (Tables 1 and 2).

Discussion

The smell of natural predator induces fear and influences the neuroendocrine system, reproductive activities, and affects the reproductive outcome in potential prey species (Apfelbach et al. 2005; Hayes et al. 2006). Among the laboratory rats, some rats readily kill mouse whereas some others never exhibit mouse killing behavior (Liu et al. 2017). Arndt et al. (2009) demonstrated that individual housing of mice with rat is highly stressful to mice. In the wild, rats are predators of mice (Bandler and Moyer 1970). It is demonstrated that predatory stress on pregnant female mice during the pre-maturation stage of oocyte development adversely affects ooplasmic maturation (Liu et al. 2012).

Kumar and Dominic (1996) demonstrated that exposure of newly inseminated

females to a male rat in the presence of the stud male significantly lowered implantation failure. The present study (Experiment 1) showed that when females were allowed to mate in the presence of a cotton ball smeared with groundnut oil and exposed to inseminated females in the presence of male rat, the majority of the females retained their pregnancy even in the absence of the stud male (Group III). The rate of pregnancy seen in these females is not significantly different from pregnancy observed in newly inseminated females exposed to male rats in the presence of the stud male (Group I) or its urine (Group II) or females left undisturbed after mating (Group V). By contrast, when females were exposed to male rats in the presence of a cotton ball without groundnut oil (Group IV) the majority of the females terminated their pregnancy and returned to estrus on the third day. These results provide additional evidence that female mice can be imprinted with the nonpheromonal odor and it acts analogous to the stud male pheromones.

Hamilton and Bronson (1985) demonstrated that severe food restriction produced pronounced defects in the reproductive processes of female mice. Archunan and Dominic (1989) reported that the presence of the stud male during starvation protected the newly inseminated female against starvation-induced implantation failure. In the present study (Experiment 2), the newly inseminated females subjected to 48 h starvation during the pre-implantation period provided a different result from the above experiment. In BALB/c females, the presence of the stud male or its urine during starvation failed to prevent implantation failure in inseminated females. The presence of the confined stud male (Group I), cotton ball smeared with the urine of stud male (Group II), cotton ball smeared with groundnut oil (Group III) and cotton ball smeared without groundnut oil (Group IV) showed significant reduction of their pregnancy. Only one out of six females exposed to stud male (Group I) and one female of Group III exposed to the nonpheromonal cue delivered pups. Control females, on the other hand,

completed their term of pregnancy and delivered healthy 8–9 pups. These results indicate that starved BALB/c females are generally refractive to the luteotrophic effect of the stud male and imprinted nonpheromonal cue is also ineffective in protecting the females from implantation block induced by starvation. The possibility of strain difference of BALB/c females employed in this experiment from that of the Parkes strain (P) of mice used by Archunan and Dominic (1989) cannot be ruled out. The imprinted luteotrophic memory of the stud male pheromones and the associated cues needs further investigation to unravel the physiological mechanisms involved in variation of the results in the present study.

Summary

The ability of a nonpheromonal cue exposed to female mice during pericopulatory period is capable of protecting inseminated females from implantation failure in the presence of two different stressful stimuli-exposure to male rat and starvation is investigated. Presence of imprinted nonpheromonal cue during exposure of inseminated female to male rat is capable of protecting the female from implantation block induced by the presence of the male rat. These results provide additional evidence that female mice can be imprinted with the nonpheromonal odor and it acts analogous to the stud male pheromones. The results indicate that starved BALB/c females are generally refractive to the luteotrophic effect of the stud male and imprinted nonpheromonal cue and is also ineffective in protecting the females from implantation block induced by starvation.

Chapter 5

Influence of social dominance on mate preference and formation of associative memory in estrus female mice

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Influence of social dominance on mate preference and formation of associative memory in estrus female mice

Introduction

Dominance can be defined as success in contests. "By killing, driving away or using other means of intimidating individuals, dominant individuals exclude at least some of their rivals from access to mates or resources crucial for attracting mates" (Qvarnström and Forsgren 1998). Like several other social mammals, mice society is also well organized and males maintain a highly linear and sharp dominance hierarchy in their social system. The ancestors of laboratory mice are said to be selected from various sub-strains of *Mus* (*Mus musculus*, *Mus domesticus*, *Mus castaneus*, *Mus moloisha*; Frazer et al. 2007). One of the major common characteristics of these subspecies is that they live in large groups with organized social structure and dominance hierarchies (Berry 1970; Crowcroft 1955). Often this social order is perpetuated by aggressive dyadic encounters and chasing behaviors. Individual males of a social group of mice can be classified as dominant, sub-dominant or subordinate (So et al. 2015).

It is reported that the social status of males plays an important role in the induction of implantation block (the Bruce effect) in newly inseminated mice. Huck (1982) reported that dominant males are more effective in blocking implantation in inseminated females than subordinates. The adaptive value of alien male-induced implantation failure is still under scrutiny. Male mice are shown to exhibit infanticidal behavior towards pups sired by another male (Labov 1980). The Bruce effect is said to be a strategy to reduce reproductive investment from the part of the female. Lukas and Huchard (2014) gathered information from 260 species of mammals out of

which 119 species exhibit infanticidal behavior and compared the phenomenon through phylogenetic analysis. They suggested that infanticide primarily evolves in mammalian social groups where reproduction is monopolized by a minority of males. The majority of mating in mice social groups is also monopolized by dominant males and females exhibit preference to mate with the dominant males. In such a situation females adopt paternity dilution strategy through promiscuity. It will be advantageous for the females to exhibit implantation failure rather than lose all her young ones after the delivery (Schwagmeyer 1979). Choosing a dominant male as the mating partner confers other selective advantages for females over other females which are less choosy in the context of mate selection. In natural conditions, the presence of another male in the territory is a sign that the original territory owner is either killed or chased away by a second male. In other words, the second male is fitter than the original territory owner. It is also an indication that the second male bears genes that may contribute to the production of better offsprings. Moreover, dominant males are expected to gain and defend greater resources (Schwagmeyer 1979).

Numerous studies show that olfactory signals produced by an individual convey genetically encoded information of the signaler. It may communicate the species identity, reproductive capability, and social and health status to the recipient (Wyatt 2003; Brennan and Kendrick 2006, Johansson and Jones 2007) all of which influence mate selection.

Male odor preference of the female mouse is based on the reproductive condition (estrus or nonestrus) of the female and the social dominance status of the male (Mossman and Drickamer 1996). Veyrac and Bakker (2008) reported in laboratory mice (C57Bl/6j) females in estrus preferred odors of dominant males; whereas nonestrus females exhibited no significant preferences for either subordinate or dominant male odors. They also found

that the odor preference of females depended on the hormonal status and prior sexual experience of the female. Roberts et al. (2010) identified a nonvolatile major urinary protein termed darcin which specifically elicits inherent sexual attraction towards a particular individual male. It is interesting to note that contact with darcin induces a learned attraction to volatile odors of the male through associative learning.

It is generally believed that pheromones are evolved chemical signals used for intraspecific communication and when perceived by another individual of the same species elicit an innate behavior in the recipient (Karlson and Butenandt 1959; Bruce 1959). VNO is said to be a specialized sense organ that conveys nonvolatile signals directly to the AOB and activate specific neural networks that elicit preprogrammed behavior patterns irrespective of individual experiences (Wyatt 2010). Recentstudies indicate that pheromones need not always induce stereotyped or innate behaviors and it may be sensed both by VNO and the MOE. Most primer pheromones are said to exert endocrine effects by direct control of GnRH neuron activity (Dulac and Wagner 2006).

The ability of female mice to distinguish the dominant male from the subordinate is shown to be mediated through pheromones associated with the male urine (Wyatt 2003; Brennan and Kendrick 2006). This study is designed to assess whether sexually inexperienced females identify social status of males through volatile or nonvolatile cues associated with male urine. The ability of females to associate nonpheromonal cues with the urine of dominant and subordinate males and the duration of associative memory in estrus females was also evaluated in this study. Retention of memory of the odor cues of the dominant male will be advantageous for the females to locate the males during estrous period.

Methodology

85 adult virgin females and 15 adult males (10–20 weeks; BALB/c) were purchased from Kerala Veterinary and Animal Sciences University. Food-grade groundnut oil and gingelly oil are used as nonpheromonal cues. Different stages of the estrous cycle were monitored daily using the vaginal smear technique.

Tube test for determining the social status of male mice

Tube test is commonly employed for testing the dominant/subordinate status of male mice (Lindzey et al. 1961; Messeri et al. 1975; Lijam et al.
1997; Shahbazian et al. 2002). In the field, dominant males generally attack or chase away subordinates. In tube test animals were not allowed to fight, but when they come face to face subordinate will be given a chance to retreat so as to avoid attack from the dominant male. A narrow transparent tube with a minimum area (3.7 cm inner diameter, 30.5 cm in length) which allows only one animal to move through it at a time was used for the test. Two adult male mice were released face to face from the two ends of the tube simultaneously. When they move through the tube and reach near, the dominant one force the other one to retreat from the tube. The individual that moves back and escapes from one end of the tube was scored as the subordinate (Hahn and Schanz 1996; Spencer et al. 2005) and the other as the dominant.

Urine collection and exposure

Two drops of fresh urine from the dominant male were placed on a glass slide. Similarly, urine from the subordinate male was also collected and placed on another glass slide. (refer general methodology for details)

Y-maze

All experiments were conducted in a Y-maze. The maze consisted of three

arms of 60 × 10 × 10 cm (see Figure1). An area of 20 × 10 ×10 cm was marked on one of the arms with a sliding door fixed at 20 cm from the opening and designated as the start box. On the other two arms, an area of 10 × 10 ×10 cm was marked as goal boxes with perforated, opaque movable doors. The triangular central area at the Y-junction of three boxes is designated as the decision-making area. The top edges of the maze were covered with Plexiglas panels. Stimuli were placed on glass slides (75 × 25 mm) in goal boxes. The maze was washed with a dilute soap solution and 70% ethanol after each test between subjects.

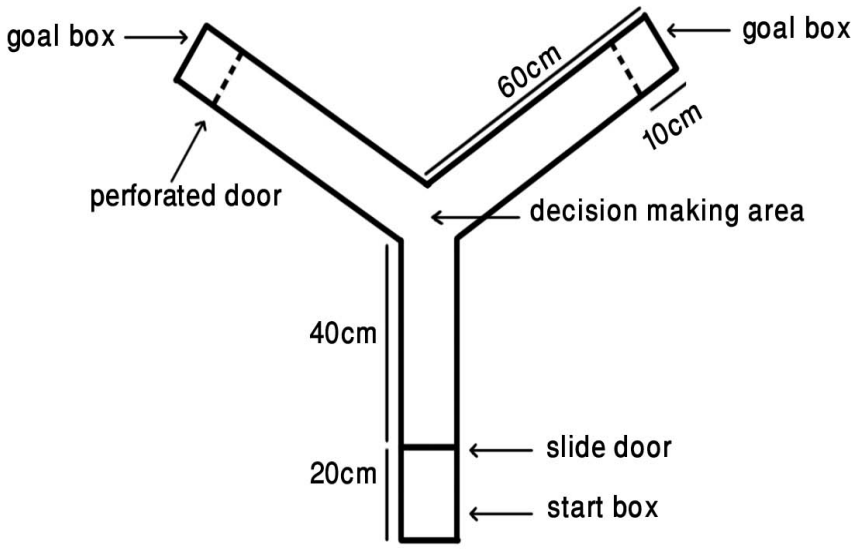

Figure 1: Diagramatic representation of Y maze

Prior to the experiment, the female mouse was released to the empty Y-maze for five minutes to familiarize itself with the apparatus and then returned to the home cage. After that, stimuli were introduced in the goal boxes for assessing the preference of the subject. Each testing session began by placing the female in the start box for 30 seconds and then opening the door, allowing the animal to approach a goal box (free trial). A choice was recorded when the female touches one of the goal box doors with its nose or front paw. The

latency to approach the goal box and the frequency of visits to each box was recorded. If the subject failed to make a choice within 120 seconds, it was returned to the start box and subjected to a guided trial. In the guided trial, the subject was placed back into the start box, the arm of the Y-maze leading to the previously chosen goal box was blocked using a Plexiglas panel and the start box door was opened, forcing the animal to approach the goal box not chosen on the previous trial. In this way, females were familiarized with the stimulus placed behind each goal box door. After a couple of trials (free and guided) the stimuli in the goal boxes were interchanged to avoid any bias influencing the choice of the females. Each day's testing session consisted of eight free trials alternating with seven guided trials (Woodley et al. 2004).

Seventy two adult virgin females that showed regular estrous cycle were used for the study. There were four experiments in this study and in each experiment there were three groups consisting of six females in each group. The olfactory preference of females to male urine was tested on the day of estrus. Dominant male urine, subordinate male urine and two nonpheromonal cues (groundnut oil and gingelly oil) were used as stimuli.

In Experiment 1, preference of estrus females to the dominant and subordinate male urines and the associated nonpheromonal cues were tested after a direct contact (both volatile and nonvolatile cues) during the pre-exposure trial. Experiment 2 followed the same procedure as in Experiment 1 but during the pre-exposure trial the females were exposed only to the volatile cues of the stimuli. In Experiment 3, the procedure was same as Experiment 2 but the nonpheromonal cues associated with the urine of dominant and subordinate males were interchanged in order to rule out the possibility of bias to any one of the nonpheromonal cues influencing the preference of experimental females. In Experiment 4, the duration of associative memory of nonpheromonal cues in the females was tested.

Experiment 1: Preference of estrus females pre-exposed to volatile and nonvolatile cues

Pre-exposure

All females were pre-exposed to both volatile and nonvolatile cues of the corresponding stimuli by allowing them to have direct physical contact with the stimuli.

The female was placed in the start box for 30 seconds and then the door of the start box was raised. The female was free to move and reach either of the goal boxes. When the female made a choice by touching its nose or front paw to any of the goal box, the door was raised and the female was allowed to investigate the stimulus allowing direct physical contact with the stimulus. After the experience with the stimulus, the female was returned to the start box for 30 seconds. The arm of the Y-maze female preferred earlier was blocked using a Plexiglas panel. After 30 seconds female was forced to move to the other goal box and allowed to investigate the stimulus in the goal box. Direct contact with the cues in the goal box is given only once during the pre-exposure trial.

Preference test

Preference of females of Group I to dominant male urine or subordinate male urine was tested on the day of estrus. Two drops of urine from the dominant male were placed in one arm of the Y-maze. In the other arm two drops of urine from the subordinate male were placed and the preference of the female to the male urine was evaluated. In Group II, females were given a choice between two different nonpheromonal cues by placing two drops of groundnut oil in one arm of the Y-maze and in the other arm two drops of gingelly oil and tested their preference. Females of Group III were given two drops of urine of dominant male paired with two drops of groundnut oil in one arm of the Y-maze and in the other arm two drops of urine of the

subordinate male paired with two drops of gingelly oil and tested the preference of females. On the next estrus day (that is, the second estrus from the day of exposure to the paired olfactory cues) females in Group III was tested for their ability for discriminating nonpheromonal cues associated with the urine of dominant and subordinate males (Group IIIa). The females were given a choice between two drops of groundnut oil (previously paired with the urine of dominant male) in one arm of the Y-maze and two drops of gingelly oil (previously paired with the subordinate male urine) in the other arm.

Experiment 2: Preference of estrus females pre-exposed only to the volatile cues

Pre-expsoure

The female was placed in the start box for 30 seconds and then the door of the start box was raised. The female was free to move and reach either of the goal boxes. The female was allowed to experience only the volatile cues of the stimuli placed in the goal box through the perforated door. The rest of the testing procedure was similar to that of Experiment 1.

Preference test

Preference of females of Group I to the dominant male urine or the subordinate male urine was tested on the day of estrus. Two drops of urine from the dominant male were placed in one arm of the Y-maze. In the other arm two drops of urine from the subordinate male were placed and the preference of the female to the male urine was evaluated. In Group II, females were given a choice between two different nonpheromonal cues by placing two drops of groundnut oil in one arm of the Y-maze and in the other arm two drops of gingelly oil and tested their preference. Females of Group III were given two drops of urine of dominant male paired with two drops of groundnut oil in one arm of the Y-maze and in the other arm two

drops of urine of the subordinate male paired with two drops of gingelly oil were placed and tested their preference. On the next estrus day (second estrus) females of Group III were tested for their ability for discriminating nonpheromonal cues associated with the urine of dominant and subordinate males (Group IIIa). The females were given a choice between two drops of groundnut oil (previously paired with the urine of dominant male) in one arm of the Y- maze and two drops of gingelly oil (previously paired with the subordinate male urine) in the other arm.

Experiment 3: Preference of estrus females after interchanging nonpheromonal cues paired with the dominant and subordinate male urine

Pre-exposure

Procedures of pre-exposure were similar to that of the treatments given to females in Experiment 2, except in Group III where the nonpheromonal cue paired with the dominant male urine was interchanged with cue paired with the subordinate male urine.

Preference test

In order to rule out the possibility of bias to any one of the nonpheromonal cues influencing the preference of experimental females, in Experiment 3 the nonpheromonal cues paired with the dominant male urine and subordinate male urine were interchanged and exposed to females in Group III. Thus the urine of dominant male was paired with two drops of gingelly oil in one arm of the Y-maze and in the other arm two drops of urine of the subordinate male was paired with groundnut oil and tested preference of females. On the next estrus day females in Group III were tested for their ability for discriminating nonpheromonal cues associated with the urine of dominant and subordinate males (Group IIIa). The treatment of Groups I and II of Experiment 3 remained the same as described in Experiment 2.

Experiment 4: Retention of memory of nonpheromonal cues associated with dominant and subordinate males.

Pre-exposure

Procedures of pre-exposure were similar to that of the treatments given to females in Group III of Experiment 2

Test for retention of associative olfactory memory

In Groups I, II and III estrus females were given a choice between male urine and an associated nonpheromonal cue by placing urine of the dominant male paired with groundnut oil in one arm of the Y-maze and urine of the subordinate male paired with gingelly oil in the other arm of the Y-maze and recorded their preference (initial trial). By the onset of next estrus (second estrus of experimental period), females of Group I were tested for retention of olfactory memory of the nonpheromonal cue associated with dominant and subordinate male urine (Group Ia). The females were given a choice between groundnut oil (previously paired with dominant male urine) in one arm and gingelly oil (previously paired with subordinate male urine) in the other arm of the Y-maze and their preference to the nonpheromonal cues associated with male urine was tested (final trial). By the onset of the third estrus, females of Group II were tested for retention of olfactory memory of nonpheromonal cues associated with male urine (Group IIa). Here also the females were given a choice between groundnut oil (previously paired with the dominant male urine) placed in one arm and gingelly oil (previously paired with the subordinate male urine) in the other arm of the Y-maze. Females of Group III were tested for any retention memory of nonpheromonal cue by the onset of their fourth estrus (Group IIIa). As in the case of Groups Ia and IIa, females of Group IIIa were given a choice between groundnut oil, (previously paired with the dominant male urine) placed in one arm and gingelly oil (previously paired with the subordinate male urine) in the other arm of the Y-maze and tested their preference to the nonpheromonal cues

associated with male urine.

Statistical analysis

Mann-Whitney *U* test, one-way ANOVA, Kruskal-Wallis test, independent sample t-test, post hoc test, paired t-test and Fisher's exact test were used for the statistical analysis of the present study.

Result

Experiment 1: Preference of estrus females pre-exposed to volatile and nonvolatile cues

Estrus females when allowed to have direct contact with the volatile and nonvolatile cues of the urine of dominant male and the urine of subordinate male showed significant preference to the cues associated with the dominant male urine. Similarly, they exhibited more preference to the nonpheromonal cue paired with the dominant male urine than the nonpheromonal cue paired with the subordinate male urine.

Table 1: Comparison of frequency of the visits between cues in various groups

Groups	Treatment		Cues		*P* Value
	Cue 1	Cue 2	1	2	
			N, mean±SD	N, mean±SD	
Group I	Dominant male urine	Subordinate male urine	6, 7.6667±0.51640	6, 0.3333±0.51640	0.003
Group II	Groundnut oil	Gingelly oil	6, 4.3333±0.81650	6, 3.6667±0.81650	0.176
Group III	Dominant male urine +groundnut oil	Subordinate male urine +gingelly oil	6, 7.3333±0.51640	6, 0.6667±0.51640	0.003
Group IIIa	Groundnut oil	Gingelly oil	6, 7.3333±0.51640	6, 0.6667±0.51640	0.003

Mann–Whitney *U* test showed that there is no significant mean difference in the frequency of visits between cues in Group II (P=0.176>0.05). Group I, III

and IIIa showed that there is a significant mean difference in the frequency of visit between cue 1 and cue 2 (P=0.003, 0.003, 0.003 respectively).

Females exposed to groundnut oil and gingelly oil (Group II) showed no significant variation in their frequency of visits to these cues indicating that there is no specific preference to any of the nonpheromonal cue. Females exposed to dominant and subordinate male urines (Group I), females exposed to dominant male urine paired with groundnut oil and subordinate male urine paired with gingelly oil (Group III), and females exposed to groundnut oil and gingelly oil alone showed significant variations in their frequency of visits to cue 1 and cue 2 (Table 1).

Table 2: Comparison of frequency of visits to cues among groups

Groups	Cue 1	*P* value	Cue 2	*P* value
	N, mean±SD		N, mean±SD	
Group I	6, 7.67±0.516	**0.000**	6, 0.33±0.516	**0.002**
Group II	6, 4.33±0.816		6, 3.67±0.816	
Group III	6, 7.33±0.516		6, 0.67±0.516	
Group IIIa	6, 7.33±0.516		6, 0.67±0.516	
Total	24, 6.67±1.494		24, 1.33±1.494	

One way ANOVA and Kruskal–Wallis test were conducted to fetch out the statistically significant mean differences in the frequency of visits to cue 1 and cue 2, respectively, among various groups and it confirmed the same (cue 1, P= 0.000 <0.05; cue 2, P= 0.002 <0.05). Corresponding post hoc tests were also performed and they showed a significant difference in cue 1 between Groups I and II (P=0.000), Groups II and III (P=0.000) and Groups II and IIIa (P=0.000); in cue 2, between Groups I and II (P=0.000), Groups II and III (P=0.004) and Groups II and IIIa (P=0.004). No significant differences were seen between other groups (Groups I and III, I and IIIa, and III and IIIa).

Females of Group II which were exposed to groundnut oil as cue 1 showed a significant variation in the frequency of visits when compared with other groups. But females of Groups I (exposed to the dominant male urine), III (exposed to the dominant male urine paired with groundnut oil) and IIIa (exposed to groundnut oil alone) exhibited no significant variation in the frequency of visits to cue 1 when compared among the groups. Females of Group II which were exposed to gingelly oil as cue 2 showed a significant variation in the frequency of visits when compared with other groups. But females of Groups I (exposed to subordinate male urine), III (exposed to subordinate male urine paired with gingelly oil) and IIIa (exposed to gingelly oil alone) exhibited no significant variation in the frequency of visits to cue 2 when compared among the groups (Table 2).

Table 3: Comparison of latency of visits between cues in various groups

Groups	Cues		*P* value
	1	2	
	N, mean±SD	N, mean±SD	
Group I	46, 3.89±1.69	2, 16.00±1.41	**0.000**
Group II	26, 21.58±6.92	22, 23.00±6.47	0.468
Group III	44, 3.98±1.72	4, 14.00±2.16	**0.000**
Group IIIa	44, 4.45±1.99	4, 16.50±4.20	**0.000**

Independent sample t-test showed that there is no significant mean difference in the latency to reach the cues in Group II (P=0.468>0.05). Groups I, III and IIIa showed that there is a significant mean difference in the latency to reach the cues (P=0.000) among the groups.

Females exposed to groundnut oil and gingelly oil (Group II) showed no significant variation in latency of visit between cues indicating that there was no specific preference for any of the nonpheromonal cues. But comparison

of latency of visit between cues by females exposed to dominant and subordinate male urines (Group I), females exposed to dominant male urine paired with groundnut oil and subordinate male urine paired with gingelly oil (Group III), and females exposed to groundnut oil and gingelly oil alone (Group IIIa) showed a significant variation (Table 3)

Table 4: Group-wise comparison of latency

Groups	Cue	*P* value
	N, mean± SD	
Group I	48, 4.3958± 2.95886	**0.000**
Group II	48, 22.2292± 6.68195	
Group III	48, 4.8125± 3.29187	
Group IIIa	48, 5.4583± 4.01040	
Total	192, 9.2240± 8.75118	

One-way ANOVA showed that there is statistically significant mean difference in latency among groups (*P*=0.000). Post hoc test (Tukey's HSD) also confirm that there is a significant mean difference between Groups I and II (*P*=0.000) but no statistically significant mean difference between Groups I and III (*P*=0.968) and Groups I and IIIa ((*P*=0.652). There is a significant mean difference between Groups II and III ((*P*=0.000) and Groups II and IIIa (*P*=0.000). But there is no significant difference between Groups III and IIIa ((*P*=0.895).

Females of Group II which were exposed to groundnut oil and gingelly oil as cue 1 and cue 2 showed a significant variation in the latency to visit the cues when they were compared with other groups. But females exposed to the dominant and subordinate male urines (Group I), females exposed to the dominant male urine paired with groundnut oil and subordinate male urine paired with gingelly oil (Group III), and females exposed to groundnut oil and gingelly oil alone (Group IIIa) exhibited no significant variation in latency to visit cues 1 and 2 when compared among the groups (Table 4).

Experiment 2: Preference of estrus females pre-exposed to the volatile cues

Estrus females when allowed to have direct contact with the volatile cues of the urine of dominant male and the urine of subordinate male, showed a significant preference for the cues associated with the dominant male urine. Similarly, they exhibited more preference to the nonpheromonal cue paired with the dominant male urine than the nonpheromonal cue paired with the subordinate male urine.

Table 5: Frequency of visits between cues by various groups

Groups	Treatment		Cues		*P* value
	Cue 1	Cue 2	1	2	
			N, mean±SD	N, mean±SD	
Group I	Dominant male urine	Subordinate male urine	6, 7.8333±0.40825	6, 0.1667±0.40825	**0.002**
Group II	Groundnut oil	Gingelly oil	6, 3.8333±0.98319	6, 4.1667±0.98319	0.545
Group III	Dominant male urine + groundnut oil	Subordinate male urine+ gingelly oil	6, 7.5000±0.54772	6, 0.5000±0.54772	**0.003**
Group IIIa	Groundnut oil	Gingelly oil	6, 7.3333±0.51640	6, 0.6667±0.51640	**0.003**

Mann–Whitney *U* test showed that there is no significant mean difference of the frequency of visits between cues 1 and 2 in Group II (P=0.545>0.05). Groups I, III and IIIa showed that there is a significant mean difference in frequency of visits between cues 1 and 2 (P=0.002, 0.003, 0.003 respectively).

Females exposed to groundnut oil and gingelly oil (Group II) showed no significant variation in their frequency of visits to cues 1 and 2. Females exposed to the dominant and subordinate male urines (Group I), females exposed to the dominant male urine paired with groundnut oil and the subordinate male urine paired with gingelly oil (Group III), and females exposed to groundnut oil and gingelly oil alone showed

significant variation in their frequency of visits to cues 1 and 2 (Table 5).

Table 6: Comparison of frequency of visits to cues among groups

Groups	Cue 1	*P* value	Cue 2	*P* value
	N, mean±SD		N, mean±SD	
Group I	6, 7.83±0.408	**0.000**	6, 0.17±0.408	**0.001**
Group II	6, 3.83±0.983		6, 4.17±0.983	
Group III	6, 7.5±0.548		6, 0.50±0.548	
Group IIIa	6, 7.33±0.516		6, 0.67±0.516	
Total	24, 6.63±1.764		24, 1.38±1.765	

One-way ANOVA and Kruskal–Wallis test were conducted to fetch out the statistically significant mean differences of the frequency of visits to cue 1 and cue 2, respectively, among various groups and it confirmed the same (cue 1, P=0.000 <0.05; cue 2, P=0.001 <0.05). Corresponding post hoc tests were also performed and they showed a significant difference in cue 1 between Groups II and I (P=0.000), Groups II and III (P =0.000) and Groups II and IIIa (P=0.000); in cue 2, between Groups II and I (P=0.000), Groups II and III (P=0.003) and Group II and IIIa (P=0.009). No significant differences were seen in other groups (Group I and III, I and IIIa, and III and IIIa).

Females of Group II which were exposed to the groundnut oil as cue 1 showed significant variation in the frequency of visit when compared with other groups. But females of Groups I (exposed to the dominant male urine), III (exposed to the dominant male urine paired with groundnut oil) and IIIa (exposed to groundnut oil alone) exhibited no significant variation in the frequency of visits to cue 1 when compared among the groups. Females of Group II which were exposed to the gingelly oil as cue 2 showed a significant variation in the frequency of visit when compared with other groups. But females of Groups I (exposed to the subordinate male urine) III (exposed to the subordinate male urine paired with gingelly oil) and IIIa (exposed to gingelly oil alone) exhibited no significant

variation in frequency of visits to cue 2 when compared among the groups (Table 6).

Table 7: Comparison of latency of visits between cues in various groups

Groups	Cues		*P* value
	1	2	
	N, mean±SD	N, mean±SD	
Group I	47, 4.00±2.35	1, 14.00±0.00	**0.000**
Group II	23, 23.43±9.33	25, 22.84±10.13	0.886
Group III	45, 3.91±2.09	3, 16.00±1.73	**0.000**
Group IIIa	44, 4.34±2.73	4, 13.75±4.92	**0.000**

Independent sample t-test showed that there is no significant mean difference in the latency of visits of the subjects between cues 1 and cue 2 in Group II (P=0.886>0.05). Groups I, III and IIIa showed that there is a significant mean difference in the latency of visits of the animals between cue 1 and 2 (P=0.000).

Females exposed to groundnut oil and gingelly oil (Group II) showed no significant variation in the latency of visit between cues, indicating that there is no specific preference for any of the nonpheromonal cues. But comparison of the latency of visit between cues by females exposed to dominant and subordinate male urines (Group I), females exposed to dominant male urine paired with groundnut oil and subordinate male urine paired with gingelly oil (Group III) and females exposed to groundnut oil and gingelly oil alone (Group IIIa) showed a significant variation (Table 7).

Table 8: Group-wise comparison of latency

Groups	Cue	*P* value
	N, mean± SD	
Group I	48, 4.2083± 2.73635	**0.000**
Group II	48, 22.6458± 9.65245	
Group III	48, 4.6667± 3.59866	
Group IIIa	48, 5.1250± 3.91193	
Total	192, 9.1615± 9.63088	

One-way ANOVA declared that there are statistically a significant mean difference in the latency among groups (*P*=0.000). Post hoc test (Tukey's HSD) also confirmed that there is a significant mean difference between Groups I and II (*P*=0.000) but no statistically significant mean difference between Groups I and III (*P*=0.979) and Groups I and IIIa (*P*=0.858). There is a significant mean difference between Groups II and III (*P*=0.000) and Groups II and IIIa (*P*=0.000). But there is no significant difference between Groups III and IIIa (*P*=0.979).

Females of Group II which were exposed to groundnut oil and gingelly oil as cues 1and 2 showed a significant variation in the latency to visit the cues when they were compared with other groups. But females exposed to the dominant and subordinate male urines (Group I), females exposed to dominant male urine paired with groundnut oil and subordinate male urine paired with gingelly oil (Group III), and females exposed to groundnut oil and gingelly oil alone (Group IIIa) exhibited no significant variation in the latency of visit to cues 1and 2 when they were compared among the groups (Table 8).

Table 9: Group-wise comparison of the frequency of visits of females between cues in Experiments 1 and 2

Groups	Experiment	Cue 1	Cue 2	*P* value
Group I	1	47	1	1.0000
	2	46	2	
Group II	1	23	25	0.6833
	2	26	22	
Group III	1	45	3	1.0000
	2	44	4	
Group IIIa	1	44	4	1.0000
	2	44	4	

P value of group-wise comparison of Experiments 1 and 2 using Fisher's exact test revealed that there is no statistically significant variation in the frequency of visits (Group I *P*=1.0000; Group II *P*=0.6833; Group III, *P*=1.0000; Group IV, *P*=1.0000).

Comparison of the frequency of visits of females in Experiments 1 and 2 between cues revealed that there is no significant variation among the groups. This indicates that virgin females in estrus exhibit higher preference to olfactory cues of the dominant male urine or nonpheromonal cues paired with its urine irrespective of whether they were pre-exposed to the nonvolatile cues or with the volatile cues (Table 9).

Results of Experiments 1 and 2 unequivocally demonstrate that direct contact with volatile and nonvolatile cues of urine from the dominant or subordinate male did not affect the preference of estrus female to dominant male urine. This indicates that the female is able to identify the social status of the male through volatile cues. These experiments also demonstrate that females form an olfactory memory of the cues of male urine or nonpheromonal cues associated with the dominant male urine.

Experiment 3: **Preference of estrus females after interchanging the nonpheromonal cues paired with dominant and subordinate male urine**

Results of this experiment demonstrate that the estrus females have no bias to any one of the nonpheromonal cues paired with the male urine. The preference exhibited by the estrus female after interchanging the nonpheromonal cues paired with the dominant and subordinate male urine failed to influence the preference of the female to the dominant male urine.

Table 10: Frequency of visits between cues by various groups

Groups	Treatment		Cues		*P* value
	Cue 1	Cue 2	1	2	
			N, mean±SD	N, mean±SD	
Group I	Dominant male urine	Subordinate male urine	6, 7.8333±0.40825	6, 0.1667±0.40825	0.002
Group II	Gingelly oil	Groundnut oil	6, 4.1667±0.98319	6, 3.8333±0.98319	0.545
Group III	Dominant male urine + gingelly oil	Subordinate male urine+ groundnut oil	6, 7.6667±0.51640	6, 0.3333±0.51640	0.003
Group IIIa	Gingelly oil	Groundnut oil	6, 7.5000±0.54772	6, 0.5000±0.54772	0.003

Mann–Whitney *U* test showed that there is no significant mean difference in the frequency of visit between cue 1 and 2 in Group II (P=0.545>0.05). Groups I, III and IIIa showed that there is a significant mean difference in the latency between cue 1 and 2 (P=0.002, 0.003, 0.003 respectively).

Females exposed to gingelly oil and groundnut oil (Group II) showed no significant variation in their frequency of visit to these cues, indicating that there is no specific preference for any of the nonpheromonal cues. Females exposed to dominant and subordinate male urine (Group I), females exposed to dominant male urine paired with gingelly oil and subordinate male urine paired with groundnut oil (Group III), and females exposed to gingelly oil and

groundnut oil alone showed a significant variation in their frequency of visit to these cues (Table 10).

Table 11: Comparison of frequency of visits to cues among groups

Groups	Cue 1	*P* value	Cue 2	*P* value
	N, mean±SD		N, mean±SD	
Group I	6, 7.83±0.408		6, 0.17±0.408	
Group II	6, 4.17±0.983	**0.000**	6, 3.83±0.983	**0.001**
Group III	6, 7.67±0.516		6, 0.33±0.516	
Group IIIa	6, 7.5±0.548		6, 0.5±0.548	
Total	24, 6.79±1.668		24, 1.21±1.668	

One-way ANOVA and Kruskal Wallis test were conducted to fetch out the statistically significant mean differences in the frequency of visits in the cue 1 and cue 2, respectively, among various groups and it confirmed the same (cue 1, P=0.000 <0.05; cue 2, P=0.001 <0.05). Corresponding post hoc tests were also performed and they showed a significant difference in cue 1 between Groups II and I (P=0.000), Groups II and III (P=0.000) and Groups II and IIIa (P=0.000); in cue 2 between Groups II and I (P=0.000), Groups II and III (P=0.002) and Groups II and IIIa (P=0.005). No significant differences were seen in other groups (Groups I and III, I and IIIa, and III and IIIa).

Females of Group II exposed to gingelly oil as cue 1 showed a significant variation in the frequency of visit when compared with other groups. But females of Groups I (exposed to dominant male urine) III (exposed to dominant male urine paired with gingelly oil) and IIIa (exposed to gingelly oil alone) exhibited no significant variation in the frequency of visit to cue 1 when compared among the groups. Females of Group II exposed to groundnut oil as cue 2 showed a significant variation in the frequency of visit when compared with other groups. But females of Groups I (exposed to subordinate male urine), III (exposed to subordinate male urine paired with groundnut oil)

and IIIa (exposed to groundnut oil alone) exhibited no significant variation in the frequency of visits to cue 2 when compared among the groups (Table 11).

Table 12: Comparison of latency of visits among various groups

Groups	Cues		*P* value
	1	2	
	N, mean±SD	N, mean±SD	
Group I	47, 4.00±2.35	1, 14.00±0.00	**0.000**
Group II	25, 22.84±10.13	23, 22.43±9.33	0.886
Group III	46, 4.09±2.17	2, 16.00±8.49	**0.000**
Group IIIa	45, 4.22±2.35	3, 16.67±3.79	**0.000**

Independent sample t-test showed that there is no significant mean difference in the latency between cues 1 and 2 in Group II (P=0.886>0.05). Groups I, III and IIIa showed a significant mean difference in the latency of visits between cue 1 and 2 (P=0.000).

Females exposed to gingelly oil and groundnut oil (Group II) showed no significant variation in latency of visit between cues indicating that there is no specific preference for any one of the nonpheromonal cues. But comparison of the latency of visit between cues by females exposed to dominant and subordinate male urines (Group I), females exposed to dominant male urine paired with gingelly oil and subordinate male urine paired with groundnut oil (Group III), and females exposed to gingelly oil and groundnut oil alone (Group IIIa) showed a significant variation (Table 12).

Table 13: Group-wise comparison of latency

Groups	Cue	*P* value
	N, mean± SD	
Group I	48, 4.2083± 2.73635	**0.000**
Group II	48, 22.6458± 9.65245	
Group III	48, 4.5833± 3.43841	
Group IIIa	48, 5.0000± 3.88121	
Total	192, 9.1094± 9.63651	

One-way ANOVA declared that there are statistically significant mean difference in the latency among groups (*P*=0.000). Post hoc test (Tukey's HSD) also confirm that there is a significant mean difference between Groups I and II (*P*=0.000) but no statistically significant mean difference between Groups I and III (*P*=0.988) and Groups I and IIIa (*P*=0.902). There is a significant mean difference between Groups II and III (*P*=0.000) and Groups II and IIIa (*P*=0.000). But there is no significant difference between Groups III and IIIa (*P*=0.984).

Females of Group II exposed to the gingelly oil and groundnut oil as cue 1 and 2 showed a significant variation in the latency of visit to cues when compared with other groups. But females exposed to dominant and subordinate male urines (Group I), females exposed to dominant male urine paired with gingelly oil and subordinate male urine paired with groundnut oil (Group III), and females exposed to gingelly oil and groundnut oil alone (Group IIIa) exhibited no significant variation in the latency of visit to cue 1and 2 when compared among the groups (Table 13).

Experiment 4: Duration of retention of olfactory memory of associated nonpheromonal cues

It is evident from the current results that the associative memory of nonpheromonal cues paired with the urine of dominant and subordinate

male were retained in the memory of the female for several days and the memory will be obliterated by the appearance of fourth estrus of the female.

Table 14: Comparison of frequency of visits among cues between initial and final trials in Group I, II and III

Groups			Cues (final trial)		Total	*P* value
			Cue 1	Cue 2		
Group I	Cues (initial trial)	Cue 1	38(90.5%)	5(83.3%)	43 (89.6%)	1.000
		Cue 2	4(9.5%)	1 (16.7%)	5 (10.4%)	
	Total		42(100.0%)	6(100.0%)	48(100.0%)	
Group II	Cues (initial trial)	Cue 1	31(91.2%)	11(78.6%)	42(87.5%)	0.057
		Cue 2	3(8.8%)	3(21.4%)	6(12.5%)	
	Total		34(100.0%)	14(100.0%)	48(100.0%)	
Group III	Cues (initial trial)	Cue 1	25(96.2%)	19(86.4%)	44(91.7%)	0.000
		Cue 2	1(3.8%)	3(13.6%)	4(8.3%)	
	Total		26(100.0%)	22(100.0%)	48(100.0%)	

McNemar test revealed that even though there are no statistically significant variation of the frequency of visits between initial and final trials of females of Group I (P=1.000) and Group II (P=0.057), it showed a statistically significant variation between initial and final trials of females of Group III (P=0.000).

Females of Groups I, II and III (initial trial) exposed to the dominant male urine paired with groundnut oil and subordinate male urine paired with gingelly oil exhibited a higher frequency of visit to dominant male urine

paired with groundnut oil than the other. Females of Group Ia exposed to groundnut oil and gingelly oil alone on the second estrus showed no significant variation in the frequency of visit to both cues when compared with Group I, females of Group IIa exposed to groundnut oil and gingelly oil alone on the third estrus showed no significant variation in the frequency of visit to both cues when compared with Group II, and females of Group IIIa exposed to groundnut oil and gingelly oil alone on the fourth estrus showed a significant variation in the frequency of visit to both cues when compared with Group III (Table 14).

Table 15: Comparison of latency of visit between initial and final trials of Groups I, II and III

Groups	Latency to visit		*P* value
	Initial trial	Final trial	
	N, mean±SD	N, mean±SD	
Groups I and Ia	48, 5.4375±4.16115	48, 6.1250±4.73388	0.451
Groups II and IIa	48, 5.6875±4.60876	48, 13.9583±6.38135	**0.000**
Groups III and IIIa	48, 5.4792±4.19214	48, 23.1042±7.72737	**0.000**

Paired sample *t*-test showed that in Group I there is no statistically significant mean difference between initial and final latency of visit (*P*=0.45). But in Group II there is a statistically significant mean difference between initial and final latency of visit (*P*=0.000). In Group III there is a statistically significant mean difference between initial and final latency of visit (*P*=0.000).

The latency of visit of estrus female to dominant male urine paired with groundnut oil and subordinate male urine paired with gingelly oil (initial trial) was compared with the latency shown by females in Groups Ia, IIa and Group IIIa on the second, third, and fourth day of estrus, respectively, during the test period and there is no significant variation in Groups I and Ia.

However, the latency of the visit of females in Groups II and IIa, and III and IIIa exhibited a significant variation (Table 15).

Discussion

The results of the present investigations show that naive females in estrus exhibit significantly more preference to the urine of the dominant male than to urine of the subordinate male. There is no significant difference in the preferences of females that had direct physical contact with male urine and preferences of females that had contact only with the volatile cues of the male urine. This indicates that BALB/c females in estrus have the ability to identify the dominant status of the male based on the volatile cues present in the male urine. Mossman and Drickamer (1996) studied olfactory preference of wild house mice living in a semi-natural field and found that estrus females preferred urinary odors of dominant male whereas non-estrus females exhibited no such preference to odor of either dominant male or subordinate male. Veyrac and Bakker (2008) studied the preference of female mice to volatile odor cues of the male and found that estrus females exhibit a higher preference for cues of the dominant male as compared to the odor of subordinate male urine. The result of the present study is in agreement with the above-mentioned report. Yano et al. (2012) demonstrated that preference of female mice to male odors varies on different stages of the estrus cycle. During estrus BALB/c females prefer to spend more time near odor cues of a male belonging to another strain of C57BL/6 than to the cues of male belonging to BALB/c males.

However, several other reports have shown that contact with the nonvolatile urinary cues is necessary for volatile odors to induce inherent sexual attraction of females to male odors (Roberts et al. 2010). It is generally believed that VNO is specialized for the detection of nonvolatile pheromones and other odorants are sensed through the MOE. The authors have shown that when the

female contacts with the nonvolatile pheromone darcin it forms an associative memory of the volatile cues of male urine. This olfactory conditioning allows the female to identify individual male through volatile pheromones and exhibit sexual attraction to a specific male.

It has been shown that highly inbred laboratory mice strains like BALB/c males produce very low levels of darcin in their urine (Cheetham et al. 2009, Roberts et al. 2010). When these females were tested for their interest in male urine they failed to show any learned attraction to the volatile cues (Roberts et al. 2010). However, the addition of recombinant darcin (r-darcin) produced a powerful attraction towards volatile cues from the same individual. Ramm et al. (2008) studied the preference of wild female mouse to the urine of male and female and reported that female mice preferred male urine to female urine only when they were allowed to contact with the urine. The females were attracted to the volatile cues of individual male urine which they had contacted previously. Several other investigators also suggested that urinary volatile signals of male may not trigger inherent or innate responses of females to volatile pheromones (Keller et al. 2006; Ramm et al. 2008; Wyatt 2009).

Hurst and Beynon (2004) suggested that volatile molecules may be indicative for the presence of a particular odor and motivate the animal for further exploration of the nonvolatile cues of the scent marks. Recent evidence indicate that volatile MHC molecules and small peptide ligands of these molecules play a significant role in mate recognition and mate choice in female mice (Boehm and Zufall 2006; Spehr et al. 2006). Wysocki et al. (2004) demonstrated that VNO-ablated female mice successfully detect urinary volatile pheromones from males of two different haplotypes, indicating the role of MOE in the detection of volatile cues. In mice, the trace-amine-associated receptors (TAARs) are expressed in MOE (Liberles

and Buck 2006). The pieces of evidence indicate that MOE is also capable of detecting volatile pheromones associated with male urine as observed in this study (Baum 2012). Baum and Keverne (2002) found that female mice are able to detect very low dilutions of volatile odors of female urine in water and suggested that this ability may give advantage for them in the context of mate choice.

Results of this investigation clearly show that estrus females are able to show preference to volatile cues of dominant male urine without any direct physical contact with the urine of the male. Hurst (1990) found that dominant males leave heavy urine marks to advertize their social status and individual identity. It is possible that detection of volatile cues associated with male urine may form a memory in the female which can be retrieved later when the female encounters male urine marks in the vicinity of its home range. It has been shown that females make use of these urine marks to identify dominant males in the particular territorial area (Hurst 1990), and in isolated family groups male countermarks are seen only in the presence of female urine marks indicating that the dominant status of the resident male is reinforced to the resident females (Hurst 1989).

The present results indicate that estrus females are able to learn a nonpheromonal cue paired with the dominant male urine and discriminate it from the other nonpheromonal cue associated with the urine of subordinate male. Estrus females spend significantly more time near the goal box of Y-maze where the dominant male urine is paired with groundnut oil. The latency to approach the stimuli was also less as compared to the latency of the female to approach the urine of subordinate male paired with gingelly oil. These evidences indicate that estrus females are capable of discriminating the nonpheromonal cues associated with the dominant male and a subordinate male based on the associative memory formed while pairing the

nonpheromonal cues with the male urine. Females show no bias to any one of the nonpheromonal cues associated with the dominant male urine or the subordinate male urine. This is indicated by the fact that when groundnut oil paired with the urine of dominant male was interchanged with gingelly oil paired with the urine of subordinate male, the females consistently identified the nonpheromonal cue associated with the urine of dominant male and exhibited a higher preference for it.

It will be advantageous for the females to retain the memory of the odor of the dominant male for several days after its first encounter. Dominant males usually countermark the urine marks of any other males perceived in its territory (Rich and Hurst 1999). In social context, in which males belonging to different social strata are cohabiting in the same area, it will be advantageous for the female to identify the male and quickly take a decision on mate choice. The present study also provides evidence for the existence of such an olfactory memory in the females. When the females were given a choice between nonpheromonal cues paired with the urine of the dominant male and another cue paired with the subordinate male the majority of females spent more time near the cue previously paired with the dominant male urine. The latency to approach the goal box scented with nonpheromonal cue associated with the dominant male urine is significantly less as compared with the latency to approach the other nonpheromonal cue paired with the subordinate male. It is observed that females can retain the memory of nonpheromonal cues until the appearance of the third estrus after their first exposure, beyond which the memory will be obliterated and the females are unable to exhibit any preference of nonpheromonal cues associated with the dominant male urine. Roberts et al. (2010) have shown that females are attracted to fresh male urine marks as well as to urine deposited 7 days before. Presence of a lasting memory of dominant male will be advantageous for the females in this context.

In conclusion, the present study demonstrates that there exists an inherent preference in estrus females to the volatile cues associated with the dominant male urine and they exhibit considerable ability to retain the memory of the olfactory cues of the dominant male for several days after their first encounter. Investigations presented in this chapter also demonstrate that estrus females form an olfactory memory of the cues of dominant male or associated nonpheromonal cues. This memory remains active in the females for several days and obliterated by the onset of the fourth estrus after the first experience with the urine of dominant male.

Summary

The ability of sexually inexperienced females for identifying social status of males through volatile or nonvolatile cues associated with male urine is investigated. The ability of females to associate nonpheromonal cues with the urine of the dominant and the subordinate males and the duration of associative memory in estrus females was also evaluated in this study. The results demonstrate that there exists an inherent preference in estrus females to the volatile cues associated with the dominant male urine and they exhibit considerable ability to retain the memory of the olfactory cues of the dominant male for several days after their first encounter. Results presented in this chapter also demonstrate that estrus females form an olfactory memory of the cues of dominant male or associated nonpheromonal cues. This memory remains active in the females for several days and obliterated by the onset of the fourth estrus after the first experience with the urine of dominant male.

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Chapter 6

Post-mating free female choice of stud vs. alien male

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Introduction

Male induced implantation failure (the Bruce effect) is well documented in several species of mammals. In almost all laboratory studies on the Bruce effect, newly inseminated females were separated from the stud male and exposed to a confined alien male or a strange male for 48 hours (Bruce 1959; Dominic 1960; Thomas 1989) and it has been noticed that majority of them exhibit implantation failure. However, very little information is available on the preference and choice of free moving inseminated females to its original mating partner (stud male) or to alien male.

Drickamer (1989) reported that during the early period of pregnancy females prefer to associate more with soiled bedding from cages of the stud male than to fresh bedding. It was also shown that newly inseminated females actively avoided bedding soiled with strange male urine and fecal matter. It is well documented that newly inseminated females are highly susceptible to the influence of alien or strange male for 48 h immediately after mating. During this period the secretion of prolactin attains a high level in the inseminated females allowing successful implantation of the fertilized ova. Inhibition of pituitary prolactin secretion is shown to be the primary endocrine response of the inseminated females on exposure to strange/alien male chemosignals (Parkes 1961; Dominic 1966b). Presence of the stud male is shown to offer a protective effect on the inseminated female (Thomas and Dominic 1987a, b) and the luteotrophic effect of chemosignals of the stud male has been demonstrated in several studies (Thomas and Dominic 1987b, c, Archunan 2014). In this context, it is not very surprising that the wild strain of house mice exhibited a low rate of implantation block as reported by Drickamer (1989). Patris and Baudoin (1998) compared the mating behavior of *Mus*

musculus domesticus and *Mus spicilegus* and found that in *Mus musculus*, estrus females familiarized with a male, but not allowed to mate, preferred an unfamiliar male and copulated with him. They suggested that it will be advantageous for females of polygamous *Mus musculus* to prefer unfamiliar males to familiar males. In females monogamous species like *Mus spicilegus* mating with familiar males is more advantageous.

Pregnant female Bank vole *Clethrionomys glareolus* when presented with the odor of the stud male and an unfamiliar male exhibited strong preference for the odor of the stud male during the early days of pregnancy. However, after implantation this olfactory preference, as indicated by significantly more time spent near the stud male odor, decreased and females showed no preference for both odors. Lactating females exhibited preference to the odor of the stud males after parturition, indicating that the olfactory memory of the stud male is often retained in them until the dispersal of the young ones (Kruczek 1998).

deCatanzaro and Murji (2004) have studied the behavior of CF1 strain of mice and showed that females preferred to inspect male belonging to a heterogenous strain than its original mating partner or another male from the strain of the stud male. It is pertinent to note that when the stud male was allowed free contact with the female, then the female attempted a lesser investigation of the novel male and thus protected its pregnancy.

Heske and Nelson (1984) studied the Bruce effect in the prairie vole *Microtus ochrogaster* in semi-natural field enclosures and reported that implantation failure occurs in this species under experimental conditions where the female is allowed to reject or avoid the strange male. There was no mitigation in pregnancy failure even if the stud male is present with the female to defend her against the strange male.

This study is designed to evaluate the hypothesis that newly inseminated female mice actively avoid strange/alien males and prefer to remain with the stud male. In most of the laboratory studies on Bruce effect females are being housed for at least 48 h with confined alien males. In such a situation active avoidance is not possible with the female. In this investigation females were free to choose between the stud male and an alien male.

Methodology

Eighteen adult virgin females and 12 adult males of 10–20 weeks old BALB/c and 6 adult Swiss males were purchased from Kerala Veterinary and Animal Sciences University, Thrissur, Kerala.

Testing apparatus (maze) and procedure

A modified guinea pig cage (Tarsons Products Pvt. Ltd.) (85×70×20 cm) was used for testing the preference of female mice. An opaque Plexiglas partition (70 × 20 cm) divided the cage into two equal compartments leaving a space as the pathway for the females to reach the stimuli placed in two goal boxes (16 × 13 × 10 cm) set at the corners in the opposite side of the cage. A temporary perforated start box (26 × 6 × 5 cm) was placed in the pathway which allowed the experimental female to receive volatile cues from both goal boxes. The top of the cage was closed using Plexiglas panels confirming free air circulation through the maze.

Twelve adult virgin females that exhibited regular estrus cycle were grouped into two, and each group consisted of 6 females. The females were paired with adult male on the day of proestrus. After the formation of vaginal plug (day 0 *post coitum*) the newly inseminated female mouse was separated from its stud male and housed in a cage with fresh bedding. After 24 h, on day 1 *post coitum* preference of females was tested in the maze. The stud and

alien males were individually housed in perforated cages and placed as goal boxes (16 × 13 × 10 cm). In order to familiarize the female with the olfactory cues in the goal boxes, the female was placed in the passage of the apparatus in a perforated start box. After 5 min the start box was lifted releasing the female to move inside the cage. The female had access to the urine and fecal matters (volatile and nonvolatile cues) of both males. The female remained in the maze for 48 h (up to day 3 *post coitum*) without any interference. Food and water were provided to all animals *ad lib*.

Females of Group I was exposed to the stud male in one goal box and the alien male in the other box. Females of Group II was exposed to the stud male in one goal box and an empty cage in the other goal box (control).

Frequency of visit and time spent near each cue was recorded using an infrared camera fixed on the top of the testing apparatus, connected to the monitor kept in the adjacent room. After day 3 *post coitum* females were individually kept in separate cages till the termination of the experiment on day 20 *post coitum*. The number of pups delivered by the females was also recorded. The maze was washed with a dilute soap solution and 70% ethanol after each test between subjects. The total period of cohabitation (48 h) of the inseminated female with the stud male and the alien male was divided into four quarters of 12 h and the time spent (in minutes) by the female near each cue was recorded.

Statistical analysis

Fisher's exact test, Wilcoxon signed-rank test, Paired sample t-test were used for the statistical analysis of the present study.

Result

The study demonstrates that inseminated females actively investigate alien males. This is indicated by the fact that the frequency of visits of inseminated females during the first quarter of their experimental period was more

towards the alien male than to the stud male.

Table 1: Preference of inseminated females to stud male and alien male

Groups	No. of females	Cue 1	Cue 2	Results		
				Pregnant *n*(%)	Pregnancy block *n*(%)	No. of pups delivered (*n*)
Group I	6	Stud male	Alien male	0(0)	6(100)	0
Group II (Control)	6	Stud male	Empty cage	6(100)	0(0)	51
P value using Fisher's exact test of Group I with Group II Group I vs. Group II=0.0022						

P value of pair-wise comparison of occurrence of pregnancy using Fisher's exact test revealed that there was statistically very significant variation between Groups I and II. Results showed that, the inseminated females in Group I (exposed to confined stud male and confined alien male), exhibited significant rate of pregnancy block as compared with females of Group II (control) (exposed to confined stud male and empty cage) (Table 1).

Table 2: Quarter-wise comparison of the frequency of visits between cue 1 and 2

Groups	Quarter	Cue		*P* value
		1	2	
		N, mean±SD	N,mean± SD	
Group I	1	6, 68.00±3.58	6, 98.33±8.82	**0.028**
	2	6, 22.17± 4.07	6, 23.17± 4.07	0.680
	3	6, 51.50±4.64	6, 49.33± 3.50	0.463
	4	6, 20.33 ±2.94	6, 18.83± 2.32	0.279
	Total	6, 162.00± 4.52	6, 189.67± 6.95	**0.028**
Group II	1	6, 91.83± 3.31	6, 18.50± 3.27	**0.027**
	2	6, 15.00± 2.83	6, 7.00± 1.55	**0.028**
	3	6, 25.00± 2.61	6, 17.33± 4.32	**0.027**
	4	6, 13.67± 2.16	6, 5.00± 1.79	**0.027**
	Total	6, 145.50± 7.50	6, 47.83 ± 4.49	**0.028**

Wilcoxon signed-rank test was conducted to find out the statistically significant difference in the frequency of visits between the cues in each group. It reveals that in Group 1, there exists a significant differences in the frequency of visits of females between cue 1 and 2 in the first quarter (P=0.028) of the experiment. The total frequency of visit towards these two cues also shows a significant difference (P=0.028). In Group II, there exists a significant difference in the frequency of visits of females between cue 1 and 2 during all the four quarters of the experiment (P=0.027, 0.028, 0.027, 0.027, respectively). Total frequency of visit of females between cue 1 and 2 in Group II is also significantly different (P=0.028).

This indicates that the frequency of visits of inseminated females in Group I during the first quarter of the experiment was more towards the alien male than the stud male. However, number of visits to stud males and alien males in other three quarters of experiment was almost equal. Total frequency of visits to alien male was also higher in females of this group. All inseminated females in Group I exhibited implantation failure. As expected, inseminated females in Group II visited the stud male more frequently than the empty cage during all the four quarters of the experiment and the total frequency of visits to stud male was also higher in Group II. All females of this group completed the term of their pregnancy and delivered 8–9 pups (Table 2).

Table 3: Quarter-wise comparison of time spent between cue 1 and 2

Groups	Quarter	Cue		*P* value
		1	2	
		N, mean±SD	N, mean±SD	
Group I	1	6, 143.50±24.34	6, 154.83±22.76	0.345
	2	6, 76.83±10.80	6,44.67±9.00	**0.001**
	3	6,137.83±15.83	6,58.33±15.21	**0.027**
	4	6,161.50±16.50	6,51.50±6.83	**0.000**
	Total	6,519.50±50.05	6,309.00±35.21	**0.028**
Group II	1	6,441.50±57.89	6,18.68±7.40	**0.028**
	2	6,122.50±19.47	6,12.50±7.64	**0.000**
	3	6,445.67±54.50	6,18.17±7.52	**0.028**
	4	6,143.00±16.89	6,14.00±9.96	**0.000**
	Total	6,1152.50±74.20	6,63.83±25.51	**0.028**

Paired sample t- test and Wilcoxon signed-rank test were conducted to find out the statistically significant difference in time spent between the cues in each group and these tests showed that there are significant differences ($P<0.05$) in all quarters of the experiment to both cues except in the first quarter of Group I ($P=0.345>0.05$).

The statistical analysis revealed that the time spent near the stud male and the alien male varied in all four quarters of the experiment in Group I except in the first quarter. In the first quarter females of Group I, spent almost equal time near both the cues compared with other quarters. In the other three quarters of the experiment females of Group I spent more time near the stud male than near the alien male and the total time spent near each cue was also the same. But in Group II females showed significant variation in the time spent near the stud male and the empty cage and it was more towards the stud male in all four quarters of the experimental period. Pregnancy failure did not occur in these females and all gave birth to 8–10 pups on completion

of the term of pregnancy (Table 3).

Discussion

Results of this investigation clearly demonstrate that BALB/c females actively investigate the alien male (Swiss) and all females tested terminated their pregnancy and returned to estrus on the fourth day in spite of the presence of the stud male. However, all inseminated females of Group II exposed to a stud male and an empty cage (control) retained their pregnancy and delivered 8–9 pups on completion of their term of pregnancy.

Heske and Nelson (1984) studied the Bruce effect in prairie vole *Microtus ochrogaster* in a semi-natural field enclosure and manipulated the presence of stud males during exposure to alien males. Prairie voles are strictly monogamous (Thomas and Birney 1979; Getz and Carter 1980; Gavish et al. 1981) and both male and female members of a mated pair would chase away any intruding strange male (Getz et al. 1981). It is reported that in this rodent pregnancy termination occurred even when the female can potentially avoid the strange male and when the stud male is present along with the inseminated female. Since the pheromone involved in the induction of implantation block is nonvolatile the possibility of occurrence of pregnancy block in a species which actively avoid strange males is not tenable. The authors highlighted their results to state that Bruce effect is not a laboratory artifact but it takes place in the natural environment.

deCatanzaro and Murji (2004) studied CF1 strain of laboratory mice and reported that inseminated females actively approached novel males (HS strain) than the stud male at the point of implantation of fertilized ova. The females spent significantly more time near a novel male (heterogenous male) than with the stud male or another male of the same strain of the stud (strange male). The majority of inseminated females that investigated the novel male (alien male) and those that spent significantly more time near the novel male

exhibited implantation block. A moderately high rate of implantation block was observed in females that spent more time near CF1 strange male than those females near the stud male. However, there is a significant rate of pregnancy block in females that spent more time near the novel male than those females that had spent lesser time near the CF1 strange male.

The present results corroborate the above findings (deCatanzaro and Murji 2004). Given a free choice, inseminated females tend to visit the alien male more frequently during the first quarter of 48 h of exposure than to visit the stud male. It is well documented that inseminated females are highly susceptible to pregnancy failure during the pre-implantation period. Paria et al. (1993) reported that uterus of inseminated female becomes receptive to implantation of blastocyst on day 4 *post coitum* and turns non-receptive for day 4 embryo by day5 *post coitum*. It is demonstrated that the primary neuroendocrine event that leads to the Bruce effect in the inseminated female is the inhibition of prolactin secretion and subsequent failure of development of corpora lutea (Parkes 1961; Dominic 1966b). Hence, it is not very surprising that inseminated females that frequently visited alien males during the first quarter of exposure to stud male and alien male terminated their pregnancy and returned to estrus on the fourth day of exposure.

The results show that the frequency of visits of inseminated females in Group I during the first quarter of the experiment was greater towards the alien male than towards the stud male. This might have been the reason for the pregnancy failure in inseminated females observed in this study. Short-term (15min) exposure of inseminated females to alien males for 7 days resulted in pregnancy failure which is not significantly different from the pregnancy failure observed in female exposed to alien male continuously for 7 days (Chipman et al. 1966). The report of Rosser et al. (1989) supports this finding that exposure of inseminated female mice to male urine for an 8 h

period during the initial surge of prolactin results in implantation block.

This result is in contrast with the observations in several previous investigations. Thomas and Dominic (1987a, b, c) demonstrated that the presence of the stud male protects the female from the implantation blocking effect of the alien male. It should be noted that in all these investigations inseminated females were exposed to the stud male and the alien male simultaneously and the females had no chance of avoiding cues of stud male and investigate alien male. In the present investigation, stud male and alien male were exposed to the female separately in two corners of the testing apparatus and the female was free to approach to any one of the males or even remain in the neutral space in the pathway.

However, in the present study, the number of visits of inseminated females to stud males and alien males in the other three quarters of the experiment was almost equal. The olfactory preference of females for male odors is shown to be dependent on the stages of the estrous cycle. In a Y-maze test Yano et al. (2012) demonstrated that BALB/c females preferred the odor cues of C57BL/6 (alien male) than males from the same strain. And during estrus, they spent equal time near both males. However, whether this observation is applicable to the preference of inseminated female is not evident at present.

There is no statistically significant variation (P=0.345>0.05) in the total time spent by inseminated females of Group I near the stud male and the alien male during the first quarter of the experimental period. This indicates that the inseminated females spent almost equal time near the stud male and the alien male. Comparing these data with the frequency of visit it is evident that there is a statistically significant difference (P=0.028) during the first quarter of the experimental period. Naturally, they may not remain longer with any one of the males during the initial period of exposure to the stud male and

the alien male. Repeated stimulation from the olfactory cues of the alien male might be one of the possible causes of pregnancy failure exhibited by the inseminated females. There are reports that multiple short-term exposure of inseminated females to strange males results in implantation failure (Chipman et al. 1966).

Unlike the monogamous rodents like prairie vole *Microtus ochrogaster* and California mouse *Peromyscus californicus*, house mouse *Mus muscuus domesticus* is highly promiscuous and mate with several males during their receptive period (Kingsbury et al. 2012). House mouse is a typical species that exhibit infanticide (Manning et al. 1995). It has been suggested that the multiple mating of female mice is a strategy of female for confusing the males on the paternity of the pups thus preventing infanticide from males other than the stud male. Moreover, females prefer the dominant male to sire its progeny. This is indicated by the fact that promiscuous female mice will accept mating from several males including the subordinate males. Rolland et al. (2003) studied the mate choice in mice and demonstrated that as a promiscuous species female mice may mate with several males; but females actively select the dominant male for the final ejaculation.

It is suggested that male infanticide is the selective force behind the evolution of the Bruce effect in mice (Hrdy 1979). In addition, Elwood et al. (1990) showed that females are more likely to undergo pregnancy block in the presence of infanticidal males rather than in presence of non-infanticidal males. In the light of these reports it is possible that inseminated females may consider the presence of an alien male as a sign of inability of the stud male to defend its territory and as the female is free to choose between two males it might have preferred the alien male. This report unequivocally demonstrates that inseminated females actively investigate alien males as suggested by deCatanzaro and Murji (2004). Further studies using more

females are needed to provide additional evidence for the preference of inseminated females to the stud male or to alien males.

Summary

This study is designed to evaluate the hypothesis that newly inseminated female mice actively avoid strange/alien males. In most of the laboratory studies on Bruce effect females were housed for at least 48 h with confined alien males. In such a situation active avoidance is not possible with the female. In this investigation, females are free to choose between the stud male and an alien male. This study shows that, inseminated females actively investigate alien male during the first quarter of the experiment. The high incidence of implantation block observed in the female may be due to the natural tendency of females of *Mus musculus* to investigate the odors of alien/strange males.

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Consolidated discussion

Consolidated discussion

The influence of chemical substances on the reproductive process of mammals was reported as early as in 1955. Hilda M. Bruce (1959) reported the male-induced implantation failure (the Bruce effect) in laboratory mice and so far it remains as one of the best investigated pheromonal effects in mammals. However, the exact definition and the role of pheromones in chemical communication in mammals is still a controversial subject. It is interesting to note that normal odors when coupled with experience may produce stereotyped behaviors similar to what is observed in the case of response to a pheromone. Unlike the pheromones in insects which trigger innate, stereotyped behaviors in recipients, the Bruce effect involves a learning process which enables the female to distinguish a strange male or its odor from that of the stud male.

The effect of exposure to the alien male, strange male, stud male and two different oils-groundnut oil and gingelly oil (nonpheromonal cues) on the pregnancy of inseminated BALB/c females show that implantation failure was observed in a large proportion of newly inseminated females on exposure to the alien male or to a strange male. Effectiveness of strange males in inducing implantation block was lesser than that of the alien males. Re-exposure of the newly inseminated females to the stud male after separation for 24 hours did not result in the implantation block. Exposure to groundnut oil or gingelly oil did not affect implantation in BALB/c females. All females exposed to these oils delivered healthy pups on completion of their term of pregnancy.

A nonpheromonal cue could possibly protect the female from alien male-induced implantation failure provided it is exposed to the female during the pericopulatory period. The majority of the females exposed to a nonpheromonal cue during the pre- or post-copulatory interaction with the

males, exhibited a higher rate of pregnancy failure on re-exposure to the same cue at the time of exposure to alien males. The protective effect of the nonpheromonal cue is observed only when the female is exposed to it during the pericopulatory sensitive period. However, imprinting of the nonpheromonal cue is possible only if the female is allowed to have direct contact (the nonvolatile and volatile cues) with the nonpheromonal cue during the pericopulatory sensitive period. Exposure of the female, only to the volatile cues associated with the groundnut oil will not imprint the odor in the inseminated females. These investigations demonstrate that a nonpheromonal cue exposed during pericopulatory period gets imprinted in the female mice and offers protection against alien male induced implantation failure.

The outcome of the experiments to evaluate whether the nonpheromonal cue imprinted during mating has any luteotrophic property in implantation-blocked females shows that, the majority of these females exhibited pseudopregnancy on re-exposure to the imprinted nonpheromonal cue irrespective of whether the original pregnancy is blocked by exposure to alien male or by injection of bromocriptine. The rate of pseudopregnancy seen in these females is similar to pseudopregnancy observed in implantation-blocked females re-exposed to the stud males. The results confirm that during mating the female mouse gets imprinted with a blend of nonpheromonal cues along with the individual pheromones of the stud male. The imprinted nonpheromonal cue acts analogous to the pheromonal cues of the stud male in inducing pseudopregnancy in implantation-blocked females.

In this study, whether a nonpheromonal cue imprinted in the female mice during pericopulatory period is capable of protecting inseminated females from implantation failure when they are exposed to two different stressful stimuli was evaluated. Presence of imprinted nonpheromonal cue during exposure of

inseminated female to a male rat is capable of protecting the female from implantation block. These results provide additional evidence that female mice can be imprinted with a nonpheromonal odor and it acts analogous to the stud male pheromones. Starved BALB/c females are generally refractive to the luteotrophic effect of the stud male and imprinted nonpheromonal cue and also ineffective in protecting the females from implantation block induced by starvation.

Preference towards urine of dominant and subordinate male by female mice in estrus and the effect of a nonpheromonal cue in the formation of olfactory associative memory was evaluated. Results revealed that the females in estrus always prefer the urine of dominant male than the subordinate male. This study also shows that when females were exposed to nonpheromonal cues paired with the urine of dominant male and subordinate male, the females prefer the cues paired with the dominant male urine. The associative olfactory memory lasts up to the fourth estrus day after the initial encounter. Estrus females are capable of identifying dominant /subordinate status of the male, based only on the volatile cues of the urine of male.

In most of the laboratory studies on the Bruce effect females were housed for at least 48 h with confined alien males. In such a situation active avoidance is not possible with the female. The results of the investigations demonstrate that if a choice is given, inseminated females actively investigate the alien male. It should be noted that in all investigations on the protective effect of the stud male, inseminated females were exposed to the stud male and the alien male simultaneously with no chance for females to avoid pheromonal cues of stud male. This study indicates that if the inseminated female is free to make a choice between the stud male and an alien male it prefers the alien male than the stud male during the first quarter of the experimental period. Promiscuous mating system seen in Mus musculus domesticus may be one of the reasons for

the observed preference of females to alien males.

Final conclusions

1. Implantation failure was observed in a large proportion of inseminated BALB/c females on exposure to alien male or to strange male. The effectiveness of strange males in inducing the implantation block was lesser than that of alien males.
2. Exposure to groundnut oil or gingelly oil does not affect implantation in BALB/c females.

3. A nonpheromonal cue imprinted in the female during the critical sensitive period of mating could possibly protect the female from alien male-induced implantation failure.
4. Pericopulatory time is the sensitive period for imprinting the olfactory memory
5. During mating the female mouse gets imprinted with a blend of nonpheromonal cues along with the individual pheromones of the stud male.
6. The imprinted nonpheromonal cue acts analogous to the pheromonal cues of the stud male in inducing pseudopregnancy in implantation blocked females
7. Presence of imprinted nonpheromonal cue during exposure to male rat is effective in protecting inseminated female from implantation failure.
8. However, the presence of imprinted nonpheromonal cue is ineffective in protecting the inseminated female from starvation-induced implantation failure.
9. BALB/c females in estrus have the ability to identify the dominant status of the male based on the volatile cues present in the male urine.
10. Estrus females are able to learn a nonpheromonal cue paired with the

dominant male urine and discriminate it from another nonpheromonal cue associate with the urine of subordinate males.

11. Females retain the memory of nonpheromonal cue until the appearance of the third estrus after their first exposure, beyond which the memory will be obliterated.

12. Inseminated females actively investigate alien male during the first quarter of the experimental period when it is given a free choice between alien male and stud male. This may be one of the reasons for the failure of pregnancy observed in inseminated females exposed to an alien male and the stud male.

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Imprinting of a Nonpheromonal Cue and Its Protective Effect on Alien Male-Induced Implantation Failure in Mice

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Abstract

Bruce effect or alien male–induced implantation failure is a well-studied phenomenon in mice. The presence of stud male during exposure to an alien male protects the female from implantation failure. The pheromones of the stud male are imprinted in the female at the time of mating and act as a luteotrophic agent. We tested whether a nonpheromonal cue exposed to the female during pericopulatory sensitive period could protect pregnancy in newly inseminated females exposed to alien males. Virgin females were allowed to mate in the presence of a cotton ball smeared with groundnut oil as a nonpheromonal cue. When these females were exposed to alien males in the presence of groundnut oil, the majority of the females retained their pregnancy. We evidenced that a nonpheromonal cue could possibly protect the female from alien male–induced implantation failure. The majority of the females exposed to the nonpheromonal cue during the pre-or post-copulatory interactions with the males, exhibited higher rate of pregnancy failure on reexposure to the same cue at the time of exposure to alien males. The protective effect of the nonpheromonal cue is observed only when the female is exposed to it during the pericopulatory sensitive period.

Key words: Bruce effect, critical period, nonpheromonal cue, olfactory imprinting, pheromones

Introduction

Pheromones are substances that are secreted externally by an individual and elicit a specific response when perceived by another individual of the same species (Karlson and Butenandt 1959). Alien male–induced implantation failure is a well-studied pheromonal effect in mice (Bruce 1959; Dominic 1987; Archunan 2014). Exposing a newly inseminated female to an alien male results in implantation failure and reverts the female to estrus stage (Bruce 1959). Reexposure to the stud male 24 h after mating did not induce implantation failure (Parkes and Bruce 1961). Pregnancy failure was not observed when the newly inseminated female was exposed to alien male in the presence of the stud male or its urine (Thomas and Dominic 1987a). It is suggested that inseminated mouse gets imprinted with the odor of the stud male and so responds differentially to a strange male than the stud male (Parkes and Bruce 1961; Thomas and Dominic 1988). This implies that each male has its signature odor to be imprinted on females.

The efficacy of the odor of the stud male in protecting pregnancy in newly inseminated female mice is well documented in several other contexts. For example, Kumar and Dominic (1996) demonstrated that the presence of the stud male significantly reduces implantation failure in newly inseminated females exposed to male rat. Nutritional stress or food deprivation during first 2 days of pregnancy significantly affects implantation ratio in laboratory mice

(Bruce 1963; McClure 1963; Sahu and Dominic 1985). But significant reduction in implantation failure was found in female mice when they were housed with stud male, even when the female was food deprived (Archunan and Dominic 1989).

Here, we addressed the question whether it is possible to imprint the female mouse with a nonpheromonal cue during the pericopulatory sensitive period. If imprinting is possible, reexposure to the imprinted nonpheromonal cue will protect pregnancy on exposure to an alien male, in the absence of the stud male. Finally, we evaluated the critical time point for imprinting the nonpheromonal cue.

Materials and methods

We purchased 60 adult virgin females and 14 adult males (10–20 weeks; BALB/c) from Kerala Veterinary and Animal Science University. All animals were housed in polypropylene cages (29 × 22 × 14 cm) with rice-husk bedding. Fourteen adult wild strain male mice were collected from houses in and around Thrissur. They were individually housed in cages and kept in a separate room. The temperature (23 °C) and reverse light:dark cycle of 12:12 h (lights on at 18:00 h) were kept constant. Animals were fed on a standard diet purchased from Small Animal Breeding Station of Kerala Veterinary and Animal Science University and water was provided ad libitum.

Different stages of estrous cycle were monitored daily using a vaginal smear technique. Stages of estrous cycle of the mice are reflected in the appearance and composition of the vaginal cells and these stages can be determined by examining the cells under a compound microscope. Vaginal smears were obtained by gentle scraping of the dorsal wall of the vagina with a steel spatula with smooth edges. The female was held in head-down position and the tip of the spatula moistened with water was introduced into the vagina and the vaginal wall was gently scraped to obtain the smear. This technique will not cause any injury to the vagina or cervix. A small sample of the cells from the vaginal epithelium was spread on a clean glass slide, dried, and examined without staining under a microscope. The approximate proportion of each cell type in the smear was recorded (McLean et al. 2012).

In each experiment, 30 adult virgin females were divided into 5 groups comprising 6 females in each group (Groups I–V). Their behavior was observed using an infrared camera connected to a monitor kept in the adjacent room. We used 2 drops of food-grade groundnut (*Arachis hypogaea*) oil (PRO PRIMIO Refined Groundnut Oil; R.R. Oomerbhoy Pvt. Ltd), smeared cotton ball to provide a nonpheromonal cue.

Urine collection and exposure

Stud male was taken out from its cage and placed on a glass plate. Holding the tail of the animal in the left hand, the abdominal region of the male was gently pressed. The expelled urine was then collected using a clean dropper. Two drops of urine were smeared on a fresh cotton ball and placed on the bedding of the cage, allowing the female to have direct contact with it.

Experiment 1: effect of a nonpheromonal cue on alien male-induced implantation failure

All females were monogamously paired with adult males and allowed to mate (Groups I–V). In Group III, a cotton ball smeared with groundnut oil was provided at time of pairing. In Group IV, the mating pair was exposed a fresh cotton ball without groundnut oil. Females with vaginal plug (day 0 *post coitum*) were separated from the stud male and housed individually in a new cage with fresh bedding. After 24 h (day 1 *post coitum*), they were subjected to the following treatments:

Group I: Female was exposed to a confined alien male in the presence of confined stud male, allowing contact with urine and excreta of both males.

Group II: Female was exposed to a confined alien male, allowing contact with its urine and excreta and exposed to a cotton ball smeared with urine of the stud male.

Group III: Female was exposed to a confined alien male allowing contact with its urine and excreta and exposed to a cotton ball smeared with groundnut oil.

Group IV: Female was exposed to a confined alien male allowing contact with its urine and excreta, and exposed to a cotton ball without groundnut oil.

Group V: Female was left undisturbed (untreated control).

After 48 h, all females were returned to their cages and housed individually till the termination of the experiments (day 5 *post coitum*).

Experiment 2: critical period of imprinting a nonpheromonal cue

Group I: The mating pair was exposed to a cotton ball smeared with 2 drops of groundnut oil during free interaction (premating exposure). On initiation of copulation, the male and the female were transferred to a new cage and allowed to mate. They were observed till the formation of the vaginal plug and after 1 h, the female was transferred to a new cage with fresh bedding.

Group II: One hour after vaginal plug formation was confirmed in female, the pair was exposed to a cotton ball smeared with groundnut oil (postmating exposure). After 2 h, the female was transferred to a new cage with fresh bedding.

Group III: Female was exposed to a cotton ball smeared with groundnut oil on initiation of copulation (pericopulatory exposure). The formation of vaginal plug was confirmed and after 1 h of postmating period, the female was transferred to a new cage with fresh bedding.

Group IV: Female was exposed to a cotton ball without groundnut oil on initiation of copulation (pericopulatory exposure). The formation of vaginal plug was confirmed and after 1 h of postmating period, the female was transferred to a new cage with fresh bedding.

Group V: After mating and formation of vaginal plug, female was separated from the stud male and kept undisturbed till the termination of experiment (untreated control).

Twenty four hours after (day 1 *post coitum*), the newly inseminated females in all groups except those in Groups IV and V, were exposed to a confined alien male in presence of a fresh cotton ball smeared with groundnut oil for 48 h. Females in Group IV were exposed to alien male in presence of a cotton ball without groundnut oil. Females in Group V were left undisturbed. Experiments were terminated on day 5 *post coitum*.

All females were housed separately in their home cages up to day 20 *post coitum* to ascertain their reproductive status. Vaginal smear was examined daily from all females of Experiments 1 and 2. Presence of abundant irregular-shaped, nonnucleated, cornified squamous epithelial cells in the smear was taken as the indication of pregnancy block and return to estrus (Thomas and Dominic 1987a; McLean et al. 2012). Vaginal smear with abundant leucocytes and

mucus was taken as the indication of pregnancy. The number of females that were pregnant after showing estrous smear and the number of pups delivered by females that did not exhibit estrous smear were recorded.

Statistical analysis

We used Fisher's Exact test for analyzing the data. This test is used when the sample sizes are small and when there are 2 nominal variables.

Ethical note

Experiments involving animals were performed in adherence to the guidelines of the Institutional Animal Ethics Committee of Jubilee Mission Medical College and Research Institute, Thrissur, Kerala, India (Reg. No.1811/PO/Re/S/15/CPCSEA: Dated 09/06/2015 of CPCSEA).

Results

Experiment 1: effect of nonpheromonal cue on alien male-induced implantation failure

P value of pair-wise comparison of incidence of pregnancy using Fisher's Exact test revealed that there was no statistically significant variation when Group III was compared with Groups I, II, and V; but Group III showed a very statistically significant variation compared with Group IV.

The nonpheromonal cue used in our model (groundnut oil) is capable of protecting the female from alien male–induced implantation failure (Group III; Table 1). This is not, however, significantly different from Group I (exposed to the alien male in the presence of the stud male), or Group II (exposed to the alien male in presence of the stud male's urine) or Group V that were left undisturbed during postmating period. The females in Group IV, which were exposed to alien male allowing free access to a cotton ball without nonpheromonal cue in the absence of the stud male or its urine showed significantly higher rate of implantation failure.

Experiment 2: critical period for imprinting nonpheromonal cue

P value of pair-wise comparison of the incidence pregnancy using Fisher's Exact test revealed that there was a very statistically significant variation when Group III compared with Groups I, II, and IV; but Group III showed no statistically significant variation compared with Group V.

The nonpheromonal cue exposed during copulation (initiated with lordosis behavior and ended in ejaculation and formation of vaginal plug) protected the pregnancy in females on reexposure to the same cue at the time of exposure to the alien male (Group III; Table 2). This is not statistically different from pregnancies observed in inseminated females which were left undisturbed after mating (Group V). Females exposed to the nonpheromonal cue during pre- (Group I) or postcopulatory periods (Group II) exhibited pregnancy failure when exposed to the alien males. Most of the females exposed to the alien males in the presence of a cotton ball without the nonpheromonal cue (Group IV) returned to estrus stage, terminating their pregnancies.

In both experiments, the females that exhibited cornified epithelial smear did not show any signs of pregnancy whereas those females that failed to show estrous smear continued their pregnancy and delivered varying number (7–9) of pups (Tables 1 and 2).

Discussion

Present results unequivocally demonstrate that the virgin females allowed to mate in the presence of a nonpheromonal cue, retained their pregnancy when they were reexposed to the nonpheromonal cue during exposure to the alien males in the absence of their stud males. The majority of these females retained their pregnancy, which is comparable with pregnancy observed in inseminated females exposed to alien males in the presence of their stud males or their urine. This indicates that a nonpheromonal cue exposed to females at the time of mating is imprinted in the females and reexposure to the same cue protects the females from alien male-induced implantation failure. In contrast, females allowed to mate with males in the presence of a cotton ball without the nonpheromonal cue exhibited significantly higher rate of pregnancy failure when exposed to alien males. It should be remembered that this effect is not specific to groundnut oil. Exposure to gingelly (*Sesamum indicum*) oil during mating period is also capable of protecting pregnancy of the inseminated females exposed to alien males (unpublished data).

It is generally believed that a female is imprinted specifically with the individual odor of the stud male and the female has the ability to discriminate him at least for a few days after the original mating and respond differentially to the odor of the stud male and to the

Table 1. Protective effect of nonpheromonal cue on alien male-induced implantation failure

Groups	Treatment	No. of females (*n*)	Results		
			Pregnancy *n* (%)	Pregnancy block *n* (%)	No. of pups delivered
Group I	Exposed to alien male along with stud male	6	6 (100)	0 (0.0)	47
Group II	Exposed to alien male along with urine of stud male	6	5 (83.3)	1 (16.7)	38
Group III	Exposed to alien male along with a cotton ball smeared with groundnut oil	6	6 (100)	0 (0.0)	48
Group IV	Exposed to alien male along with a cotton ball without groundnut oil	6	0 (0.0)	6 (100)	0
Group V	Untreated control	6	6 (100)	0 (0.0)	49

P value using Fisher's Exact test of Group III with other groups.
Group III with Group I = 1.000.
Group III with Group II = 1.000.
Group III with Group IV = 0.0022.
Group III with Group V = 1.000.

Table 2. Critical period for imprinting of a nonpheromonal cue

Groups	Treatment	No. of females (*n*)	Results		
			Pregnancy *n* (%)	Pregnancy block *n* (%)	No. of pups delivered
Group I	Premating exposure to a cotton ball smeared with groundnut oil	6	1 (16.7)	5 (83.3)	7
Group II	Postmating exposure to a cotton ball smeared with groundnut oil	6	0 (0.0)	6 (100)	0
Group III	Pericopulatory exposure to a cotton ball smeared with groundnut oil	6	6 (100)	0 (0.0)	44
Group IV	Pericopulatory exposure to a cotton ball without groundnut oil	6	1 (16.7)	5 (83.3)	8
Group V	Untreated control	6	6 (100)	0 (0.0)	47

P value using Fisher's Exact test of Group III with other groups.
Group III with Group I = 0.0152.
Group III with Group II = 0.0022.
Group III with Group IV = 0.0152.
Group III with Group V = 1.000.

odor of other males (Parkes and Bruce 1961; Thomas and Dominic 1987c). It has been demonstrated that pheromones other than the stud male can interfere with the imprinting of the odor of the stud in the inseminated female. Thomas and Dominic (1989) reported that the ability of alien males to induce implantation failure in inseminated females is significantly reduced when they were exposed to alien males immediately after insemination.

Presence of the stud male during exposure to the alien male considerably lowers the incidence of implantation failure in inseminated females (Thomas and Dominic 1986, 1987a). It is suggested that the stud male could protect the newly inseminated female from implantation failure because its pheromones have a luteotrophic effect in inseminated females (Thomas and Dominic 1987b, 1987c; Archunan 2014).

The luteotrophic effect of the cues associated with the stud male is further demonstrated in a study in which a majority of the pregnancy-blocked females when reexposed to confined stud males exhibited pseudopregnancy (Thomas and Dominic 1987c), indicating that the olfactory memory of the stud male is retained in the females for a long time after the actual mating.

The protective effect of the nonpheromonal cue against the implantation blocking influence of the alien male shows that nonpheromonal cue can be imprinted in the memory of the inseminated female provided the female is exposed to it during mating process. In other words, the female identifies the stud male and shows differential response to the stud male and another male, not because of the specificity of the individual odor of the stud male, but because of the associative learning that takes place during the mating time.

A critical period or sensitive period is one of the major characteristic features of imprinting and in most cases it takes place during certain unique experience of the individual. Precocial birds are shown to get imprinted with any moving object that they see first and exhibit "following behavior," an instinctive behavior usually exhibited by the chicks to their mother (Lorenz 1961; Spalding 1872), indicating that animals can be imprinted with any stimulus with certain characteristic features, if they are exposed to it during the sensitive period. In precocial birds, movement is the characteristic feature to which the young pay attention to than the shape or the color of the stimulus.

In this study, the protective effect of the nonpheromonal cue against the pregnancy blocking influence of the alien male is observed only when the nonpheromonal cue is exposed to females during pericopulatory sensitive period (Experiment 2; Group III). The majority of the females exposed to the nonpheromonal cue during the pre- or postcopulatory interactions, (Experiment 2; Groups I and II), exhibited higher rate of pregnancy failure on reexposure to the same cue at the time of exposure to the alien males. It seems that vaginocervical stimulation is the key factor in imprinting the nonpheromonal cue in these females. In sheep, studies demonstrate that sudden rise in estrogen in females just before parturition and vaginocervical stimulation during the expulsion of the fetus plays significant role in imprinting the odor of the neonate in the mother (Kendrick et al. 1991; Nowak et al. 2011). It was revealed that in mice the formation of pheromone-induced olfactory memory in accessory olfactory bulb depends on vaginocervical stimulation during mating with the stud male (Otsuka et al. 2001; Ichikawa 2003).

This study on the protective effect of a nonpheromonal cue against implantation failure induced by an alien male indicates that the nonpheromonal cue of groundnut oil, which the females experience during pericopulatory sensitive period has the ability to act as a luteotrophic signal in inseminated mice. To the best of our knowledge, this is the first report that demonstrates the effectiveness of an imprinted nonpheromonal cue in protecting pregnancy of a female against male-induced implantation failure (the Bruce effect). This study shows that ambient chemical cues or nongenetically determined odors that the female mouse perceives at the time of mating may blend with the genetically determined pheromonal cues of the stud male in forming a chemical signature of the stud male (Wyatt 2010). The female learns or gets imprinted with this chemical signature, rather than only with the pheromones of the stud male. It is important to note that the nonpheromonal cue, once imprinted, acts analogous to the stud male pheromones, and may form a memory in the olfactory system offering protection against the Bruce effect. However, the mechanism involved in the protective effect of nonpheromonal cue is presently unknown.

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CPCSEA APPROVAL

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Government of India
Ministry of Environment, Forests & Climate Change
Animal Welfare Division
O/o Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)
******

5th Floor, Vayu Block, Indira Paryavaran Bhawan,
Jor Bagh Road, New Delhi - 110003.
9th June, 2015

To

Dr. V. K. Ramankutty,
Principal,
Jubilee Mission Medical College & Research Institute,
Jubilee Mission Hospital Trust
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Subject: Registration of Establishment with the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) - reg.

Sir,

This is with reference to your application on the above mentioned subject. Your establishment has been registered with CPCSEA for the purpose of *'Research for educational purpose on small animals'*.

2. The Registration number is 1811/PO/Re/S/15/CPCSEA and the registration is valid up to three years i.e., from the date of issue of this letter. Henceforth, the above registration number may be quoted in all your future correspondence with this office.

3. Kindly acknowledge the receipt of this letter.

Yours faithfully,

(S. Gowri Shankar)
Deputy Secretary (AW) & Member Secretary (CPCSEA

Encl.: As above.

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JUBILEE MISSION HOSPITAL TRUST

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Ref No :

Date .....

JMMC&RI/SARF/ IAEC/02/2015

October 28, 2015

Proceedings of the Institutional Animal Ethics Committee (IAEC) meeting, JMMC & RI.

To

Ms. Preeji K.P
Research Scholar
Department of Zoology
Christ College
Irinjalakuda. Thrissur

Madam,

Subject: Approval of your research project – regarding.

This is to certify that the research project entitled "*The Bruce effect: Investigations on the luteotrophic olfactory mnemonic in laboratory mice*" has been approved by the Institutional Animal Ethics Committee.

You are permitted to proceed with your project at the Small Animal Research Facility of this Institution.

28/10/15

Dr. V.K. Ramankutty
Chairman, IAEC
Jubilee Mission Medical College & Research Institute
Thrissur

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CERTIFICATE

This is to certify that the project title "...The Bruce effect: Investigations on the luteotrophic olfactory mnemonic in laboratory mice..." has been approved by the IAEC.

Name of Chairman/Member Secretary IAEC

Dr. V. K. RAMANKUTTY, M. D.
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& RESEARCH INSTITUTE,
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Signature with date

28/10/15

Chairman/Member Secretary IAEC

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