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Original Article

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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Olfactory discrimination and transitive inference in mice

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Abstract

Female mice are able to discriminate the social status of males based on the pheromone content of the urine. We tested whether this ability of female mice is innate or whether it develops through learning during contact with the male urine. Further we tested the ability for transitive inference of the mice by pairing the male urine with odorous edible oil. The results showed that without prior contact with male urine, virgin females were able to discriminate urine of dominant male from that of the subordinate male and thus, it is an innate ability of female mice. It was found that virgin females exhibit significant preference to the odour of oil paired with the dominant male urine as compared with that of the odour of the oil paired with the subordinate male urine. The preference shown by virgin females to the oil associated with dominant male urine was not significantly different from response shown to the urine of dominant male urine. The present study shows that the virgin female mice are able to transfer the value of dominant male urine to odour of the edible oil through associative memory. It was found that this associative memory lasts for two oestrous cycles and then fades off.

Keywords: Keywords: Dominant male urine, subordinate male urine, pheromone, olfactory discrimination, transitive inference

Introduction

Olfactory information is essential for a wide range of mouse behaviors, including navigating, foraging, avoiding predators, kin recognition, bond formation, mate selection, sexual and parental behaviors[1,2,3,4,5,6]. The onset of estrus and successful fetal implantation in female mice are both modulated by male odors [7]. Mice have the ability to discriminate conspecifics that differ in MHC odor type [8] and can determine whether others of their species are infected by viruses or parasites, presumably a skill useful in selecting a healthy mate [9,10]. Females of several species of mammals exhibit preference for dominant male and olfactory signals have been shown to be involved in this type of identification in hamsters, lemmings, mice and European rabbits[11]. In common with many species, male house mice advertise their location, successful territory ownership and dominance through scent marks continually deposited around their defended territory[12]. Such social odors are typically produced in urine or secreted from scent glands distributed over the body. Both volatile and nonvolatile cues are known to be produced[13,14,15,16]. Olfactory memory refers to the recollection of odors. Studies have found various characteristics of common odor memory including persistence and high resistance to interference. Recently several tests have been adapted to assess olfactory memory in mice[17,18,19,20]. Among different laboratory tasks used to investigate

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recognition memory in rats and mice, the social recognition/social discrimination task has several advantages. For instance, this task does not require the application of additional stimuli in order to provoke the behavioral response used as an index for the memory performance[21,22]. More specifically, the task takes advantage of the innate drive of an adult animal to investigate nonfamiliar over familiar conspecifics. Both rats and mice are macrosmates for which store and recall of information acquired by olfaction and this is a prerequisite for a successful interaction with the living and nonliving world[23,24]. Performance on the social recognition/social discrimination task requires olfactory information and male mice are able to store the "olfactory signature" of a given conspecific for at least 7 days[25].

It has been demonstrated that female mice are able to identify its stud male based on olfactory cues and the presence of the stud male or its urine protects the female from alien male induced implantation failure (The Bruce effect)[26]. Further it has been shown that when implantation blocked females are re-exposed to the stud males the majority of the females exhibited pseudo pregnancy, indicating that the activation of the olfactory memory by pheromones of the stud male releases the hormones essential for the maintenance of pregnancy in this species[27].

Transitive inference (TI) is a form of deductive reasoning that allows one to derive a relation between items that have not been explicitly compared before. In a general form, TI is the ability to deduce that if Item B is related to Item C and Item C is related to Item D, then Item B must be related to Item D.TI assumes that the animal assigns each stimulus with a value. The stimulus gets its own value, independent of the other stimuli. According to the theory of value transfer, in any simultaneous discrimination task, some of the value associated with the S+ is transferred to the accompanying S- The value transfer theory, predicts that the animal solves the TI task by assessing the value of the items being presented[28]. These values are acquired through training. The positive value of an item is transferred to the other item with which it is being presented[29]. Previous studies on mice have reported mixed results on whether this species is capable of associative inference[30],[31] and one study has reported that mice show transitive inference in the hierarchical series variant of the task[32].

The present study is designed to evaluate the ability of the oestrous female to discriminate the social status (dominant/ subordinate) of the male through pheromones present in the urine and whether the female is able to transfer the value of information they gained to any other substances through associative memory and transitive inference.

Materials and Methods

All studies were conducted in Balb/c mice purchased from Veterinary College, Mannuthy, Trissur, Kerala. Animals were housed under standard laboratory conditions of constant temperature of 23°C; 12:12 reverse light/dark cycle (lights on at 5 p.m.) and food and water were provided *ad lib*. Estrous cycle of female mice was assessed using standard vaginal smear method. Selected females were grouped into four, GP I, GP II, GP III and GP IV. Discrimination test was done in the first three sets. Associative memory test was conducted in the remaining GP IV. There were 3 mice in each group except the IVth group which had 8 mice. All tests were done during the estrous day (cornified+leucocyte cells in the smear) of females.

Study of the estrous cycle by examination of the vaginal smear

Stages of oestrous cycle of the mice are reflected in the changes in appearance and composition vaginal cells. The oestrous cycle of the animal can very easily be determined by examining the vaginal cells under a microscope. Vaginal smears are obtained by gentle scraping of the dorsal wall of the vagina with a steel spatula with smooth edges. The female is held in head down position and the tip of the spatula moistened with water is introduced into the vagina and the vaginal wall is gently scraped to obtain the smear. The 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.

Y-maze Tests for olfactory discrimination

Dominant and subordinate status of the male mice is determined through a dyadic encounter across a Plexiglas partition. Male showing higher aggressive behaviour indicated by higher frequency of aggressive postures and tail rattling is assumed to be the dominant male.

The maze consisted of a Plexiglas box (120 x 90 x30cm) plus two goal boxes and one start box (45 x30 x 30cm). A Plexiglas triangle at one end of the box divided it into a Y-shaped maze. The top edges of the Y-maze were covered with Plexiglas panels. Stimuli were placed in goal boxes, separated from the rest of the maze by perforated, opaque doors. The maze and start box were washed with a dilute soap solution and 70% ethanol between subjects.

Prior to the experiment, subjects were placed in the empty Y-maze for five min to familiarize them with the apparatus. Each testing session began by placing the subject in the start box for 30 seconds and then raising the door, allowing the animal to approach a goal box (free trial). A choice was recorded when the subject touches one of the goal box doors with her nose or front paw and the time spent near the goal box and latency to approach the goal box was recorded. If the subject failed to make a choice within 120s, it was returned to the start box and the trial was repeated.

After completion of a free 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 off using a wooden block and the start box door was raised, forcing the animal to approach the goal box not chosen on the previous trial (guided trial). In this way subjects were reminded of which stimulus lay behind each goal box door. Each day's testing session consisted of eight free trials alternating with seven guided trials. The locations of the stimuli were alternated on each test. The tests were done as follows;

Discrimination test

GP I – Urine of dominant male/ Urine of subordinate male (D/S)

GP II – Urine of dominant male + Groundnut oil/ Urine of subordinate male + Gingili oil (D+GN/S+GG)

GP II a – Groundnut oil/ Gingili oil (GN/GG)

GP III – Urine of dominant male+ Gingili oil/ Urine of subordinate male+ Groundnut oil (D+GG/S+GN)

GP III a - Gingili oil/ Groundnut oil (GG/GN)

Virgin female mice of 30-32g body weight were tested for their preference to approach Y-maze goal boxes containing the stimulus. In GPI the preference of the subjects to urine of dominant/subordinate male urine is tested. In GII one drop of the urine of dominant male was paired with ground nut oil and urine of the subordinate male was paired with gingili oil and tested their preference. One drop of male urine (Dominant/subordinate male) is placed on one end of a glass slide and one drop of oil was placed on the other side to avoid mixing up. In order to test the associative memory and value transfer ability of the females GP II were exposed to one drop of ground nut oil/gingili oil and tested their preference (GPII a). It is assumed that if the animal is able to associate the smell of the oil with that of the male urine paired with it, the subjects will exhibit prefetence to the respective oil in the absence of the urine of the male. To rule out the possibility of any natural preference of the animals to the oil, in GP III, urine of dominant male is paired with gingili oil and the urine of the subordinate male is paired with ground nut oil (D+GG/S+GN) and tested the preference of the subjects. In GP IIIa the same subjects were exposed to oil alone and their preference is tested. Frequency of visits to the stimuli, latency to approach the stimuli and the time spent near the stimuli were recorded. Proportion of equality test was used to assess statistical significance of the preference of the female mice.

Test for duration of associative memory

GP IV - Urine of Dominant male+ Groundnut oil/ Urine of Subordinate male + Gingili oil (D+GN/S+GG)

GP IV a- Groundnut oil/ Gingili oil (GN/ GG)

GP IV b- GN/GG

GP IV c- GN/ GG

GP IV d- GN/GG

The eight mice in the GP IV were initially exposed to the urine of Dominant male+ Groundnut oil/ urine of Subordinate male+ Gingili oil and tested their preference. All tests were conducted during the oestrous day

Y - maze

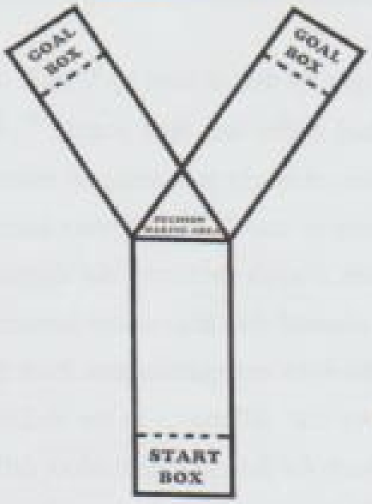

Fig.1. Model of Y-maze

of the female mice in a Y maze, as explained earlier. They were then divided into four sub groups (GP IV a, GP IV b, GP IV c and GP IV d) consisting of two mice in each group. In order to test the duration of associative memory of the four subgroups, olfactory preference of the subjects were tested by presenting only oil, in the absence of male urine. Test was done 6 days after first exposure in the case of GP IV a. In GP IV b, the experiment was done on the 12^{th} day, leaving one oestrous day in between. In GP IV c, test was done on the 18^{th} day after leaving two consecutive oestrous days without experiment. In GP IV d, the test was done after leaving three consecutive oestrous days without test ie., on 24^{th} day after the first exposure. Here also the frequency of visits, latency to reach the stimuli, and the time spent by the subjects near the stimuli during the free trials were noted. Proportion of equality test was done to analyse the statistical significance.

Result and Discussion

The results demonstrate that given a choice between urine of dominant and subordinate male, virgin female mice preferred the urine of dominant male. This is indicated by the fact that the frequency of visit of the female and time spent near the stimulus containing the urine of dominant male was significantly higher than that of the stimulus containing urine of subordinate male (Fig.2and 4) and the latency to reach the preferred stimuli was lower in all cases (Fig. 3). It is evident that virgin female is able to discriminate dominant male urine from that of the subordinate male and exhibited preference to it. Individual recognition is very crucial among social species in which each member depends on the identity of the other. It is suggested that one of the important functions of vertebrate pheromones is to designate the identity of the emitter[33]. These urinary scents provide a broad range of individual-specific information on a male's genetic compatibility and quality that is used by female mice in mate selection, influencing the relative attractiveness of individual males and their scents[34,35]. Female mice range over several male territories under natural conditions, strongly preferring to mate with dominant territory owners, and approach selected males when ready to mate[36,37,38]. In Syrian hamsters given a choice between dominant and subordinate males the females always preferred the dominant male[39]. In the house mouse (*Mus musculus*), where females were allowed free movement between confined dominant and subordinate males, they accepted more intromissions and ejaculations from the dominant than from the subordinate males[40]. In mice it has been shown that difference in the H-2 major histocompatibility locus and Tla regions of chromosome 17 is the basis for the individual odour difference[41]. Laboratory evidences suggest that female house mice choose males based on variation in traits such as the major histocompatibility locus and t alleles[42,43,44,45].

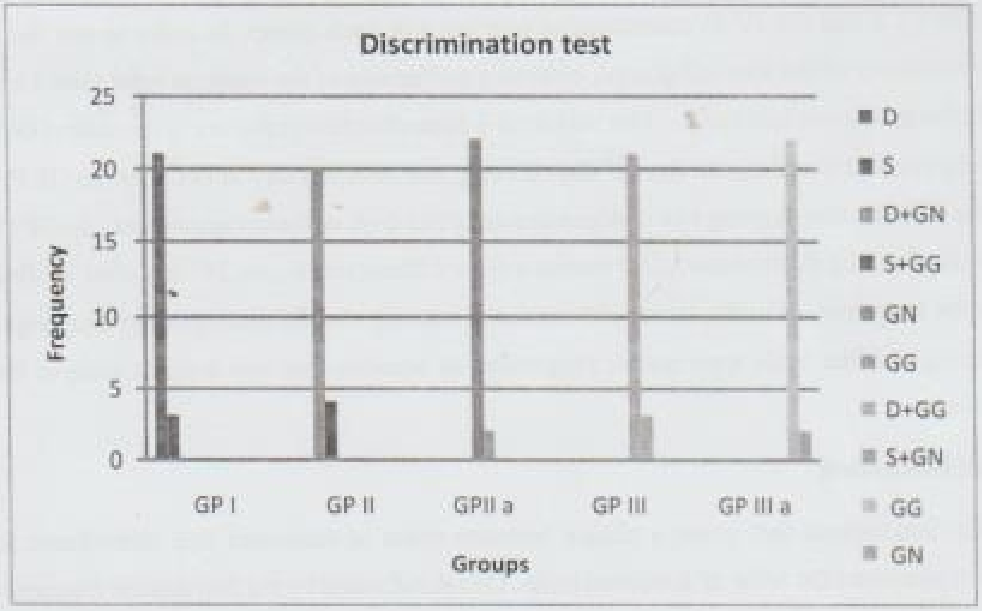

Fig. 2. Showing the frequency of visits by the female mice towards the stimuli during free trials.

(The table value for proportion of equality test is 1.645 and if we get values greater than table value means that the proportions are not equal and has significant variation. If the calculated values are lower or equal to table value means that the proportions are equal or has no significant variation. Calculated values for fig.2, GP I=5.55, GP II=4.38, GP II a=7.385, GP III=5.55 and GP III a=7.385, for fig.3, GP I=1.88, GP II=1.94, GP II a=1.94, GP III=2.08 and GP III a=2.04 and for fig.4, GP I=2.86, GP II=2.88, GP II a=2.72, GP III=2.73 and GP III a=2.76. The result shows that all values are greater than the table value means that proportions are not equal and have significant variation).

The present study shows that naive oestrous females always preferred dominant male urine and its associated stimuli, edible oil to that of the subordinate male and also take lesser time to reach the preferred stimuli. Likewise, time spent near the goal post presented with the urine of dominant male or oil associated the urine of dominant male indicates that the female associates the smell of the edible oil with olfactory signals emanating from the dominant male urine. The discrimination tests revealed that, preference towards groundnut oil or gingili oil is based on their association with the urine of dominant male and not depended on the quality of the oil (Fig. 2, 3 and 4).

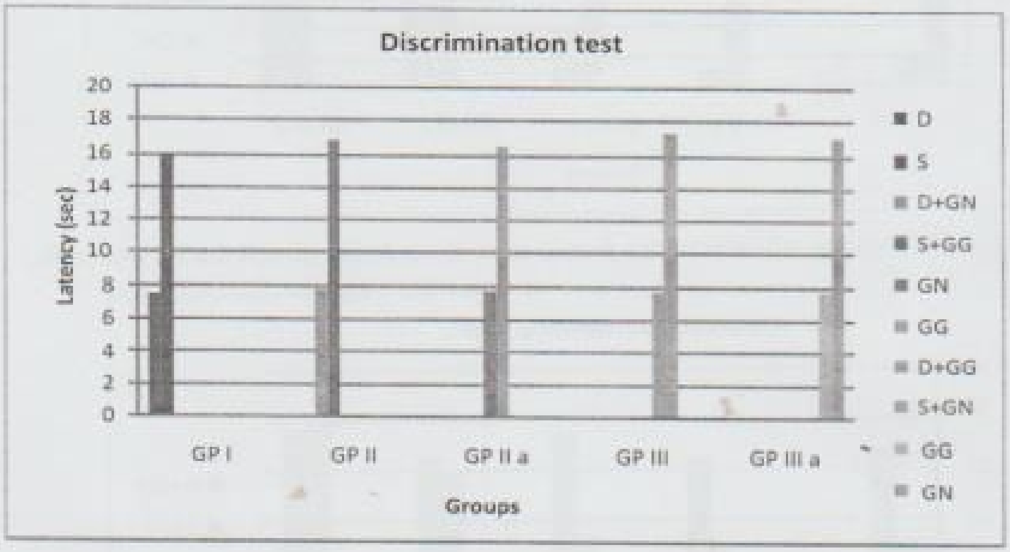

Fig. 3. Showing the time taken by the female mice to reach the stimuli during free trials.

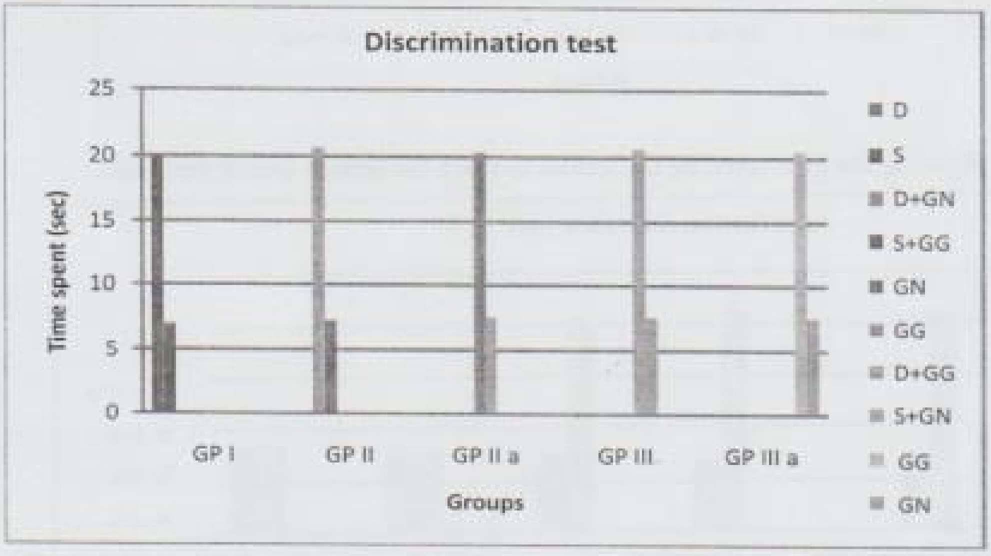

Fig. 4. Showing the time spent by the female mice near the stimuli during free trials

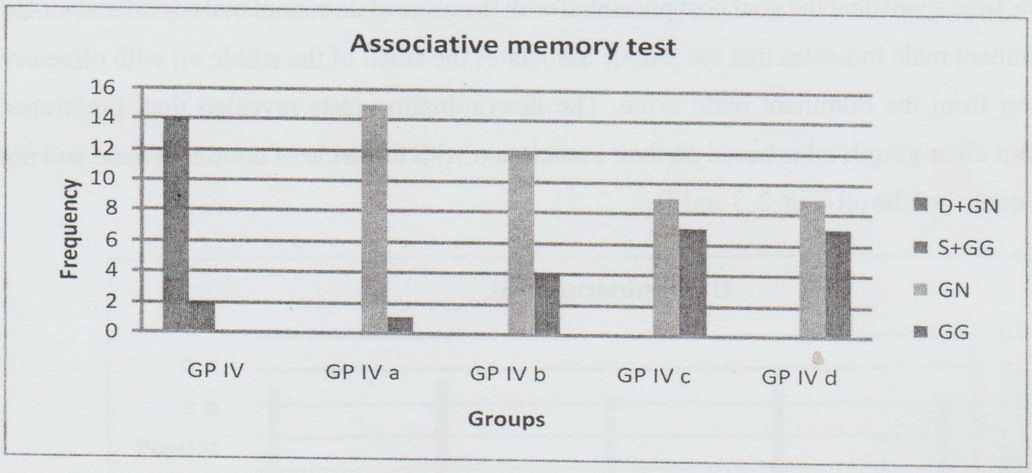

Fig. 5. Showing the frequency of visits by the female mice towards the stimuli during free trials.

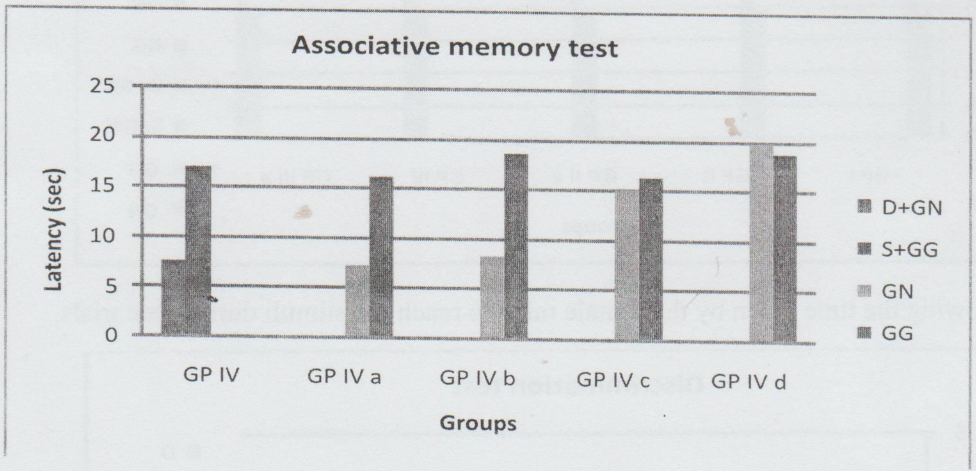

Fig. 6. Showing the time taken by the female to reach the stimuli during free trials

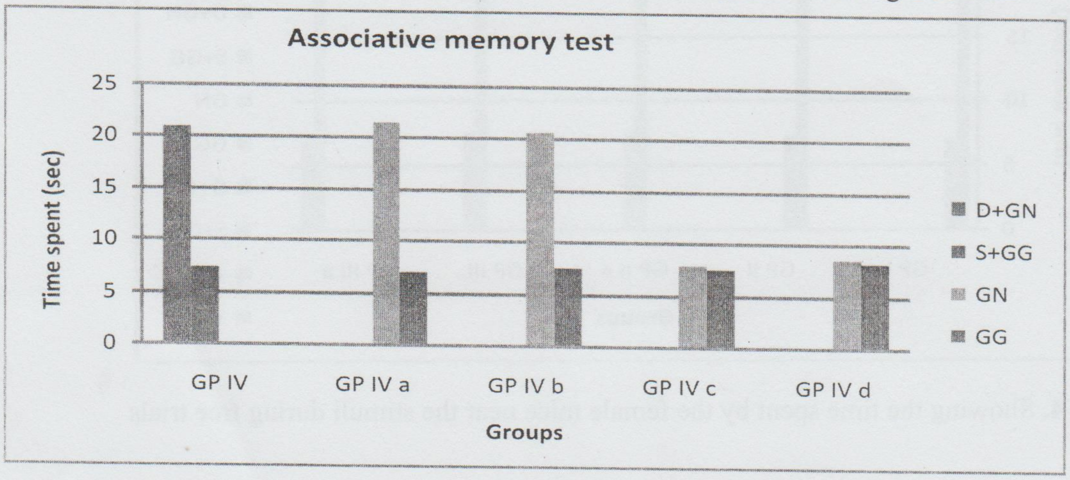

Fig.7. Showing the time spent by the female mice near the stimuli during free trials

(Table value is 1.645. Calculated values for fig.5, GP IV=4.53, GP IV a=7.2295, GP IV b=2.31, GP IV c=0.5039 and GP IV d=.0.5039. Result shows that there is significant variation in the proportions of GP IV, GP IV a and GP IV b but variation in the proportions are not significant in the case of GP IV c and GP IV d. For fig.6, GP IV=2.07, GP IV a=1.99, GP IV b=2.17, GP IV c=0.21 and GP IV d=0.18 indicates that GP IV c and GP IV d shows insignificant variation in their proportions but others shows significant variation in their proportions. For fig.7, GP IV=2.93, GP IV a=3.13, GP IV b=2.81, GP IV c=0.12 and GP IV d=0.09 shows that variafion in the proportions are not significant in GP IV c and GP IV d but significant in the remaining groups).

The test for the duration of retention of associative olfactory memory shows that the female preferred the smell of the edible oil associated with the dominant male urine till the end of second oestorus cycle (about 12-18 days). The sub groups of GP IV (Fig. 5,6 and 7) showed that, from the third oestrous day onwards, there is no significant difference in the frequency of visits, and duration of time spent near the associated oil of urine of both males. Moreover, there is no significant difference in the latency to reach the stimuli from the third oestrous day onwards, indicating that the olfactory memory of the female mice is higher during the early days of exposure and decrease significantly as the time passes on. The results indicate that female mice retains the memory of the urine of dominant male and the associated oil, only up to the third oestrous day.

Selecting dominant male as the mate provides several advantages to the female. In wild baboons (*Papio ursinus*) the age structure of MHC-*DRB* heterozygosity suggested higher longevity for heterozygotes, which should favour preferences for MHC dissimilarity[46]. It has been shown that female house mice accrue viability benefits for their offspring when they mate with males they prefer versus with males they do not prefer[47]. In another study in deer mice, *Peromyscus maniculatus*,[48] has shown that through four generations, the sons of dominant males generally were dominant to the sons of subordinate males.

It is interesting to note that the female mice have the ability to form associative memory of olfactory signals of the edible oil paired with the male urine and exhibit differential response to the oil paired with male urine even in the absence of male urine. It has been shown that neonatal mice, as young as 3 days old exhibit associative learning ability and learned preference for the conditioned odorant over a novel control odorant after removal of the dam from the home cage for 2 h, pre-`weanling CD-1 mice of ages 3, 5, and 10 days postnatal[49]. One of the interesting findings of the present study is that odour of edible oils can be conditioned by pairing with biologically important olfactory signal of urinary pheromone from a male and through this learned association; same odour can elicit situation specific and appropriate behavioural responses. Further studies are needed to elucidate the role of associative memory in individual recognition and mate choice in mammals.

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