



Ageing and sex attraction of *Teratorhabdits* nematode

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ABSTRACT. Sex attraction was observed in the free - living nematode *Teratorhabdits palmarum* (Gerber & Giblin-Davis, 1990). Attraction by means of pheromones provides the ability to find and distinguish appropriate individual with which to mate. In the present study, we performed sex attraction assay to examine whether males of different age groups or reproductive states respond to volatile sex attractants of females. In *T. palmarum*, young virgin males were strongly attracted to young virgin females but declined with their age. Young non - virgin males responded to young virgin females while they did not respond to ten - day old virgin female. Similarly, old but not young virgin males failed to respond to non - virgin females. Attraction of virgin and non - virgin males declined with their age and also declined or did not occur with increasing age of females. We also investigated the pheromone system of the gonochoristic nematode *Teratorhabdits palmarum* that produces sex - specific attractants of the opposite sex. Mass Spectrometric Analysis of the *T. palmarum* exometabolome revealed that females but not males, produce the male attracting ascr#1 and ascr#9, ascaroside previously identified from *C. elegans* and other nematodes. The specific function of these ascarosides would not be analysed, because these pheromones play different roles in different nematode species.

Keywords: Age; ascarosides; free-living; pheromones; reproduction; virgin.

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Introduction

Nematodes exhibit diverse modes of reproduction. Most species are dioecious (reproduce by cross fertilization), some are hermaphroditic (self - fertilization) or parthenogenetic (reproduce without fertilization). Most of the gonochoristic species attract the opposite sex by releasing sex pheromones (attractants). These attractants increase the chances of male - female encounters for cross fertilization. Greet (1964), was the first to observe sex attraction in free - living nematode *Panagrolaimus rigidus*. Duggal (1978) observed the effect of age and reproduction on sex attraction in *Panagrellus redivivus*. Ahmad and Jairajpuri (1980ab & 1981ab) demonstrated sex attraction in *Chiloplacus symmetricus* and *Cruznema lambdiensis* and suggested that continuous fall of males' attraction towards females was due to degenerative changes in reproductive state with age. Green and plumb (1970) demonstrated that females of *Globodera* and *Heterodera* produced attractant of more than one type and their males responded to more than one attractant. However, Chasnov et al. (2007) found that males of *C. elegans* are attracted to volatile of females of the gonochoristic *C. brenneri* and *C. remanei*. *G. rostochiensis* males were attracted by the sex pheromones of female *G. rostochiensis* but not attracted to female sex pheromone of firmly related *G. pallida* (Riga et al., 1996). According to White et al. (2007) sexual attraction requires both male - specific and core sensory neurons. Here, we present the sex attraction behaviour of *T. palmarum* and discuss the detail of attraction in homosexual as well as heterosexual mode.

Material and methods

Sampling

Farmyard manure samples collected from near the fort (27.8°N, 78°E) in Aligarh were processed by the decanting and sieving and the modified Baermann's funnel technique (Flegg, 1967).

Culturing the nematodes

Teratorhabdits palmarum was cultured in the laboratory on Nematode Growth Medium (NGM). First started the seed culture; *T. palmarum* culture was initiated by transferring nematodes collected from manure

into plain 1% water agar supplemented with milk powder. A single gravid female from the seed culture was transferred into a 0.1% streptomycin sulphate solution and an incision was made in the posterior region. The cut caused the release of eggs into the streptomycin sulphate solution. The eggs were left for five minutes and then repeatedly washed in sterile water. A monoxenic culture was initiated by transferring the sterilized eggs into 5 cm Petri dishes containing NGM which had been inoculated with *E. coli* (OP50) and left overnight at room temperature to allow bacteria to multiply.

The sex attraction assay was done in 5 - cm Petri dishes. The bottom of the dishes was marked with two concentric circles, 0.5 cm and 1.5 cm in diameter thus dividing the dishes into three zones, and outer, middle and inner zone. Sex attractants or pheromones were obtained by placing 20 females in 2 ml of water for 24 hr in a micro centrifuge tube. One drop of the attractant was placed in the inner zone of the Petri dishes containing a 3mm thick layer of 1% water agar and left for 12 hr. Ten males were then released at various points on the middle circle and their distribution was recorded after 30, 60 and 90 min. All experiments were replicated five times.

Attraction of males towards the females were tested using different age groups of males and females and also using individuals isolated (virgin) or in reproductive phases (non - virgin). The various combinations of heterosexual attraction studied were:

A) Attraction of virgin males towards virgin females' attractants

i) Two - day old males: Attraction between 2 - day old virgin males towards 2 - day old virgin females; 2 - day old virgin males towards 10 - day old virgin females.

ii) Ten - day old males: Attraction between 10 - day old virgin males towards 2 - day old virgin females; 10 - day old virgin males towards 10 - day old virgin females.

B) Attraction of non - virgin males towards virgin females' attractants

i) Two - day old males: Attraction between 2 - day old non - virgin males towards 2 - day old virgin females; 2 - day old non - virgin males towards 10 - day old virgin females.

ii) Ten - day old males: Attraction between 10 - day old non - virgin males towards 2 - day old virgin females; 10 - day old non - virgin males towards 10 - day old virgin females.

C) Attraction of virgin males towards non - virgin females' attractants

i) Two - day old males: Attraction between 2 - day old virgin males towards 2 - day old non - virgin females; 2 - day old virgin males towards 10 - day old non - virgin females.

ii) Ten - day old males: Attraction between 10 - day old virgin males towards 2 - day old non - virgin females; 10 - day old virgin males towards 10 - day old non - virgin females.

D) Homosexual attraction of young (2 - days old) males and females were tested towards the male and female secretions.

Pheromone extraction

Teratorhabditis palmarum was maintained on nematode growth media agar plates containing *Escherichia coli* (OP50) at $25 \pm 1^\circ\text{C}$. For exometabolome collection, approximately 500 females were picked with needle and washed twice with distilled water. The worms were transferred into a 1.5 mL tube containing 500 μL of water and stored at -70°C . A frozen 500 μL sample was thawed at ambient temperature and solution was filtered to remove worms. Sample was added with 1 ml of methanol and mixed in a vortexer for several seconds. The combined extract was again filtered, concentrated to dryness at 40°C under reduced pressure, and reconstituted in methanol for HPLC - MS analysis.

Mass spectrometric analysis

HPLC - MS analysis was performed using an Agilent 1100 Series HPLC system equipped with an Agilent Eclipse XDB - C18 column (9.4×250 mm, 5 μm particle diameter) connected to a Quattro II spectrometer (Micromass/Waters) using a 10:1 split ratio. Samples were analyzed using 0.1% acetic acid - water or 0.1% acetic acid - acetonitrile solvent gradient at a flow rate of 3.6 mL min^{-1} . Acetonitrile content was held at 5% for the first 5 min then increased to 100% over 40 min. Samples were analyzed by HPLC - ESI - MS in negative and positive ion modes. Single ion monitoring (SIM) in negative - ion ionization mode was used to detect the molecular ions ($[M - H]^-$) of ascr#9 and ascr#1 at m/z 247.2 and 275.2, respectively. Relative ascaroside content was quantified by integration of LC - MS signals from corresponding ion traces.

Results

A) Attraction of virgin males towards virgin females' attractants

i) Two - day old males: Young two days old males responded positively towards two - day female attractants (Figure 1A) and within 60 min a significantly larger number were in the inner zone as compared to the outer or middle zones ($p < 0.05$). After ninety min more males had migrated from the outer and middle zones to the inner zone ($p < 0.05$). Nearly 80% of the males had reached the inner zone while the rest were distributed approximately equally in the other two zones.

There was no positive response of two - day old males towards the secretion of 10 - day old female (Figure 1B). After 60 min there were significantly fewer males in the inner or middle zones as compared to the outer zone ($p < 0.05$). After 90 min, although the number of males was slightly more in the inner zones as compared to the other two zones, the increase was not significant ($p > 0.05$).

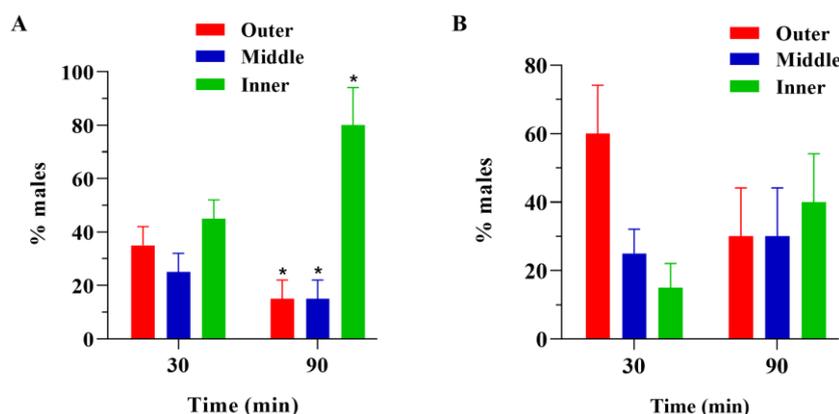


Figure 1. A: Attraction of 2-day virgin males towards 2-day virgin female secretions; B: 2-day virgin males towards 10-day virgin female secretions. Significant differences are indicated by a single asterisk ($p < .05$).

ii) Ten - day old males: Ten - day old males showed a relatively weaker response towards two - day old female attractants (Figure 2A). Attraction was observed only after 90 min ($p < 0.05$). There was no positive attraction of ten - day old males towards 10 - day old female attractants (Figure 2B). Males were unevenly distributed in the three zones after 90 min ($p > 0.05$).

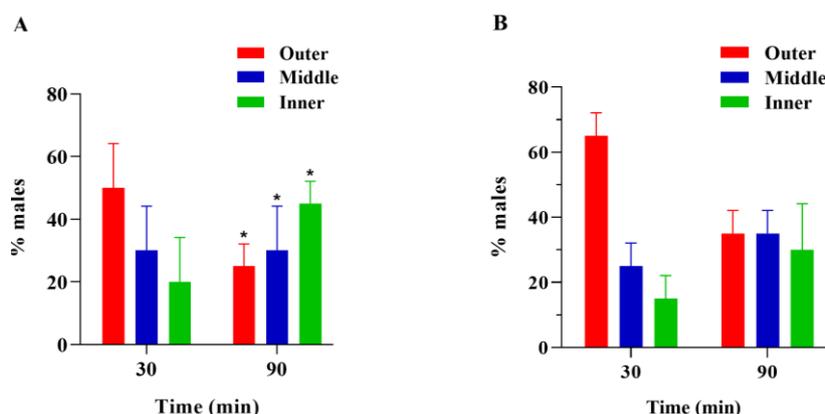


Figure 2. A: Attraction of 10 - day virgin males towards 2 - day virgin female secretions; B: 10 - day virgin males towards 10 - day virgin female secretions. Significant differences are indicated by a single asterisk ($p < .05$).

B) Attraction of non - virgin males towards virgin females' attractants

i) Two - day old non - virgin males: Two - day old non - virgin males were attracted towards the secretion of two - day old virgin female (Figure 3A). Attraction was only evident after 90 min when significantly more males ($p < 0.05$) were present in the inner zone as compared to the middle and outer zones.

There was no positive response of two - day old non - virgin males towards the secretion of 10 - day old virgin female (Figure 3B) after 30, 60 or 90 min ($p > 0.05$).

ii) Ten - day old non - virgin males:

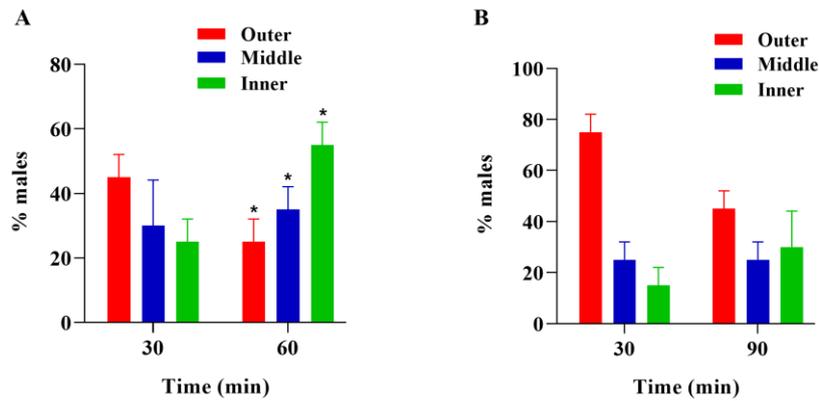


Figure 3. A: Attraction of 2 - day non - virgin males towards 2 - day virgin female secretions; B: 2 - day non - virgin males towards 10 - day virgin female secretions. Significant differences are indicated by a single asterisk ($p < .05$).

Ten days old non - virgin males showed no attraction ($p > 0.05$) towards two - and ten - days old virgin female secretions (Figure 4A & B).

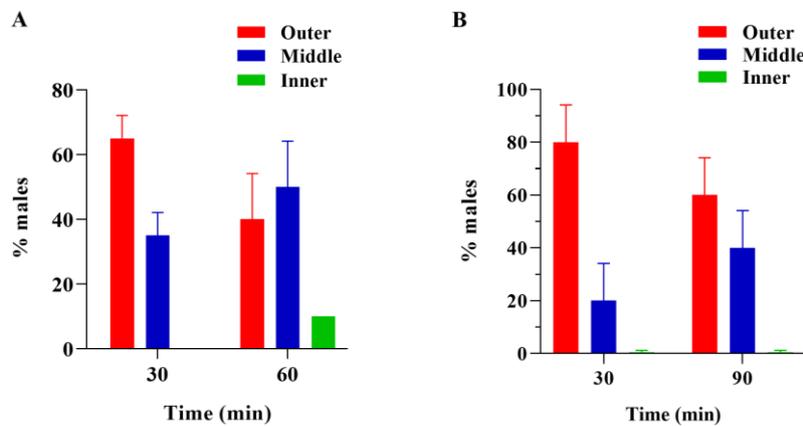


Figure 4. A: Attraction of 10 - day non - virgin males towards 2 - day virgin female secretions; B: 10 - day non - virgin males towards 10 - day virgin female secretions.

C) Attraction of virgin males towards non - virgin females' attractants

i) Two - day old virgin males: Two - day old virgin males responded positively ($p < 0.05$) towards the two - day old non - virgin female attractants (Figure 5A) only after 90 min.

No attraction was evident towards secretion of ten - day old non - virgin female (Figure 5B).

ii) Ten - day old virgin males:

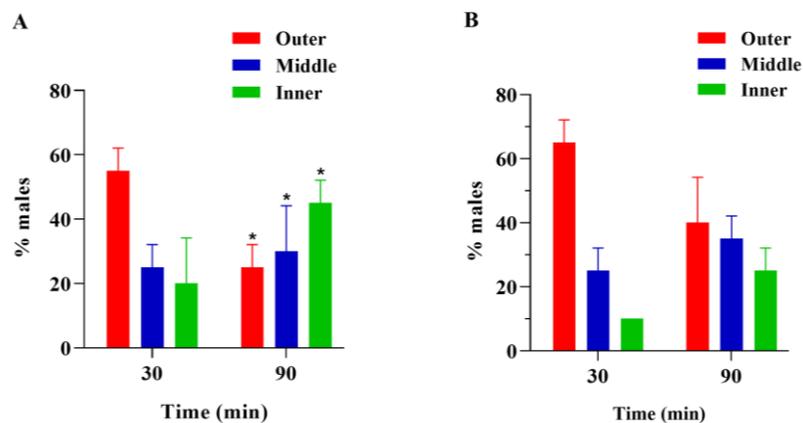


Figure 5. A: Attraction of 2 - day virgin males towards 2 - day non - virgin female secretions; B: 2 - day virgin males towards 10 - day non - virgin female secretions. Significant differences are indicated by a single asterisk ($p < 0.05$).

Ten days old virgin males did not show ($p > 0.05$) attraction towards two days and ten days old non - virgin female secretions (Figure 6A & B).

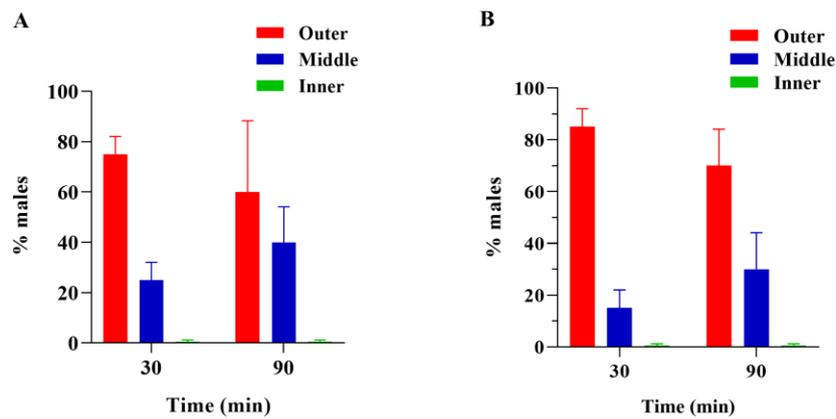


Figure 6. A: Attraction of 10 - day virgin males towards 2 - day non - virgin female secretions; B: 10 - day virgin males towards 10 - day non - virgin female secretions.

D) Homosexual attraction

Males did not show any significant attraction ($p > 0.05$) when tested towards male attractants and females also did not respond ($p > 0.05$) towards the female attractants.

HPLC - MS analysis of female secretions

Quantitative LC - MS analysis confirmed the presence of ascr#1 and ascr#9 in the exometabolome of the female. Ion chromatograms for ascr#1 and ascr#9 from negative - ion ESI - HPLC - MS analysis were obtained at 275.2 and 247.2 m/z ratio (Figure 7).

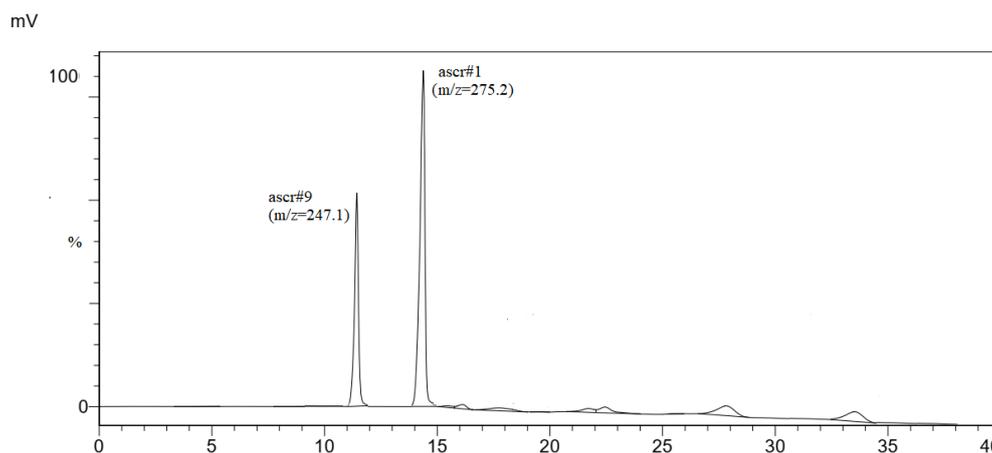


Figure 7. SIM - HPLC - MS analyses showing molecular ion traces for ascr#9 (left) and ascr#1 (right) in exo - metabolome sample of females.

Discussion

Ever since Greet (1964) first demonstrated the phenomenon of sex attraction in *Panagrolaimus rigidus*, several other soil and animal parasitic nematodes have been shown to produce and respond to sex attractants (Green, 1966; Bone & Shorey, 1977; Duggal, 1978; Ahmad & Jairajpuri, 1980a; Kiyohara, 1982). Sex attractants undoubtedly enhance the chances of male and female encounters, increasing mating possibilities. In *T. palmarum*, only females produced water soluble sex attractants that attracted males. Male attractants are also known in *Pelodera teres* (Jones, 1966); *Rhabditis pellio* (Somers et al., 1977); *C. elegans* Hedgecock, 1976); *Cylindrocorpus longistoma*, *C. cruzii* (Chin & Taylor, 1969); *Panagrolaimus silusiae* (Cheng & Samoiloff, 1971) and *C. symmetricus* (Ahmad & Jairajpuri, 1980a). There are however, several species where both males and females produce attractants for the opposite sex viz., *Panagrolaimus rigidus* (Greet, 1964); *Pelodera strongyloides* (Stringfellow, 1974); *P. redivivus* (Samoiloff et al., 1974; Duggal, 1978) and *C. tripartitum* (Ahmad & Jairajpuri, 1981a). Species showing unisexual attraction outnumber those showing bisexual attraction 2:1 and may represent, if the term is permissible, an older trait. It may be reasonable to assume that when both sexes attract one another, the chances of encountering one another and hence successful mating are enhanced. On the other hand, species where only females produce attractants appear to be as successful in

reproduction as those producing both types of attractants. In the sedentary tylenchid plant parasites, *Heterodera* spp and *Globodera* spp, it is understandable that only male attractants are produced (Green, 1966; Green & Plumb, 1970), while in the sedentary *Meloidogyne* species which are parthenogenetic, there were no attractants and no male response (Santos, 1972). If mutual attraction of the sexes is a more efficient mechanism, then this may be tempting to suggest that longevity could be one factor. In those species with a short lifespan, this may ensure a rapid aggregation of individuals leading to cross fertilization and successful reproduction. While in *C. lambdiense*, with an average life span of 6.5 days in mated individuals and 10 days in virgin may support such a hypothesis, information of a large number of species is needed to substantiate this relationship.

A decline in the attraction of mated females has been reported by Duggal (1978) in *P. redivivus* and Ahmad and Jairajpuri (1981a) in *C. lambdiense*. In *T. palmarum*, only two - day virgin males responded to two - day non - virgin females while ten - day virgin males failed to respond to all age groups of non - virgin females. Such a decline would be beneficial as females copulate only a few times in their lives and males could more efficiently locate the more actively secreting non - mated females. The cause of this decline has been suggested to be the changes in the female reproductive tract post copulation (Ahmad & Jairajpuri, 1981a). More recently, it has been shown that *C. remanei* females became unattractive after copulation but regained their attractiveness after mating (Chasnov et al., 2007). This would suggest that there may not be any significant change in the reproductive tract after mating but only the secretory pathway is inhibited for some time. The gonads have been implicated as the most likely source of attractants in *P. silusiae* (= *redivivus*) wherein inhibition of gonad development inhibited sex attraction (Cheng & Samoiloff, 1972). Conclusive evidence of the gonad as a source of attractants in females of *C. remanei* was provided by Chasnov et al. (2007). The behaviour of virgin and non - virgin males towards virgin females was nearly similar barring the response of six - day virgin males to all age groups of virgin females. This inconsistency is perplexing/confusing and is somewhat difficult to explain. Attraction of virgin and non - virgin males declined with their age and also declined or did not occur with increasing age of the females. As females were virgins, the postulate that changes in gonad after mating may have inhibited or reduced attractant secretion (Ahmad & Jairajpuri, 1981a) does not hold. It may seem likely that age related changes in the female gonad may have reduced or inhibited attractant secretion. An alternate possibility of a reduced male response could be a reduced perceptibility of attractants by chemosensory structures. We are inclined to believe that a reduced male response may be due to a combination of factors. A reduced secretion of attractants by females and an increased perception threshold of the sensory apparatus of males.

Recently Pungalija et al. (2009) showed that ascarosides regulates mating behaviour in *C. elegans*. In *P. redivivus*, *ascr#1* was the potent male attractant (Choe et al., 2012) while in *C. elegans*, *ascr#1* was originally thought to be a dauer inducing pheromone (Jeong et al., 2005) but later *ascr#2* and *ascr#3* were found to be more potent in inducing dauer larvae (Butcher et al., 2007). The presence of *ascr#1* and *ascr#9* shows that pheromones, similar to those found in other nematode species, also occur in *T. palmarum*. The specific activity of these ascarosides would not be determined and because similar pheromone play different roles in different nematode species. The specific role of *ascr#1* and *ascr#9* on the behaviour of *T. palmarum* remain unknown.

Conclusion

Observations on sex attraction in *T. palmarum* showed that only the males were attracted towards female secretions. Males showed no significant attraction towards male secretions. The females also did not respond to their own secretions. Sex attraction of ageing males and females was dependent on the age and reproductive state of the worms. All age groups of virgin males were responsive to all age groups of virgin females except that there was no positive attraction of ten - day old males towards six - days old or 10 - days old female attractants. The response of young males to older females decreased gradually and similarly the response of ageing males to younger females also decreased gradually. The attraction between non - virgin males and virgin females showed that only two - days old non - virgin males were attracted towards the secretion of two days old or six - days old virgin females and six - days old non - virgin males towards the two - days old virgin females' attractants while the females of other age groups were not attractive. Young non - virgin males responded to young virgin females but not to old virgin females. Males of all virgin age groups did not show positive response towards non - virgin females. Only two - day old virgin males were attracted towards the secretion of two - day old non - virgin females. Pheromone secretion is known to decline with age and also with physiological state of the females. Reproducing females generally show reduced pheromone production.

Data availability

All data generated or analyzed during this study are included in this article.

Acknowledgements

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