Effects of *Neozygites floridana* Infection in *Tetranychus urticae* Females on Sexual Behavior of Males

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Master thesis

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Preface

This master thesis is a part of the BERRYSYS project, run by the Norwegian Institute for Agriculture and Environmental Research (Bioforsk) and funded by Agricultural Agreement Research Funds (Forskningsmidler over jordbruksavtalen, JA) and Norwegian Foundation of Research Levy on Agricultural Products (Fonded for forskningsavgift på landbruksprodukter, FFL). Collaborating partners are Koppert Biological Systems, The Netherlands, and scientists at the Agricultural Faculty of São Paulo University (USP-ESALQ), Brazil.

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Ås, October 2012

Upendra Raj Bhattarai

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Abstract

The entomopathogenic fungus *Neozygites floridana* infects and kills the two-spotted spider mite, *Tetranychus urticae*, an important herbivorous pest worldwide. The fungal development stages in/ on the cadaver (mite infected and killed with *N. floridana*) might be described in three stages as: 1) non-sporulating cadaver 2) sporulating cadaver with primary conidia 3) sporulating cadaver with secondary (capilli) conidia. The last stage is the only stage which is infective to *T. urticae*. I tested the effects of these three cadaver stages on sexual behavior of *T. urticae* males in a series of experiments where males could choose between two non-moving females: a healthy quiescent deutonymph and a cadaver, placed individually on each of two slightly overlapping leaf discs. Results showed that cadaver stage had significantly affected the leaf disc choice of males. *T. urticae* males showed preference to leaf discs with non-sporulating cadavers and sporulating cadavers with primary conidia compared to healthy females. Conversely, *T. urticae* males preferred healthy females to sporulating cadavers with secondary conidia.

The frequency of two specific male behaviors, touching (male touching a female for less than 30 sec) and guarding (male mounted upon or within a body length of a female for 30 sec or longer) was also observed in this experiment. For touching, no significant difference between cadaver and healthy female was found for any of the three cadaver stages. Further, no significant difference in guarding of non-sporulating cadavers and healthy females was found. On the other hand, guarding rarely happened to primary conidia cadavers, and was never observed in secondary conidia cadavers.

To differentiate between the effect of fungal and female odor, an experiment where *T. urticae* males could choose between a non-sporulating male cadaver and a non-sporulating female cadaver was conducted. Results showed that males were more attracted to leaf discs with female cadavers than to leaf discs with male cadavers, and female cadavers were more often touched than male cadavers. Further, guarding was only observed for female cadavers.

Results presented above suggest that to *T. urticae* males, non-sporulating female cadavers are more attractive than healthy females. Moreover, males often approach and even touch cadavers that are full of infective conidia. This time-wasting and risky behavioral pattern demonstrates that the males are not well adapted to avoid *N. floridana* infection. However, they have the ability to avoid guarding cadavers full of spores. Sensory mechanisms of the male mites, together with texture and smell of the cadavers, may explain the results, but further studies are needed to conclude. If these results apply to biological control of *T. urticae* under larger-scale field conditions, female cadavers will spread *N. floridana* infection much faster with multifold mechanisms than male cadavers.

1 Introduction

The two-spotted spider mite [*Tetranychus urticae* (Acari: Tetranychidae)] is of high economic importance in many horticultural, agronomic and ornamental crops globally. It is extremely polyphageous and feeds on hundreds of plant species across many families (Jeppson *et al*., 1975). *T. urticae* infestations are most serious under hot and dry conditions. Due to its rapid proliferation and very short life cycle when climatic conditions are favorable, many generations of *T. urticae* can be completed in one growing season (Crooker, 1985). *T. urticae* sucks up leaf fluid and photosynthetic pigment like chlorophyll, which causes browning of the leaves, reduced photosynthesis, and eventually premature leaf death. In berries, fruit size may decrease and fruit set can be reduced the following year (Polk, 1994). Further, even a small amount of *T. urtica* feeding can lead to high economic losses in ornamentals like cut flowers (Croft & Van de Bann, 1988).

T. urticae often reaches pest population levels following pesticide treatments and many authors suggest that increases in *T. urticae* populations after pesticide treatment is caused by the destruction of predator natural enemies or mite pathogenic fungi such as *Neozygites floridana* (Zygomycetes: Entomophthorales). The use of chemical pesticides may also have profound direct effects on spider mite dispersal, reproduction, developmental rate, diapause and resistance (Penman and Chapman, 1988; James and Price, 2002; Klingen and Westrum, 2007).

T. urticae has five different life stages (Figure 1). Egg changes to a larva, which is colorless and after it starts eating the body color changes to either light green, brownish yellow or dark green. After sufficient feeding it enters into a quiescent stage [period of inactivity with slow metamorphosis also called protochrysalis (Crooker, 1985)]. During this phase it develops to a protonymph with four pairs of legs, the body size increases and the body color turns darker. The protonymph then starts to feed on the host plant and after sufficient feeding it goes into a second quiescent phase (deutochrysalis) and develops into a deutonymph which also feeds. Males and females can be differentiated at this stage since females are bigger and rounder in shape than the males which are comparatively smaller with a pointed abdomen. After enough feeding the deutonymph goes into a last quiescent phase (teleiochrysalis) before it finally molts into an adult.

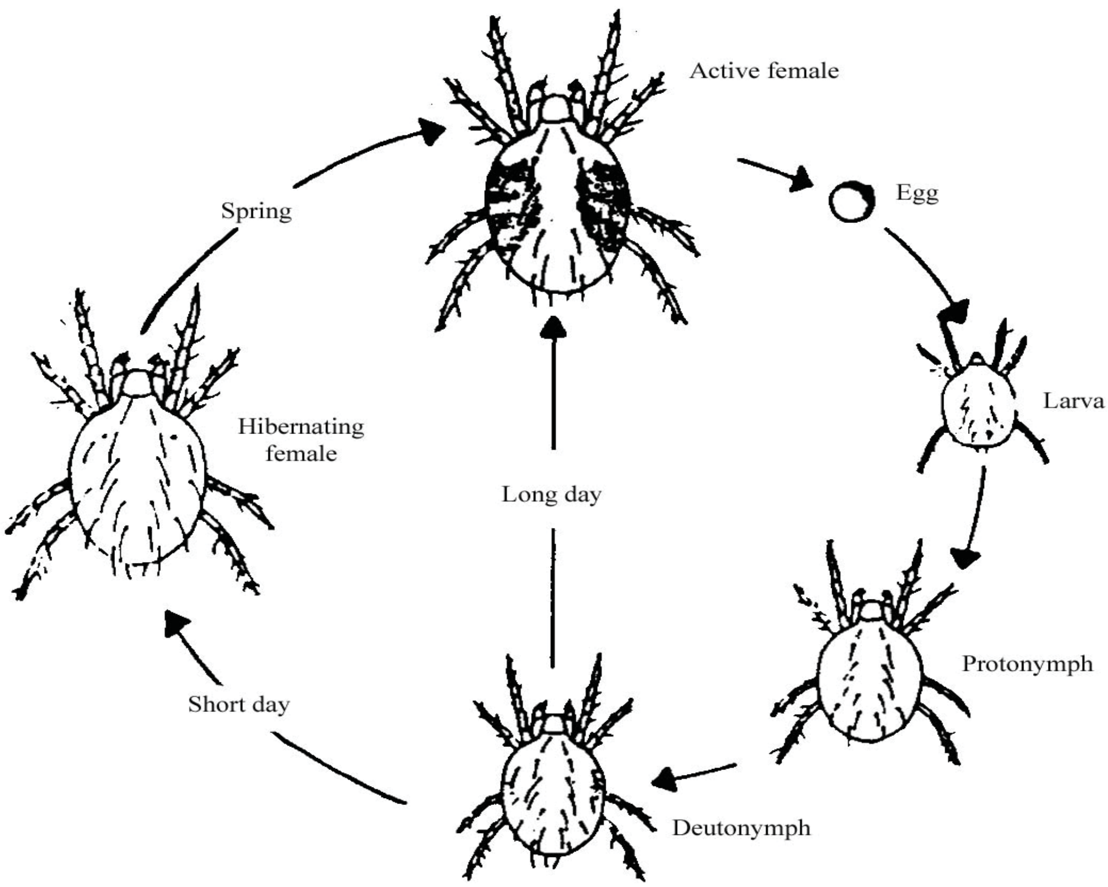

Figure 1: Life cycle of *Tetranychus urticae* (from Stenseth, 1993)

N. floridana is a fungus in the order Entomophthorales. It is an obligate pathogen of spider mites (Keller, 1997). *N. floridana* develops inside *T. urticae* as hyphal bodies, kills its host, penetrates the cuticle and produces spores (primary conidia). Primary conidia are actively ejected from swollen brown cadavers (Klingen and Westrum 2007). These conidia germinate to form the infective and more persistent capilli conidia that infect new mites (Carner, 1976; Elliot, 1998; Delalibera *et al*, 2006). A capilli conidium may adhere to the legs or other parts of the hosts (e.g. *T. urticae*) that pass by (Elliot *et al*., 2002). The conidium attached to the host body then germinates, penetrates the cuticulae of the host and enters into the host body before it further develops into hyphal bodies and starts a new fungal life cycle. It has been documented that a single capilli conidium of *N. floridana* is sufficient to kill and infect an adult female mite (Odour *et al*., 1997).

Epizootiology is the science of causes and forms of the mass phenomena of diseases at all levels of intensity in a host population (Fuxa and Tanada, 1987). In insect and mite epizootiology five key factors are mentioned as important for the development of an epizootic. These are 1) The host population (density, susceptibility, behavior) 2) The pathogen population (latency, biological properties, density and dispersion) 3) Transmission (routes, rates, method of dissemination) 4) Environment (different factors (temperature, humidity, moisture, desiccation, light, nutritional factors etc.) at host and pathogen populations) 5) Patterns over place and time (global distribution, innate and introduced insects and diseases, patterns over time). To be able to induce an epidemic level of a microbial control agent in a pest population and hence use it successfully, we need to understand these factors and know how to manipulate them.

A host-pathogen association can be viewed as an evolutionary arms race, in which complex and interacting factors can result in outcomes ranging from highly antagonistic to commensalism (May *et al.*, 1990). "The pathogen and host adapt to maximize their reproductive output and ultimate fitness by adopting physiological, ecological, and behavioral adaptations that are as diverse as the organisms themselves (Roy *et al.,* 2006)." Most of the research on entomopathogenic fungi and their arthropod hosts examining such relationship in the past has concentrated on aspects of host physiology only (Roy *et al.,* 2006), but pathogen induced and host mediated behavioral changes are also of great scientific interest to understand the co-evolution between a pathogen and its host. Those behavioral alterations may affect other important parameters in pathogen and host evolution such as transmission (Moore, *et.al.,* 1992).

T. urticae is a precopulatory mate guarding species which means that the adult male guard females in the last quiescent phase and mate with them soon after the ecdysis (i.e. shedding of old exoskeleton). The reason for such guarding could be that only the first mating is effective and results in progeny even an adult female may mate with the same or different males 2-3 times (Wrensch & Young, 1975). Besides guarding, hovering and touching with male positioned over the deutonymph and moving its appendages is described as one of the important stage in mate finding process of *T. urticae* (Sonenshine, 1985). Fighting or aggression between males are common in *T. urticae* when there are more than one male per female. These competitive interactions between males occur more frequently and last longer with increasing male: female ratio (Potter *et al.,* 1976).

Guarding behavior is time-consuming and exposes the male to predators, diseases and competitors, so the male should have a way of identifying females worth guarding, using chemical, visual and tactile cues. *T. urticae* vision is not well developed (Oku *et al.,* 2003) and males may sense the female sex pheromones through contact chemoreception only (Royalty *et al.,* 1992). Getting very close to the female, and also to touch her, may thus be a necessary part of the selection process, and unless males have a way of detecting *N. floridana* infection from a longer distance, mating behavior will entail a risk of getting infected with *N. floridana*. To my knowledge, there are no studies on the effect of *N. floridana* on sexual attraction and behavior of any mite species even though there are studies that suggest that entomopathogenic fungi may affect the sexual behavior of insects and that this might be important for the spread of the fungus in the host population (Møller, 1993). Observations in the laboratory at Bioforsk Plant Health and Plant Protection Institute indicated, however, that *T. urticae* males were guarding *N. floridana* killed *T. urticae* cadavers (pers. comm. Karin Westrum) and I therefore wanted to study this closer.

The purpose of this study was to investigate the effect of *N. floridana* infection in *T. urticae* females on *T. urticae* male premating and mating behavior. Males were given a choice between a healthy quiescent deutonymph and an *N. floridana* killed female (cadaver). To differentiate between the effect of fungal and female odor in the male choice an experiment where males were given the choice between a *N. floridana* killed female (cadaver) and a *N. floridana* killed male (cadaver) was also carried out. Detailed knowledge on male behavior towards infected females will help us to reveal if this is a factor that may influence the epidemic development of *N. floridana* in *T. urticae* populations.

2 Materials and methods

2.1 T. urticae stock culture

T. urticae was collected in a commercially strawberry field (cultivar Zephyr) at Ås, in southeastern Norway (59^0 42” N, 10^0 44” E) in 2003. The *T. urticae* stock culture was maintained on bean plants in a climatic chamber at 25 ±1^0C, 60 % RH and L16:D8.

2.2 N. floridana isolate

The *N. floridana* isolate ESALQ 1420 used in these experiments was collected in Piracicaba, São Paulo, Brazil (22^0 42’ 30” S, 47^0 38’ 00” W) from its natural host *T. urticae* on Jack bean, *Canavalia ensifromis* (Fabales: Fabaceae).

2.3 Production of cadavers

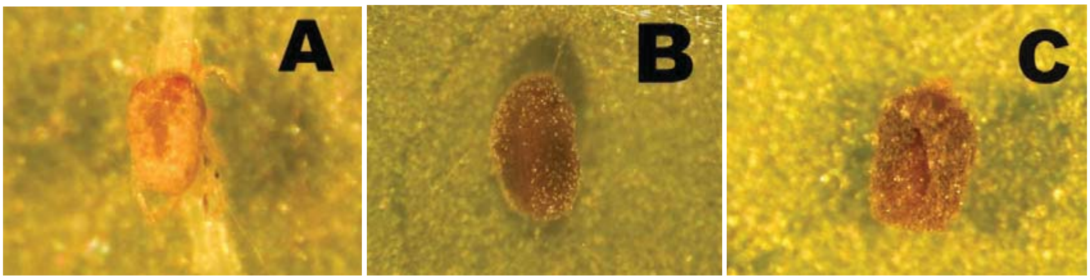

Figure 2: Different stages of female cadaver (*T. urticae*) A: non-sporulated, B: with primary conidia, C: with secondary (capilli) conidia. (Photo: Karin Westrum)

Three non sporulating *N. floridana* killed *T. urticae* cadavers were placed with their dorsal side up on a bean leaf disc (15 mm diameter) with axial surface down onto 1.5% water agar in a Petri dish (5cm diameter and 2 cm high). Six such Petri dishes with water agar, leaf discs and cadavers were placed in a plastic box (22x16x7 cm) with the lid slightly open, to provide the right RH, and wrapped with aluminum foil for darkness. The box was kept in a climatic chamber at 20 ±1^{0}C, 90% RH for 24 hours, for the cadavers to sporulate. Thirty healthy adult females were then transferred to each leaf disc with sporulating cadavers and placed at the conditions described above for 24 hours for *N. floridana* inoculation. The next day the leaf discs with *N. floridana* inoculated *T. urticae* were transferred to a 3 weeks old bean plant at ambient laboratory conditions (21-25 ^{0}C, 20-35% RH and 24 h of light). As leaf disc with inoculated mites started to wilt, mites walked on to the bean plant and established there. After 8-9 days, infected *T. urticae* died and dry non-sporulating cadavers (Figure 2A) were collected, and stored in a cotton cloth in a NUNC Cryo Tube ™ (1.8 ml) and stored in the refrigerator at 3-4 ^{0}C for 25-35 days before used in the experiment.

Production of cadavers with only primary conidia was done by first placing a non-sporulating cadaver on one of the two leaf discs as shown in Figure 3. The Petri dish was placed under the moist and dark conditions described above for 3 hours only. After these 3 hours the cadavers had produced primary conidia on conidiophores but the primary had not been ejected yet and certainly no secondary capilli conidia had been produced (Figure 2B) which was directly used in the experiment.

Cadavers with secondary conidia (capilli conidia) were produced by first placing a non-sporulating cadaver on one of the two leaf discs as described in Figure 3. The Petri dish was placed under the moist and dark conditions described above for 8 hours only. After these 8 hours the cadavers had produced secondary conidia (Figure 2C), the same Petri dish with secondary conidia bearing cadaver was directly used in the experiment.

2.4 Production of quiescent T. urticae female deutonymphs

Adult *T. urticae* females were placed individually on bean leaf discs (15 mm diameter) with the axial surface down onto 1.5% water agar in a 30 ml plastic vial with lid. Nine holes were made in the lid with insect pin no. 2 for aeration. Vials with leaves and *T. urticae* females were placed in a climatic chamber at 21 ±1^0C, 60 % RH, L16:D8 and a light intensity of 46 µmol $m^{-2}s^{-1}$ for 24 hours to lay eggs.

T. urticae females were then removed and eggs left to hatch and develop into quiescent female deutonymphs at the same climatic conditions as mentioned above. Pilot studies were conducted to establish the exact time needed for eggs to develop into quiescent female deutonymphs. These established that quiescent female deutonymphs were obtained 7-8 days after egg laying in the climatic conditions mentioned above. These quiescent deutonymphs were used in the choice experiment immediately after they were observed. During the production of quiescent deutonymphs daily observation was done to watch for undesired growth of microbes on the leaf disc or the water agar. If any were seen, mites were transferred to a new vial with a fresh leaf disc and water agar.

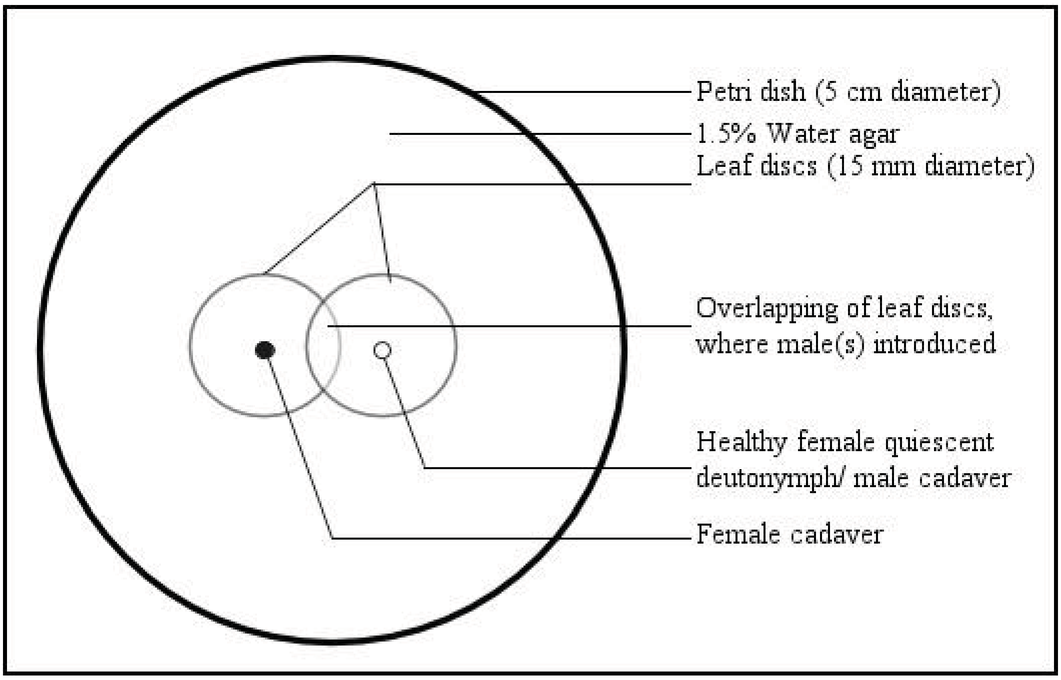

Figure 3: Experimental setup for choice experiment in a Petri dish

2.5 Choice experiment between quiescent female deutonymph and non-sporulating cadaver

Two bean leaf discs (15 mm in diameter) were placed on top of 1.5% water agar in a Petri dish (5cm diameter and 2 cm high) so that they were slightly overlapping. A non-sporulating *N. floridana* killed *T. urticae* cadaver was placed in the center of one of the leaf discs and a quiescent *T. urticae* female deutonymph was placed in the center of the other leaf disc (Figure 3) so that males could choose between a healthy quiescent *T. urticae* female and a *N. floridana* killed non-sporulating *T. urticae* cadaver. Male *T. urticae*, freshly picked from the stock culture, were then introduced where the two leaf discs overlapped. Few drops of water was added to the water agar to prevent males from walking off the leaf discs and into the water agar. Three male densities were tested: One male, two males and three males. The One male density treatment was repeated eight times (giving n=8 males choosing) the Two and Three male density treatment were repeated four times (giving n=8 and n=12 males choosing respectively). The experiment was repeated three times over time (17, 18, and 19 October 2011) at ambient laboratory conditions (21-25^{o} C, 20-35% RH) during day time between 10:00 – 17:00.

2.6 Choice experiment between quiescent female deutonymph and sporulating cadaver with primary conidia only

This experiment was conducted as described for the experiment in 2.5, except that sporulating cadavers with primary conidia only were used. Petri dishes with leaf discs with cadavers with primary conidia prepared as in 2.3 were taken into ambient laboratory conditions to conduct the experiment. In the laboratory, the conidial production stopped due to the dry laboratory conditions (20-35% RH). Another leaf disc was then added to the Petri dish and a quiescent *T. urticae* female deutonymph was placed in the center of that leaf disc so that males could choose between a healthy quiescent *T. urticae* female and a *N. floridana* killed *T. urticae* cadaver with primary conidia. The rest of the experiment was conducted as described under 2.5. The experiment was repeated three times over time (3, 4, and 5 April 2012).

2.7 Choice experiment between quiescent female deutonymph and sporulating cadaver with secondary conidia

Petri dishes with leaf discs with cadavers with secondary conidia prepared as in 2.3 were taken into ambient laboratory conditions to conduct the experiment. In the laboratory, the conidial production stopped due to the dry laboratory conditions (20-35% RH). Another leaf disc was then added to the Petri dish and a quiescent *T. urticae* female deutonymph was placed in the center of that leaf disc so that males could choose between a healthy quiescent *T.*

urticae female and a *N. floridana* killed *T. urticae* cadaver with secondary conidia. The rest of the experiment was conducted as described under 2.5. The experiment was repeated three times over time (23, 24, and 25 May 2012).

2.8 Choice experiment between non sporulating male and female cadavers

To differentiate the effect of fungus and female odor as an important factor involved in the male choice, an experiment where males were given the choice between a *N. floridana* killed female (cadaver) and *N. floridana* killed male (cadaver) was conducted. This experiment was conducted as described for the experiment in 2.5 except that male cadaver was used in place of a healthy quiescent *T. urticae* female so that males could choose between a *N. floridana* killed *T. urticae* non-sporulating female cadaver and a *N. floridana* killed *T. urticae* non-sporulating male cadaver. Both the cadavers were produced as described in 2.3. The experiment was repeated three times over time (25 April, 10 and 11 July 2012).

2.9 Observation of male behavior in the four choice experiments

Each Petri dish was observed through a stereo microscope (magnification 20x) once every hour for 6 hours after introduction of males.

2.9.1 Leaf disc choice

Each male was categorized and scored as present on leaf disc with healthy female/ male cadaver or female cadaver.

2.9.2 Touching behavior

A male was considered to be touching if he touched the healthy female or cadaver in any way and the contact did not last continuously for up to 30 seconds at the time of observation. Males were observed for 10 seconds if seen moving towards healthy female or cadaver, to check if touching occurred.

The incidence of touching was scored 6 times for each male (once an hour) and categorized as touching healthy female/male cadaver, touching female cadaver and not touching any of them.

2.9.3 Guarding behavior

"A male was considered to be guarding if he remained motionless for 30 s or longer while mounted upon or within one body length of the quiescent female (Collins *et al*., 1993)." But in presence of more than one male, if one tries to disturb or take over a guarding male than he will try to defend himself and his guarding position, at that time even male is moving little bit but still not leaving female and is continuously guarding her. So guarding behavior in our study is defined as: "A male was considered to be guarding if he remained mounted upon or within one body length of the quiescent female or cadaver and did not leave it for 30 seconds or longer."

The incidence of guarding was scored 6 times for each male (once an hour) and categorized as guarding healthy female/male cadaver, guarding female cadaver and not guarding any of them.

2.10 Statistical analysis

All the data were statistically analyzed with Minitab® Statistical Software 16. Stage of cadaver (without spores, with primary conidia only, with secondary conidia) and male density (1, 2, 3 males) were the explanation variables whereas leaf disc choice, guarding and touching behaviors were response variables.

Data were analyzed with each Petri dish as a single unit to avoid pseudo-replication. As a measure of cadaver attractiveness, an index for each dish was made by summing up the 6 hourly observations (for each hour a score of "1" was given for every male seen on disc with cadaver, and "0" for every male seen on disc with healthy female, giving a maximum sum of 6, 12 and 18 in Petri dishes with one, two and three males respectively), and then divide the sum by number of males introduced in the dish to give equal weight to each Petri dish. The resulting index ranged between 0 and 1, with 0.5 denoting Petri dishes with equal number of observations of males on the two leaf discs. Some quiescent females (n= 11) developed into adults during the experiment. Observations of these were excluded (but keeping the hourly observations before ecdysis, ensuring that all Petri dishes were indexed). The indexed data on leaf disc choice (Appendix 1 and 4) yielded normally distributed residuals when analyzed with the general linear model (GLM). When a variable was significant, post-hoc comparison among means were carried out using Tukey tests at 95.0% confident interval. In addition Wilcoxon signed rank test was used to test if the index was significantly different from 0.5, i.e. to test whether males had the same preference for cadavers and healthy females (or for both cadavers in the last experiment).

Observations of guarding and touching behavior were indexed similarly for each Petri dish, but these were not normally distributed. Two different indices had to be calculated for each behavior: [1] index for guarding and touching female cadaver (Appendix 2 and 5), [2] index for guarding and touching healthy female (or male cadaver) (Appendix 3 and 6 respectively). The two indices for each behavior, e.g. guarding cadaver Vs guarding healthy female, were compared using Mann-Whitney *U-test* for each cadaver stage separately, pooling the three male densities.

3 Results

3.1 Effect of cadaver stage on choice of leaf disc

Cadaver stage significantly affected the leaf disc choice of males ($F_{2,135}$= 3.98, P = 0.021 GLM). No significant effect of male density ($F_{2,135}$=1.94, P = 0.15) or any interaction between cadaver stage and male density was observed ($F_{4,135}$=0.91, P=0.46) and this was not analyzed further.

Table 1. Male attraction to leaf discs with different stages of *Neozygites floridana* killed *Tetranychus urticae.*

Cadaver stage	N	Mean index of attraction	Grouping*
Non-sporulating cadaver	48	0.6	A
Cadaver with primary conidia	48	0.6	A
Cadaver with secondary conidia	48	0.4	B

*Different letters in a column denote significant differences using Tukey (P < 0.5)

3.2 Behavior in choice test between non-sporulating cadaver and healthy female

Significantly more males were attracted to leaf discs with non-sporulating cadavers (P = 0.030 Wilcoxon signed rank test) than healthy females (Figure 4A). Males were touching a similar percentage of non-sporulating cadavers and healthy females and no significant difference (P = 0.093, Mann-Whitney *U-test*) in the male touching index between non-sporulating cadavers and healthy females were seen (Figure 4B). The same was true for the guarding index (P = 0.33, Mann-Whitney *U-test*) (Figure 4C).

3.3 Behavior in choice test between sporulating cadaver with primary conidia and healthy female

Significantly more males were attracted to leaf discs with sporulating cadavers with primary conidia (P = 0.014 Wilcoxon signed rank test) than to healthy females (Figure 4A). Males were touching a similar percentage of sporulating cadavers with primary conidia and healthy females and no significant difference (P = 0.51 Mann-Whitney *U-test*) in touching behavior between sporulating cadavers with primary conidia and healthy females were seen (Figure 4B). However, males were significantly more often (P = 0.048, Mann-Whitney *U-test*) guarding healthy females than sporulating cadavers with primary conidia (Figure 4C).

3.4 Behavior in choice test between sporulating cadaver with secondary conidia and healthy female

Significantly more males were attracted to leaf discs with healthy females (*P* = 0.048 Wilcoxon signed rank test) than to sporulating cadavers with secondary conidia (Figure 4A). Males were touching a similar percentage of sporulating cadavers with secondary conidia and healthy females and no significant difference (*P* = 0.185 Mann-Whitney *U-test*) in touching behavior between sporulating cadavers with secondary conidia and healthy females were seen (Figure 4B). However, guarding behavior was only observed with healthy females (Figure 4C).

3.5 Behavior in choice test between non-sporulating male and female cadavers

Males were significantly attracted to leaf discs with non sporulating female cadavers (*P* = 0.003 Wilcoxon signed rank test) than non sporulating male cadavers. Male density had no significant effect in the male choice of the leaf disc ($F_{2,45} = 0.08$, $P = 0.92$, GLM). However, significantly (*P* = 0.0011, Mann-Whitney *U-test*) more female cadavers than male cadavers were touched and males were only seen guarding female cadavers, i.e. no guarding of male cadavers were seen (Figure 5).

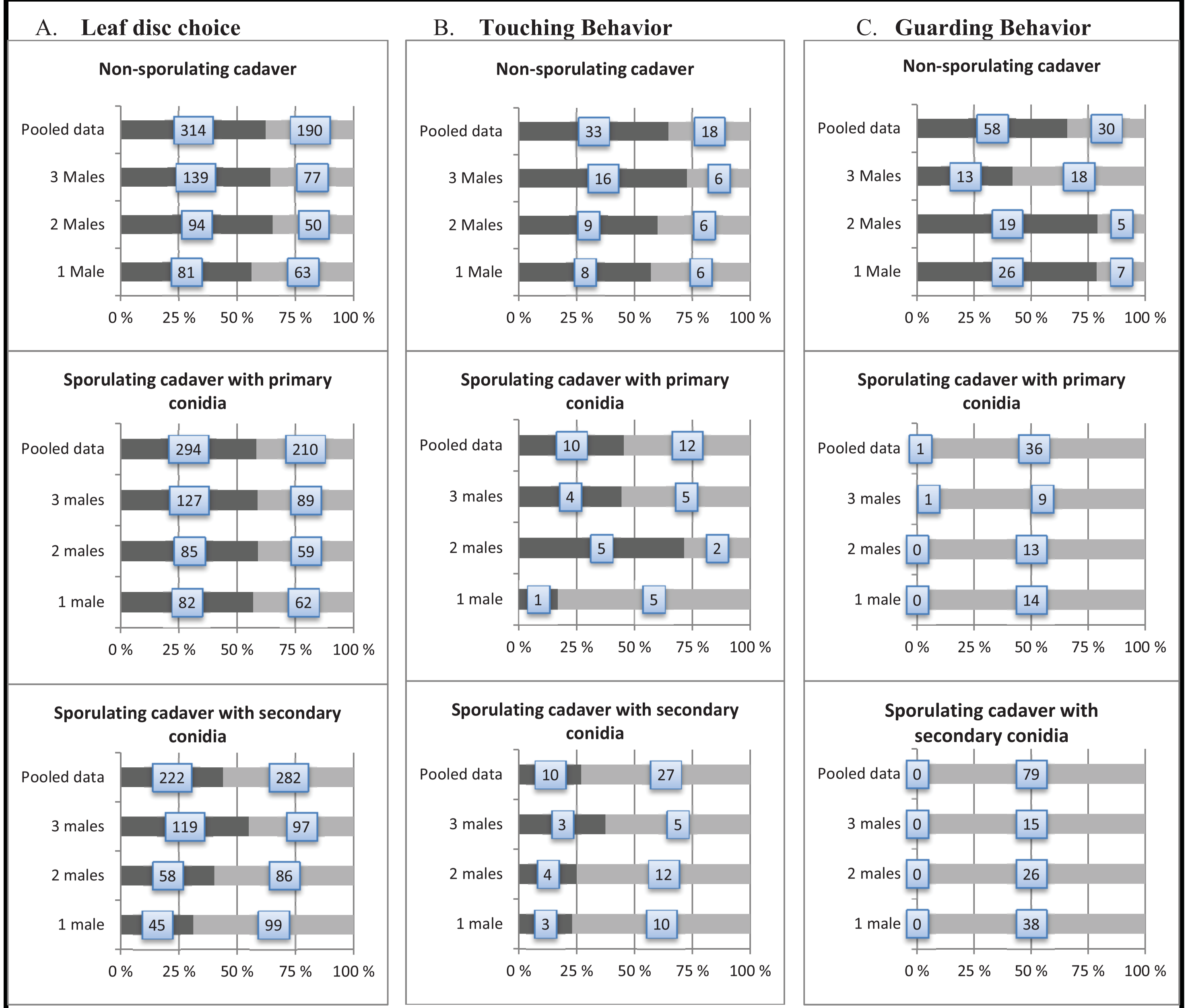

Figure 4: Percentage and number of times *T. urticae* males choosing different stages of female cadaver (■) or healthy female quiescent deutonymph (■) in leaf disc choice or for touching and guarding.

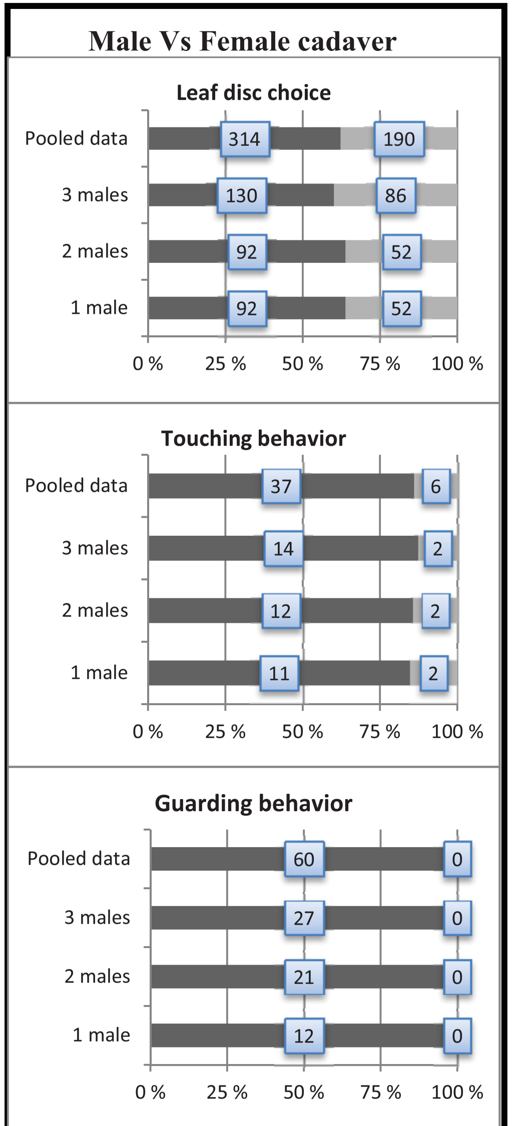

Figure 5: Percentage and number of times *T. urticae* males choosing non-sporulating female cadaver (■) or non-sporulating male cadaver (■) in leaf disc choice or for touching and guarding.

4 Discussion

Males given a choice between leaf discs with healthy females and non-sporulating cadavers and cadavers sporulating with primary conidia had a preference for leaf discs with cadavers. Males given a choice between leaf discs with healthy females and sporulating cadavers with secondary conidia, however, showed a preference towards healthy females.

Males were touching a similar percentage of healthy females compared to non-sporulating cadavers and sporulating cadavers with primary and secondary conidia. As cadavers matured (sporulated with primary and secondary conidia), there was a tendency of less touching behavior, however. This means that males seemed to have a similar preference for touching healthy and cadaver females of any stage.

Males were guarding a similar percentage of healthy females compared to non-sporulating cadavers. In contrast, more males guarded a healthy female than a sporulating cadaver with primary conidia and none of the males guarded cadaver with secondary conidia which is the only infective units in the asexual life cycle of *N. floridana* (Carner, 1976; Smitley *et al.,* 1986).

Different male densities had no effects in attraction of males in any of the experiment. Some agonistic interactions between males to guard healthy as well as infected females were observed in experiment with more than 1 male. But no attempts to kill or hurt another male were seen. Extremes of these interactions could have been observed if we had even higher male densities (Potter *et al.,* 1976) and fighting and killing of males for a female (healthy or infected) was common in the cultured populations of *T. urticae* observed in our pilot study.

The overall pattern of leaf disc choice was identical when males had the choice between two non-sporulating cadavers, one male and one female, to the one when males choose between a non-sporulating female cadaver and a healthy female. Thus it may seem that from a distance, male non-sporulating cadavers are as attractive to males as healthy females, while female non-sporulating cadavers are even more attractive than these two. The observed non-avoidance of both male and female non-sporulating cadavers indicates that males are unable to distinguish these from a healthy female until they come very close. When close to a male cadaver, however, the males were better at spotting the mistake, since they were much less inclined to touch or guard a male cadaver than a female one.

Clearly, *T. urticae* males are not well adapted to avoid *N. floridana* contamination during mating. Because of their regular touching behavior to cadavers even with secondary conidia is enough to infect the host as capilli conidia are present all over the leaf surface. They waste time and risk their life exploring targets that are dead, contagious and even of the wrong sex. This must be due to restrictions in their sensory capabilities and perhaps also to manipulating stimuli induced by the pathogen, *N. floridana*.

Since Oku *et al*. (2003) reported that spider mites use chemicals to get information about the surroundings; Cone *et al*. (1971) found female sex pheromones that were attractive to males and Sonenshine (1985) stated sex attractants compounds like nerolidol, farnesol or geraniol are actively secreted from quiescent female deutonymphs of *T. urticae,* we might suggest that attractive fungal odors mimicking the *T. urticae* female sex pheromone are involved in attracting males to fungal killed *T. urticae* and this is the reason that active males choose the leaf disc with the healthy female and male cadaver identically when each of them were tested with female cadaver. But in both experiments males prefer female cadaver may be because they contain more fungal hypha. This is true for the choice between sporulating cadaver with primary conidia and healthy female. In contrast, males were more attracted to the healthy female when given a choice with female cadaver with secondary conidia. This might suggest that the fungus plus the female (female cadaver) smells more attractive than the healthy quiescent female when the fungal development is not in a too advanced stage. At an advanced stage (secondary conidia cadaver) the smell from the healthy quiescent female is more attractive than the smell of the cadaver. In additions comes that the secondary conidia, that rises 60-100 µm on or from the leaf surface surrounding the cadaver and also emerging from the cadaver, might make it difficult for the active male to move around. When the active male gets closer, however, and starts to touch the cadavers, it feels that the shape of the male cadaver (small and pointed abdomen) is not as desirable as the shape of the female cadaver (big and round abdomen) and the shape choice might be more important than the odor choice at this stage. To reveal this a more detailed study, maybe a Y-tube study, needs to be conducted in which visual and contact cues can be excluded and only volatile signals are carried by the air stream to the mite. Since Royalty *et al*. (1992) argued that spider mite female sex pheromones are perceived only through contact chemoreception a second explanation to the change of the choice situation as soon as the active male starts to touch might be that, when the male starts to touch the contact chemoreception of the active male responds since there are still some remnants of the female sex pheromone on the cuticle on the fungal killed but non-sporulating female cadaver. To reveal this, may be a detailed study on odor analysis from different stages of cadaver and healthy female needs to be conducted.

When the active male gets closer, however, and starts to touch and perform guarding behavior, the male feels that the shape of sporulating cadavers (bumpy and coarse with protruding conidia and spores) are not as attractive as the shape of the healthy quiescent female and non sporulating cadaver; and the shape choice might be more important than the odor choice at this stage. In addition, the male chemo reception of the most developed fungal stage cadaver (secondary conidia) might not "taste" well (see arguments above) and the active male choose no guarding behavior of the secondary conidia cadavers. In additions comes that the secondary conidia, in and around the cadaver (see arguments above) might make it difficult for the active male to move and hence also perform touching and guarding behavior. In additions comes that the smell of the infective stage of the fungus (secondary conidia cadaver) might evolutionary be so bad that the spider mites avoids it to not get infected. To reveal this, more detailed behavioral studies, maybe with Etho-Vision need to be conducted.

Many pathogens and parasites bring alterations in the physiology and behavior of their hosts. Such changes may be adaptive responses by the host to stop growth and development of the pathogen or the changes can also be induced by the pathogen in its host to enhance its own growth, development, reproduction or dissemination. An intermediate hypothesis of selective advantages: where neither host nor pathogen are completely dominating but are able to change the behavior of another to some extent for their own benefit, has also been proposed to describe the changed behavior of infected host for the benefit of the hosts (Evans, 1989; Watson *et al*., 1993), and for the benefit of the pathogens (Goulson, 1997) of other systems. As seen in this study, neither host nor pathogen have the complete upper hand: males did not guard cadavers in infective stage, but they were not completely avoiding the fungus for leaf disc choice and touching even in infective stage. , which is enough to infect the host as capilli conidia are present all over the leaf surface. So, pathogen seems ahead of the host in this evolutionary arms race.

Further studies on sex pheromones and volatile compounds present on cadavers and healthy female quiescent deutonymphs and the mechanisms as well as consequences of the behavioral modification may eventually lead to more accurate predictions of the population dynamics and epizootic development of the fungus; and helps to formulate a better and more effective fungal bio pesticide for the control of the mites. If these results apply to biological control of *T. urticae* under larger-scale field conditions, female cadavers will spread more infection much faster with multifold mechanisms and would be an effective biological control agent.

5 References

Carner, G. R. (1976). Description of the life cycle of *Entomophthora* sp. in the Two-Spotted Spider Mite. *Journal of Invertebrate Pathology*, 28 (2): 245–254.

Collins, R. D., Margolies, D. C. & Rose, S. (1993). Guarding behavior and reproductive isolation in two tetranychid mite species (Acari: Tetranychidae). *Annals of the Entomological Society of America*, 86 (1): 111-116.

Cone, V. W., McDonough, L. M., Maitlen, J. C. & Burdajewicz, S. (1971). Pheromone studies of the Two-Spotted Spider Mite. 1. Evidence of a sex pheromone. *Journal of Economic Entomology*, 64 (2): 355–358.

Croft, B. A. & Van de Baan, H. E. (1988). Ecological and genetic factors influencing evolution of pesticide resistance in tetranychid and phytoseiid mites. *Experimental and Applied Acarology*, 4: 277-300.

Crooker, A. (1985). Embryonic and Juvenile Development. In Sabelis, M.W. & Helle, W. (eds) vol. 1A, *Spider mites: their biology, natural enemies and control.* Amsterdam: Elsevier.

Delalibera Jr, I., Deme´trio, C. G. B., Manly, B. F. J. & Hajek, A. E. (2006). Effect of relative humidity and of isolates of *Neozygites tanajoae* (Zygomycetes: Entomophthorales) on production of conidia from cassava green mite, *Mononychellus tanajoae* (Acari: Tetranychidae) cadavers. *Biological Control*, 39: 489–496.

Elliot, S. L. (1998). Ecology and epizootiology of *Neozygites floridana*, a pathogen of the cassava green mite. Ph.D. Thesis. Berkshire, United Kingdom: Imperial College at Silwood Park.

Elliot, S. L., De Moraes, G. J. & Mumford, J. D. (2002). Importance of ambient saturation deficits in an epizootic of the fungus *Neozygites floridana* in cassava green mites (*Mononychellus tanajoa*). *Experimental and Applied Acarology*, 27: 11-25.

Evans, H. C. (1989). Mycopathogens of insects of epigeal and aerial habitats. In Wilding, N., Collins, N. M., Hammond, P. M. & Webber, J. F. (eds), *Insect-Fungus Interactions*, pp. 205-238. London: Academic Press.

Fuxa, J. R. & Tanada, Y. (1987). Epizootiology of insect diseases. A Wiley-interscience publication, New York. Part 2: 71-239.

Goulson, D. (1997). *Wipfelkrankheit*: modification of host behaviour during baculoviral infection. *Oecologia*, 109 (2): 219–228.

James, D. G., Price, T. S. (2002). Fecundity in two spotted spider mite (Acari: Tetranychidae) is increased by direct and systemic exposure to imidacloprid. *Journal of Economic Entomology*, 95: 729–732.

Jeppson, L. R., Keifer, H. H. & Baker, E. W. (1975). Mites injurious to economic plants. University of California Press, Berkeley, CA, USA, 614 pp.

Keller, S. (1997). The genus *Neozygites* (Zygomycetes: Entomophthorales) with special reference to species found in tropical regions. *Sydowia*, 49 (2): 118–146.

Klingen, I. & Westrum, K. (2007). The effect of pesticides used in strawberries on the phytophagous mite *Tetranychus urticae* (Acari: Tetranychidae) and its fungal natural enemy *Neozygites floridana* (Zygomycetes: Entomophthorales). *Biological Control*, 43: 222-230.

May, R. M. & Anderson, R. M. (1990). Parasite-host coevolution. *Parasitology*, 100: 89-101.

Minitab Inc. (2010). *Minitab 16.1.1*. Enterprise drive, State College, PA, USA.

Moore, D., Redd, M., Le Patourel, G., Abraham, Y. J. & Prior, C. (1992). Reduction of feeding by the desert locust, *Schistocerca gregaria,* after infection with *Metarhizium flavoviride*. *Journal of Invertebrate Pathology,* 60 (3): 304-307.

Møller, A. (1993). A fungus infecting domestic flies manipulates sexual behavior of its host. *Behavioral ecology and sociobiology*, 33 (6): 403-407.

Odour, G. I., Yaninek, J. S., De Moraes, G. J. & Van der Geest, L. P. S. (1997). The effect of pathogen dosage on the pathogenicity of *Neozygites floridana* (Zygomycetes: Entomopthorales) to *Mononychellus tanajoa* (Acari: Tetranychidae). *Journal of Invertebrate Pathology*, 70: 127-130.

Oku, K., Yano, S., Osakabe, M. & Takafuji, A. (2003). Spider mites assess predation risk by using the odor of injured conspecifics. *Journal of Chemical Ecology*, 29 (11): 2609–2613.

Penman, D. R., Chapman, R. B. (1988). Pesticide-induced mite outbreaks: pyrethroids and spider mites. *Experimental and Applied Acarology*, 4: 265–276.

Polk, D. (1994). Insects and Mite Management. In Barkette, L. P. Management Guide for Low-Input Sustainable Apple Production. Diane Publishing Co.

Potter, D. A., Wrensch, D. L. & Johnston, D. E. (1976). Guarding, aggressive behavior, and mating success in male twospotted spider mites. *Annals of the Entomological Society of America*, 69 (4): 707–711.

Roy, H. E., Steinkraus, D. C., Eilenberg, J., Hajek, A. E. & Pell, J. K. (2006). Bizarre interactions and endgames: entomopathogenic fungi and their arthropod host. *Annual Review of Entomology*, 51: 331-57.

Royalty, R. N., Phelan, P. L. & Hall, F. R. (1992). Arrestment of male two spotted spider mite caused by female sex pheromone. *Journal of Chemical* Ecology, 18 (2): 137-153.

Smitley, D. R., Brooks, W. M. & Kennedy, G. G. (1986). Environmental Effects on Production of Primary and Secondary Conidia, Infection and Pathogenesis of *Neozygites floridana*, a Pathogen of the Two-spotted Spider Mite, *Tetranychus urticae*. *Journal of Invertebrate Pathology*, 47 (3): 325–332.

Sonenshine, D. E. (1985). Pheromones and other semiochemicals of the acari. *Annual review of Entomology,* 30: 1-28.

Stenseth, C. 1993. Diagnose av skadegjørere i bær. Etterutdanningkurs i 1993. Faginfo nr 4: 6-33.

Watson, D. W., Mullens, B. A. & Peterson, J. J. (1993). Behavioral fever response of *Musca domestica* (Diptera: Muscidae) to infection by *Entomophthora muscae* (Zygomycetes: Entomophthorales*). Journal of invertebrate Pathology*, 61 (1): 10–16.

Wrensch, D. L. & Young, S. S. Y. (1975). Effects of quality of resource and fertilization status on some fitness traits in the two-spotted spider mite (*Tetranychus urticae* Koch). *Oecologia*, 18: 259-267.

6 Appendix

Appendix I: Data indexing for males on leaf disc in experiment: Female cadaver Vs Healthy female quiescent deutonymph.

Appendix II: Data indexing for guarding and touching female cadavers in the experiment: Female cadaver Vs Healthy female quiescent deutonymph.

Appendix III: Data indexing for guarding and touching healthy female quiescent deutonymph in the experiment: Female cadaver Vs Healthy female quiescent deutonymph.

Appendix IV: Data indexing for males on leaf disc in the experiment: Male cadaver Vs Female cadaver.

Appendix V: Data indexing for guarding and touching female cadaver in the experiment: Female cadaver Vs male cadaver.

Appendix VI: Data indexing for guarding and touching male cadaver in the experiment: Female cadaver Vs male cadaver.

Here,

Stage:	Stage of cadaver.
Date:	Date of experiment (within Stage).
Male density:	Number of males within the actual dish.
Dish:	Dish number.
n:	Number of observed mite in a Petri dish.
Sum:	Sum of the n 1 or 0's coding for mite position and behavior.
Mean:	Sum / n.

Appendix: I

Data indexing for males on leaf disc in experiment: Female cadaver Vs Healthy female quiescent deutonymph.

Row	Stage	Date	Male density	Dish	n	Sum	Mean
1	1	1	1	1	6	6	1.0000
2	1	1	1	2	6	0	0.0000
3	1	1	1	3	6	4	0.6667
4	1	1	1	4	6	1	0.1667
5	1	1	1	5	6	6	1.0000
6	1	1	1	6	6	3	0.5000
7	1	1	1	7	6	5	0.8333
8	1	1	1	8	6	2	0.3333
9	1	1	2	9	12	9	0.7500
10	1	1	2	10	12	5	0.4167
11	1	1	2	11	12	10	0.8333
12	1	1	2	12	12	9	0.7500
13	1	1	3	13	18	13	0.7222
14	1	1	3	14	18	17	0.9444
15	1	1	3	15	18	4	0.2222
16	1	1	3	16	18	4	0.2222
17	1	2	1	1	6	6	1.0000
18	1	2	1	2	6	1	0.1667
19	1	2	1	3	6	6	1.0000
20	1	2	1	4	6	2	0.3333
21	1	2	1	5	6	3	0.5000
22	1	2	1	6	6	1	0.1667
23	1	2	1	7	6	5	0.8333
24	1	2	1	8	6	6	1.0000
25	1	2	2	9	12	10	0.8333
26	1	2	2	10	12	12	1.0000
27	1	2	2	11	12	12	1.0000
28	1	2	2	12	12	9	0.7500
29	1	2	3	13	18	4	0.2222
30	1	2	3	14	15	10	0.6667
31	1	2	3	15	18	10	0.5556
32	1	2	3	16	18	13	0.7222
33	1	3	1	1	6	0	0.0000
34	1	3	1	2	6	1	0.1667
35	1	3	1	3	6	6	1.0000
36	1	3	1	4	6	2	0.3333
37	1	3	1	5	6	6	1.0000
38	1	3	1	6	6	0	0.0000
39	1	3	1	7	6	3	0.5000
40	1	3	1	8	6	6	1.0000
41	1	3	2	9	12	1	0.0833
42	1	3	2	10	12	7	0.5833
43	1	3	2	11	12	4	0.3333
44	1	3	2	12	12	6	0.5000
45	1	3	3	13	18	18	1.0000
46	1	3	3	14	18	15	0.8333
47	1	3	3	15	18	15	0.8333
48	1	3	3	16	18	16	0.8889
49	2	1	1	1	6	3	0.5000
50	2	1	1	2	6	4	0.6667
51	2	1	1	3	6	3	0.5000
52	2	1	1	4	6	3	0.5000
53	2	1	1	5	6	5	0.8333
54	2	1	1	6	6	3	0.5000

55	2	1	1	7	6	6	1.0000
56	2	1	1	8	6	4	0.6667
57	2	1	2	9	12	6	0.5000
58	2	1	2	10	12	11	0.9167
59	2	1	2	11	12	5	0.4167
60	2	1	2	12	12	3	0.2500
61	2	1	3	13	18	15	0.8333
62	2	1	3	14	18	11	0.6111
63	2	1	3	15	18	10	0.5556
64	2	1	3	16	18	11	0.6111
65	2	2	1	1	6	3	0.5000
66	2	2	1	2	6	1	0.1667
67	2	2	1	3	6	3	0.5000
68	2	2	1	4	6	3	0.5000
69	2	2	1	5	6	4	0.6667
70	2	2	1	6	6	1	0.1667
71	2	2	1	7	6	2	0.3333
72	2	2	1	8	6	5	0.8333
73	2	2	2	9	12	11	0.9167
74	2	2	2	10	12	6	0.5000
75	2	2	2	11	12	8	0.6667
76	2	2	2	12	12	6	0.5000
77	2	2	3	13	18	8	0.4444
78	2	2	3	14	18	14	0.7778
79	2	2	3	15	18	5	0.2778
80	2	2	3	16	18	10	0.5556
81	2	3	1	1	6	4	0.6667
82	2	3	1	2	6	0	0.0000
83	2	3	1	3	6	3	0.5000
84	2	3	1	4	6	4	0.6667
85	2	3	1	5	6	4	0.6667
86	2	3	1	6	3	4	1.3333
87	2	3	1	7	6	4	0.6667
88	2	3	1	8	6	6	1.0000
89	2	3	2	9	12	8	0.6667
90	2	3	2	10	12	3	0.2500
91	2	3	2	11	12	10	0.8333
92	2	3	2	12	12	8	0.6667
93	2	3	3	13	18	9	0.5000
94	2	3	3	14	18	11	0.6111
95	2	3	3	15	18	10	0.5556
96	2	3	3	16	18	13	0.7222
97	3	1	1	1	6	1	0.1667
98	3	1	1	2	6	0	0.0000
99	3	1	1	3	5	3	0.6000
100	3	1	1	4	6	1	0.1667
101	3	1	1	5	6	2	0.3333
102	3	1	1	6	5	1	0.2000
103	3	1	1	7	6	1	0.1667
104	3	1	1	8	6	0	0.0000
105	3	1	2	9	12	5	0.4167
106	3	1	2	10	12	0	0.0000
107	3	1	2	11	12	2	0.1667
108	3	1	2	12	8	7	0.8750
109	3	1	3	13	18	3	0.1667
110	3	1	3	14	18	6	0.3333
111	3	1	3	15	18	14	0.7778
112	3	1	3	16	18	12	0.6667
113	3	2	1	1	6	4	0.6667
114	3	2	1	2	5	3	0.6000
115	3	2	1	3	6	3	0.5000

116	3	2	1	4	6	2	0.3333
117	3	2	1	5	6	2	0.3333
118	3	2	1	6	6	1	0.1667
119	3	2	1	7	6	2	0.3333
120	3	2	1	8	6	3	0.5000
121	3	2	2	9	12	4	0.3333
122	3	2	2	10	12	11	0.9167
123	3	2	2	11	12	2	0.1667
124	3	2	2	12	10	11	1.1000
125	3	2	3	13	18	11	0.6111
126	3	2	3	14	12	16	1.3333
127	3	2	3	15	18	15	0.8333
128	3	2	3	16	18	13	0.7222
129	3	3	1	1	5	1	0.2000
130	3	3	1	2	6	5	0.8333
131	3	3	1	3	6	4	0.6667
132	3	3	1	4	5	3	0.6000
133	3	3	1	5	6	2	0.3333
134	3	3	1	6	6	0	0.0000
135	3	3	1	7	6	0	0.0000
136	3	3	1	8	6	1	0.1667
137	3	3	2	9	12	2	0.1667
138	3	3	2	10	12	5	0.4167
139	3	3	2	11	12	4	0.3333
140	3	3	2	12	10	5	0.5000
141	3	3	3	13	18	6	0.3333
142	3	3	3	14	18	9	0.5000
143	3	3	3	15	18	4	0.2222
144	3	3	3	16	18	10	0.5556

Appendix: II

Data indexing for guarding and touching female cadavers in the experiment: Female cadaver Vs Healthy female quiescent deutonymph.

Row	Stage	Date	Male density	Dish	n	Guarding		Touching	
						Sum	Mean	Sum	Mean
1	1	1	1	1	6	0	0.0000	0	0.0000
2	1	1	1	2	6	0	0.0000	0	0.0000
3	1	1	1	3	6	0	0.0000	0	0.0000
4	1	1	1	4	6	0	0.0000	0	0.0000
5	1	1	1	5	6	5	0.8333	1	0.1667
6	1	1	1	6	6	0	0.0000	0	0.0000
7	1	1	1	7	6	0	0.0000	0	0.0000
8	1	1	1	8	6	0	0.0000	1	0.1667
9	1	1	2	9	12	0	0.0000	0	0.0000
10	1	1	2	10	12	0	0.0000	0	0.0000
11	1	1	2	11	12	0	0.0000	0	0.0000
12	1	1	2	12	12	0	0.0000	0	0.0000
13	1	1	3	13	18	0	0.0000	0	0.0000
14	1	1	3	14	18	3	0.1667	1	0.0556
15	1	1	3	15	18	0	0.0000	1	0.0556
16	1	1	3	16	18	0	0.0000	0	0.0000
17	1	2	1	1	6	2	0.3333	3	0.5000
18	1	2	1	2	6	0	0.0000	0	0.0000
19	1	2	1	3	6	3	0.5000	1	0.1667
20	1	2	1	4	6	0	0.0000	0	0.0000
21	1	2	1	5	6	0	0.0000	0	0.0000

22	1	2	1	6	6	0	0.0000	0	0.0000
23	1	2	1	7	6	4	0.6667	1	0.1667
24	1	2	1	8	6	0	0.0000	0	0.0000
25	1	2	2	9	12	5	0.4167	1	0.0833
26	1	2	2	10	12	4	0.3333	3	0.2500
27	1	2	2	11	12	4	0.3333	0	0.0000
28	1	2	2	12	12	2	0.1667	0	0.0000
29	1	2	3	13	18	0	0.0000	0	0.0000
30	1	2	3	14	15	0	0.0000	1	0.0667
31	1	2	3	15	18	0	0.0000	0	0.0000
32	1	2	3	16	18	4	0.2222	7	0.3889
33	1	3	1	1	6	0	0.0000	0	0.0000
34	1	3	1	2	6	0	0.0000	0	0.0000
35	1	3	1	3	6	5	0.8333	1	0.1667
36	1	3	1	4	6	0	0.0000	0	0.0000
37	1	3	1	5	6	0	0.0000	0	0.0000
38	1	3	1	6	6	0	0.0000	0	0.0000
39	1	3	1	7	6	0	0.0000	0	0.0000
40	1	3	1	8	6	6	1.0000	0	0.0000
41	1	3	2	9	12	0	0.0000	0	0.0000
42	1	3	2	10	12	4	0.3333	2	0.1667
43	1	3	2	11	12	0	0.0000	1	0.0833
44	1	3	2	12	12	0	0.0000	2	0.1667
45	1	3	3	13	18	0	0.0000	1	0.0556
46	1	3	3	14	18	5	0.2778	4	0.2222
47	1	3	3	15	18	1	0.0556	1	0.0556
48	1	3	3	16	18	0	0.0000	0	0.0000
49	2	1	1	1	6	0	0.0000	0	0.0000
50	2	1	1	2	6	0	0.0000	0	0.0000
51	2	1	1	3	6	0	0.0000	0	0.0000
52	2	1	1	4	6	0	0.0000	0	0.0000
53	2	1	1	5	6	0	0.0000	0	0.0000
54	2	1	1	6	6	0	0.0000	0	0.0000
55	2	1	1	7	6	0	0.0000	1	0.1667
56	2	1	1	8	6	0	0.0000	0	0.0000
57	2	1	2	9	12	0	0.0000	0	0.0000
58	2	1	2	10	12	0	0.0000	1	0.0833
59	2	1	2	11	12	0	0.0000	0	0.0000
60	2	1	2	12	12	0	0.0000	1	0.0833
61	2	1	3	13	18	0	0.0000	2	0.1111
62	2	1	3	14	18	0	0.0000	2	0.1111
63	2	1	3	15	18	1	0.0556	0	0.0000
64	2	1	3	16	18	0	0.0000	0	0.0000
65	2	2	1	1	6	0	0.0000	0	0.0000
66	2	2	1	2	6	0	0.0000	0	0.0000
67	2	2	1	3	6	0	0.0000	0	0.0000
68	2	2	1	4	6	0	0.0000	0	0.0000
69	2	2	1	5	6	0	0.0000	0	0.0000
70	2	2	1	6	6	0	0.0000	0	0.0000
71	2	2	1	7	6	0	0.0000	0	0.0000
72	2	2	1	8	6	0	0.0000	0	0.0000
73	2	2	2	9	12	0	0.0000	0	0.0000
74	2	2	2	10	12	0	0.0000	0	0.0000
75	2	2	2	11	12	0	0.0000	0	0.0000
76	2	2	2	12	12	0	0.0000	1	0.0833
77	2	2	3	13	18	0	0.0000	0	0.0000
78	2	2	3	14	18	0	0.0000	0	0.0000
79	2	2	3	15	18	0	0.0000	0	0.0000

80	2	2	3	16	18	0	0.0000	0	0.0000
81	2	3	1	1	6	0	0.0000	0	0.0000
82	2	3	1	2	6	0	0.0000	0	0.0000
83	2	3	1	3	6	0	0.0000	0	0.0000
84	2	3	1	4	6	0	0.0000	0	0.0000
85	2	3	1	5	6	0	0.0000	0	0.0000
86	2	3	1	6	3	0	0.0000	0	0.0000
87	2	3	1	7	6	0	0.0000	0	0.0000
88	2	3	1	8	6	0	0.0000	0	0.0000
89	2	3	2	9	12	0	0.0000	1	0.0833
90	2	3	2	10	12	0	0.0000	0	0.0000
91	2	3	2	11	12	0	0.0000	1	0.0833
92	2	3	2	12	12	0	0.0000	0	0.0000
93	2	3	3	13	18	0	0.0000	0	0.0000
94	2	3	3	14	18	0	0.0000	0	0.0000
95	2	3	3	15	18	0	0.0000	0	0.0000
96	2	3	3	16	18	0	0.0000	0	0.0000
97	3	1	1	1	6	0	0.0000	0	0.0000
98	3	1	1	2	6	0	0.0000	0	0.0000
99	3	1	1	3	5	0	0.0000	0	0.0000
100	3	1	1	4	6	0	0.0000	0	0.0000
101	3	1	1	5	6	0	0.0000	0	0.0000
102	3	1	1	6	5	0	0.0000	0	0.0000
103	3	1	1	7	6	0	0.0000	0	0.0000
104	3	1	1	8	6	0	0.0000	0	0.0000
105	3	1	2	9	12	0	0.0000	1	0.0833
106	3	1	2	10	12	0	0.0000	0	0.0000
107	3	1	2	11	12	0	0.0000	1	0.0833
108	3	1	2	12	8	0	0.0000	0	0.0000
109	3	1	3	13	18	0	0.0000	0	0.0000
110	3	1	3	14	18	0	0.0000	0	0.0000
111	3	1	3	15	18	0	0.0000	0	0.0000
112	3	1	3	16	18	0	0.0000	0	0.0000
113	3	2	1	1	6	0	0.0000	1	0.1667
114	3	2	1	2	5	0	0.0000	1	0.2000
115	3	2	1	3	6	0	0.0000	0	0.0000
116	3	2	1	4	6	0	0.0000	0	0.0000
117	3	2	1	5	6	0	0.0000	0	0.0000
118	3	2	1	6	6	0	0.0000	0	0.0000
119	3	2	1	7	6	0	0.0000	0	0.0000
120	3	2	1	8	6	0	0.0000	0	0.0000
121	3	2	2	9	12	0	0.0000	0	0.0000
122	3	2	2	10	12	0	0.0000	1	0.0833
123	3	2	2	11	12	0	0.0000	0	0.0000
124	3	2	2	12	10	0	0.0000	0	0.0000
125	3	2	3	13	18	0	0.0000	0	0.0000
126	3	2	3	14	12	0	0.0000	1	0.0833
127	3	2	3	15	18	0	0.0000	1	0.0556
128	3	2	3	16	18	0	0.0000	1	0.0556
129	3	3	1	1	5	0	0.0000	0	0.0000
130	3	3	1	2	6	0	0.0000	1	0.1667
131	3	3	1	3	6	0	0.0000	0	0.0000
132	3	3	1	4	5	0	0.0000	0	0.0000
133	3	3	1	5	6	0	0.0000	0	0.0000
134	3	3	1	6	6	0	0.0000	0	0.0000
135	3	3	1	7	6	0	0.0000	0	0.0000
136	3	3	1	8	6	0	0.0000	0	0.0000
137	3	3	2	9	12	0	0.0000	0	0.0000

138	3	3	2	10	12	0	0.0000	0	0.0000
139	3	3	2	11	12	0	0.0000	0	0.0000
140	3	3	2	12	10	0	0.0000	1	0.1000
141	3	3	3	13	18	0	0.0000	0	0.0000
142	3	3	3	14	18	0	0.0000	0	0.0000
143	3	3	3	15	18	0	0.0000	0	0.0000
144	3	3	3	16	18	0	0.0000	0	0.0000

Appendix: III

Data indexing for guarding and touching healthy female quiescent deutonymph in the experiment: Female cadaver Vs Healthy female quiescent deutonymph.

Row	Stage	Date	Male density	Dish	n	Guarding		Touching	
						sum	Mean	sum	Mean
1	1	1	1	1	6	0	0.0000	0	0.0000
2	1	1	1	2	6	2	0.3333	0	0.0000
3	1	1	1	3	6	0	0.0000	0	0.0000
4	1	1	1	4	6	0	0.0000	0	0.0000
5	1	1	1	5	6	0	0.0000	0	0.0000
6	1	1	1	6	6	0	0.0000	0	0.0000
7	1	1	1	7	6	0	0.0000	0	0.0000
8	1	1	1	8	6	0	0.0000	0	0.0000
9	1	1	2	9	12	0	0.0000	0	0.0000
10	1	1	2	10	12	0	0.0000	0	0.0000
11	1	1	2	11	12	0	0.0000	0	0.0000
12	1	1	2	12	12	0	0.0000	0	0.0000
13	1	1	3	13	18	0	0.0000	0	0.0000
14	1	1	3	14	18	0	0.0000	0	0.0000
15	1	1	3	15	18	3	0.1667	1	0.0556
16	1	1	3	16	18	0	0.0000	3	0.1667
17	1	2	1	1	6	0	0.0000	0	0.0000
18	1	2	1	2	6	0	0.0000	0	0.0000
19	1	2	1	3	6	0	0.0000	0	0.0000
20	1	2	1	4	6	0	0.0000	0	0.0000
21	1	2	1	5	6	0	0.0000	0	0.0000
22	1	2	1	6	6	3	0.5000	0	0.0000
23	1	2	1	7	6	0	0.0000	0	0.0000
24	1	2	1	8	6	0	0.0000	0	0.0000
25	1	2	2	9	12	0	0.0000	0	0.0000
26	1	2	2	10	12	0	0.0000	0	0.0000
27	1	2	2	11	12	0	0.0000	0	0.0000
28	1	2	2	12	12	0	0.0000	0	0.0000
29	1	2	3	13	18	5	0.2778	0	0.0000
30	1	2	3	14	15	3	0.2000	1	0.0667
31	1	2	3	15	18	5	0.2778	1	0.0556
32	1	2	3	16	18	2	0.1111	0	0.0000
33	1	3	1	1	6	1	0.1667	3	0.5000
34	1	3	1	2	6	1	0.1667	3	0.5000
35	1	3	1	3	6	0	0.0000	0	0.0000
36	1	3	1	4	6	0	0.0000	0	0.0000
37	1	3	1	5	6	0	0.0000	0	0.0000
38	1	3	1	6	6	0	0.0000	0	0.0000
39	1	3	1	7	6	0	0.0000	0	0.0000

40	1	3	1	8	6	0	0.0000	0	0.0000
41	1	3	2	9	12	0	0.0000	2	0.1667
42	1	3	2	10	12	4	0.3333	0	0.0000
43	1	3	2	11	12	0	0.0000	0	0.0000
44	1	3	2	12	12	1	0.0833	4	0.3333
45	1	3	3	13	18	0	0.0000	0	0.0000
46	1	3	3	14	18	0	0.0000	0	0.0000
47	1	3	3	15	18	0	0.0000	0	0.0000
48	1	3	3	16	18	0	0.0000	0	0.0000
49	2	1	1	1	6	0	0.0000	0	0.0000
50	2	1	1	2	6	0	0.0000	0	0.0000
51	2	1	1	3	6	0	0.0000	0	0.0000
52	2	1	1	4	6	0	0.0000	0	0.0000
53	2	1	1	5	6	0	0.0000	0	0.0000
54	2	1	1	6	6	0	0.0000	0	0.0000
55	2	1	1	7	6	0	0.0000	0	0.0000
56	2	1	1	8	6	0	0.0000	0	0.0000
57	2	1	2	9	12	0	0.0000	0	0.0000
58	2	1	2	10	12	0	0.0000	0	0.0000
59	2	1	2	11	12	2	0.1667	0	0.0000
60	2	1	2	12	12	2	0.1667	1	0.0833
61	2	1	3	13	18	0	0.0000	0	0.0000
62	2	1	3	14	18	3	0.1667	1	0.0556
63	2	1	3	15	18	0	0.0000	1	0.0556
64	2	1	3	16	18	3	0.1667	1	0.0556
65	2	2	1	1	6	0	0.0000	1	0.1667
66	2	2	1	2	6	2	0.3333	0	0.0000
67	2	2	1	3	6	3	0.5000	0	0.0000
68	2	2	1	4	6	0	0.0000	0	0.0000
69	2	2	1	5	6	0	0.0000	0	0.0000
70	2	2	1	6	6	3	0.5000	1	0.1667
71	2	2	1	7	6	0	0.0000	1	0.1667
72	2	2	1	8	6	0	0.0000	0	0.0000
73	2	2	2	9	12	0	0.0000	0	0.0000
74	2	2	2	10	12	0	0.0000	0	0.0000
75	2	2	2	11	12	0	0.0000	0	0.0000
76	2	2	2	12	12	2	0.1667	0	0.0000
77	2	2	3	13	18	0	0.0000	0	0.0000
78	2	2	3	14	18	0	0.0000	0	0.0000
79	2	2	3	15	18	3	0.1667	1	0.0556
80	2	2	3	16	18	0	0.0000	0	0.0000
81	2	3	1	1	6	0	0.0000	1	0.1667
82	2	3	1	2	6	6	1.0000	0	0.0000
83	2	3	1	3	6	0	0.0000	0	0.0000
84	2	3	1	4	6	0	0.0000	0	0.0000
85	2	3	1	5	6	0	0.0000	1	0.1667
86	2	3	1	6	3	0	0.0000	0	0.0000
87	2	3	1	7	6	0	0.0000	0	0.0000
88	2	3	1	8	6	0	0.0000	0	0.0000
89	2	3	2	9	12	3	0.2500	0	0.0000
90	2	3	2	10	12	4	0.3333	1	0.0833
91	2	3	2	11	12	0	0.0000	0	0.0000
92	2	3	2	12	12	0	0.0000	0	0.0000
93	2	3	3	13	18	0	0.0000	1	0.0556
94	2	3	3	14	18	0	0.0000	0	0.0000
95	2	3	3	15	18	0	0.0000	0	0.0000
96	2	3	3	16	18	0	0.0000	0	0.0000
97	3	1	1	1	6	5	0.8333	0	0.0000

98	3	1	1	2	6	6	1.0000	0	0.0000
99	3	1	1	3	5	2	0.4000	0	0.0000
100	3	1	1	4	6	5	0.8333	0	0.0000
101	3	1	1	5	6	2	0.3333	0	0.0000
102	3	1	1	6	5	0	0.0000	0	0.0000
103	3	1	1	7	6	0	0.0000	0	0.0000
104	3	1	1	8	6	0	0.0000	0	0.0000
105	3	1	2	9	12	0	0.0000	1	0.0833
106	3	1	2	10	12	5	0.4167	1	0.0833
107	3	1	2	11	12	6	0.5000	0	0.0000
108	3	1	2	12	8	0	0.0000	0	0.0000
109	3	1	3	13	18	2	0.1111	2	0.1111
110	3	1	3	14	18	0	0.0000	0	0.0000
111	3	1	3	15	18	0	0.0000	0	0.0000
112	3	1	3	16	18	1	0.0556	0	0.0000
113	3	2	1	1	6	0	0.0000	0	0.0000
114	3	2	1	2	5	0	0.0000	0	0.0000
115	3	2	1	3	6	0	0.0000	0	0.0000
116	3	2	1	4	6	0	0.0000	0	0.0000
117	3	2	1	5	6	2	0.3333	1	0.1667
118	3	2	1	6	6	2	0.3333	3	0.5000
119	3	2	1	7	6	0	0.0000	0	0.0000
120	3	2	1	8	6	0	0.0000	0	0.0000
121	3	2	2	9	12	4	0.3333	2	0.1667
122	3	2	2	10	12	0	0.0000	0	0.0000
123	3	2	2	11	12	3	0.2500	1	0.0833
124	3	2	2	12	10	0	0.0000	0	0.0000
125	3	2	3	13	18	1	0.0556	0	0.0000
126	3	2	3	14	12	0	0.0000	0	0.0000
127	3	2	3	15	18	0	0.0000	0	0.0000
128	3	2	3	16	18	1	0.0556	0	0.0000
129	3	3	1	1	5	3	0.6000	1	0.2000
130	3	3	1	2	6	0	0.0000	0	0.0000
131	3	3	1	3	6	0	0.0000	0	0.0000
132	3	3	1	4	5	1	0.2000	1	0.2000
133	3	3	1	5	6	0	0.0000	0	0.0000
134	3	3	1	6	6	5	0.8333	1	0.1667
135	3	3	1	7	6	3	0.5000	0	0.0000
136	3	3	1	8	6	2	0.3333	3	0.5000
137	3	3	2	9	12	2	0.1667	3	0.2500
138	3	3	2	10	12	0	0.0000	0	0.0000
139	3	3	2	11	12	4	0.3333	2	0.1667
140	3	3	2	12	10	2	0.2000	2	0.2000
141	3	3	3	13	18	4	0.2222	0	0.0000
142	3	3	3	14	18	2	0.1111	1	0.0556
143	3	3	3	15	18	2	0.1111	2	0.1111
144	3	3	3	16	18	2	0.1111	0	0.0000

Appendix: IV

Data indexing for males on leaf disc in the experiment: Male cadaver Vs Female cadaver.

Row	Stage	Date	Male density	Dish	n	sum	Mean
1	1	1	1	1	6	2	0.3333
2	1	1	1	2	6	1	0.1667
3	1	1	1	3	6	3	0.5000

4	1	1	1	4	6	4	0.6667
5	1	1	1	5	6	6	1.0000
6	1	1	1	6	6	1	0.1667
7	1	1	1	7	6	4	0.6667
8	1	1	1	8	6	4	0.6667
9	1	1	2	9	12	3	0.2500
10	1	1	2	10	12	10	0.8333
11	1	1	2	11	12	8	0.6667
12	1	1	2	12	12	10	0.8333
13	1	1	3	13	18	17	0.9444
14	1	1	3	14	18	7	0.3889
15	1	1	3	15	18	10	0.5556
16	1	1	3	16	18	14	0.7778
17	1	2	1	1	6	6	1.0000
18	1	2	1	2	6	3	0.5000
19	1	2	1	3	6	2	0.3333
20	1	2	1	4	6	6	1.0000
21	1	2	1	5	6	5	0.8333
22	1	2	1	6	6	6	1.0000
23	1	2	1	7	6	5	0.8333
24	1	2	1	8	6	3	0.5000
25	1	2	2	9	12	12	1.0000
26	1	2	2	10	12	2	0.1667
27	1	2	2	11	12	7	0.5833
28	1	2	2	12	12	8	0.6667
29	1	2	3	13	18	12	0.6667
30	1	2	3	14	18	11	0.6111
31	1	2	3	15	18	16	0.8889
32	1	2	3	16	18	11	0.6111
33	1	3	1	1	6	4	0.6667
34	1	3	1	2	6	5	0.8333
35	1	3	1	3	6	2	0.3333
36	1	3	1	4	6	2	0.3333
37	1	3	1	5	6	6	1.0000
38	1	3	1	6	6	6	1.0000
39	1	3	1	7	6	4	0.6667
40	1	3	1	8	6	2	0.3333
41	1	3	2	9	12	9	0.7500
42	1	3	2	10	12	10	0.8333
43	1	3	2	11	12	10	0.8333
44	1	3	2	12	12	3	0.2500
45	1	3	3	13	18	4	0.2222
46	1	3	3	14	18	6	0.3333
47	1	3	3	15	18	7	0.3889
48	1	3	3	16	18	15	0.8333

Appendix: V

Data indexing for guarding and touching female cadaver in the experiment: Female cadaver Vs male cadaver.

Row	Stage	Date	Male density	Dish	n	Guarding		Touching	
						sum	Mean	sum	Mean
1	1	1	1	1	6	0	0.0000	0	0.0000
2	1	1	1	2	6	0	0.0000	0	0.0000
3	1	1	1	3	6	0	0.0000	0	0.0000

4	1	1	1	4	6	1	0.1667	0	0.0000
5	1	1	1	5	6	0	0.0000	0	0.0000
6	1	1	1	6	6	0	0.0000	0	0.0000
7	1	1	1	7	6	0	0.0000	0	0.0000
8	1	1	1	8	6	0	0.0000	1	0.1667
9	1	1	2	9	12	2	0.1667	0	0.0000
10	1	1	2	10	12	6	0.5000	2	0.1667
11	1	1	2	11	12	0	0.0000	0	0.0000
12	1	1	2	12	12	1	0.0833	0	0.0000
13	1	1	3	13	18	5	0.2778	5	0.2778
14	1	1	3	14	18	1	0.0556	0	0.0000
15	1	1	3	15	18	0	0.0000	0	0.0000
16	1	1	3	16	18	11	0.6111	0	0.0000
17	1	2	1	1	6	0	0.0000	0	0.0000
18	1	2	1	2	6	0	0.0000	1	0.1667
19	1	2	1	3	6	0	0.0000	0	0.0000
20	1	2	1	4	6	6	1.0000	0	0.0000
21	1	2	1	5	6	0	0.0000	1	0.1667
22	1	2	1	6	6	0	0.0000	1	0.1667
23	1	2	1	7	6	0	0.0000	2	0.3333
24	1	2	1	8	6	1	0.1667	1	0.1667
25	1	2	2	9	12	0	0.0000	3	0.2500
26	1	2	2	10	12	0	0.0000	1	0.0833
27	1	2	2	11	12	0	0.0000	0	0.0000
28	1	2	2	12	12	1	0.0833	1	0.0833
29	1	2	3	13	18	1	0.0556	2	0.1111
30	1	2	3	14	18	0	0.0000	1	0.0556
31	1	2	3	15	18	6	0.3333	2	0.1111
32	1	2	3	16	18	0	0.0000	1	0.0556
33	1	3	1	1	6	1	0.1667	1	0.1667
34	1	3	1	2	6	1	0.1667	2	0.3333
35	1	3	1	3	6	0	0.0000	0	0.0000
36	1	3	1	4	6	0	0.0000	0	0.0000
37	1	3	1	5	6	2	0.3333	0	0.0000
38	1	3	1	6	6	0	0.0000	0	0.0000
39	1	3	1	7	6	0	0.0000	1	0.1667
40	1	3	1	8	6	0	0.0000	0	0.0000
41	1	3	2	9	12	3	0.2500	2	0.1667
42	1	3	2	10	12	4	0.3333	2	0.1667
43	1	3	2	11	12	4	0.3333	0	0.0000
44	1	3	2	12	12	0	0.0000	1	0.0833
45	1	3	3	13	18	0	0.0000	0	0.0000
46	1	3	3	14	18	0	0.0000	0	0.0000
47	1	3	3	15	18	1	0.0556	1	0.0556
48	1	3	3	16	18	2	0.1111	2	0.1111

Appendix: VI

Data indexing for guarding and touching male cadaver in the experiment: Female cadaver Vs male cadaver.

Row	Stage	Date	Male density	Dish	n	Guarding		Touching	
						Sum	Mean	Sum	Mean
1	1	1	1	1	6	0	0	0	0.0000
2	1	1	1	2	6	0	0	0	0.0000
3	1	1	1	3	6	0	0	0	0.0000

4	1	1	1	4	6	0	0	0	0.0000
5	1	1	1	5	6	0	0	0	0.0000
6	1	1	1	6	6	0	0	1	0.1667
7	1	1	1	7	6	0	0	0	0.0000
8	1	1	1	8	6	0	0	0	0.0000
9	1	1	2	9	12	0	0	0	0.0000
10	1	1	2	10	12	0	0	0	0.0000
11	1	1	2	11	12	0	0	0	0.0000
12	1	1	2	12	12	0	0	1	0.0833
13	1	1	3	13	18	0	0	0	0.0000
14	1	1	3	14	18	0	0	1	0.0556
15	1	1	3	15	18	0	0	0	0.0000
16	1	1	3	16	18	0	0	0	0.0000
17	1	2	1	1	6	0	0	0	0.0000
18	1	2	1	2	6	0	0	0	0.0000
19	1	2	1	3	6	0	0	1	0.1667
20	1	2	1	4	6	0	0	0	0.0000
21	1	2	1	5	6	0	0	0	0.0000
22	1	2	1	6	6	0	0	0	0.0000
23	1	2	1	7	6	0	0	0	0.0000
24	1	2	1	8	6	0	0	0	0.0000
25	1	2	2	9	12	0	0	0	0.0000
26	1	2	2	10	12	0	0	0	0.0000
27	1	2	2	11	12	0	0	0	0.0000
28	1	2	2	12	12	0	0	0	0.0000
29	1	2	3	13	18	0	0	0	0.0000
30	1	2	3	14	18	0	0	0	0.0000
31	1	2	3	15	18	0	0	0	0.0000
32	1	2	3	16	18	0	0	0	0.0000
33	1	3	1	1	6	0	0	0	0.0000
34	1	3	1	2	6	0	0	0	0.0000
35	1	3	1	3	6	0	0	0	0.0000
36	1	3	1	4	6	0	0	0	0.0000
37	1	3	1	5	6	0	0	0	0.0000
38	1	3	1	6	6	0	0	0	0.0000
39	1	3	1	7	6	0	0	0	0.0000
40	1	3	1	8	6	0	0	0	0.0000
41	1	3	2	9	12	0	0	0	0.0000
42	1	3	2	10	12	0	0	0	0.0000
43	1	3	2	11	12	0	0	0	0.0000
44	1	3	2	12	12	0	0	1	0.0833
45	1	3	3	13	18	0	0	0	0.0000
46	1	3	3	14	18	0	0	1	0.0556
47	1	3	3	15	18	0	0	0	0.0000
48	1	3	3	16	18	0	0	0	0.0000