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The copulation behaviour of the western flower thrips

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Abstract

The western flower thrips *Frankliniella occidentalis* (Pergande) (Thysanoptera: Thripidae) is a major pest of agricultural and horticultural importance in most parts of the world. It damages crops by feeding and transmission of viruses. There is a need for sustainable IPM due to development of resistance to insecticides. Understanding the reproductive biology of this pest may suggest novel approaches to pest control. The aim of this research was to study the copulation behaviour of *F. occidentalis* and the possible role of pheromones. Female *F. occidentalis* copulated multiple times with virgin males either in rapid succession or over days, contrary to previous reports that females will not copulate again until after fifteen days. Subsequent copulations were, however, shorter than the first, probably due to detection of a barrier by males that may prevent sperm transfer. Culture reared males copulated with virgin females, but rarely with copulated females, and virgin males copulated with dead virgin females more often than with dead mated females of similar age, which showed that males could detect whether females had copulated. Observation suggested that during copulation, males were applying antiaphrodisiac pheromone by stroking or antennation, which may have been used for assessing females' copulation status. This antiaphrodisiac pheromone may be the contact pheromone, 7-methyltricosane. Culture reared males were probably limited in reproductive resources due to old age or multiple copulation, thus they could have changed strategy to show preference for virgin females, copulating more with them than already copulated females, whereas, most young virgin males (with more resources) were not mate selective. It is also possible that the increased preference for virgin females resulted from male mating experience. Choice based on male copulation status was not observed in virgin females.

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List of species cited

Thrips species
Caliothrips indicus (Bagnall)
Apterothrips apteris (Daniel) formally *Sericothrips apteris* Daniel
Caliothrips (Heliothrips) fasciatus (Pergande)
Arorathrips mexicanus (Crawford) *formally Chirothrips mexicanus* Crawford
Dunatothrips aneurae Mound
Echinothrips americanus Morgan
Elaphrothrips tuberculatusor (Hood) formally *Idolothrips tuberculatus* Hood
Frankliniella intonsa (Trybom)
Frankliniella schultzei (Trybom)
Frankliniella fusca (Hinds)
Frankliniella occidentalis (Pergande)
Franklinothrips orizabensis Johansen
Hoplothrips karnyi (Hood)
Hoplothrips pedicularius (Haliday)
Limothrips denticornis (Haliday)
Megalurothrips sjostedti (Trybom)
Kladothrips habrus (Mound) *Oncothrips* Mound
Kladothrips tepperi (Karny) formally *Oncothrips tepperi* Karny
Parabaliothrips newmani Gillespie, Mound & Wang
Ponticulothrips diospyrosi Haga & Okajima
Retithrips syriacus (Mayet) formally *Thrips (Heliothrips) syriacus* Mayet

Rhipiphorothrips cruentatus Hood

Scirtothrips aurantii Faure

Scolothrips sexmaculatus (Pergande)

Suocerathrips linguis Mound & Marullo

Pezothrips *dianthi* (Priesner) formally *Taeniothrips dianthi* Priesner

Thrips fuscipennis Haliday

Thrips major Uzel

Thrips palmi Karny

Thrips tabaci Lindeman

Other arthropods including fish

Amblyseius (*Neoseiulus*) *cucumeris* (Oudemans)

Anoplophora malasiaca Thomson

*Drosophila melanogaster*Meigen

Helicoverpa zea (Boddie)

Helicoverpa zea (Fabricius)

Nysius huttoni White

Orius tristicolor (White)

Panorpa cognata Rambur

Pemphigus bursarius (Linnaeus)

Pseudomugil signifier Kner (Fish)

Requena verticalis Walker

Tenebrio molitor Linnaeus

Trichogramma turkestanica Meyer

Utetheisa ornatrix (Linnaeus)

Plants
Calystegia sepium (Linnaeus)
Camellia sinensis (Linnaeus) Kuntze
Phaseolus vulgaris Linnaeus
Pinus thunbergii Parl.

Chapter 1

General introduction

1.1 Introduction

Thrips (insects in the order Thysanoptera) are tiny, slender insects that are widespread throughout the world in both tropical and temperate regions (Lewis, 1973). They have delicately fringed wings with long cilia (Lewis, 1997b; Moritz, 1997) and an arolium (Moritz, 1997). The arolium is a small bladder at the end of the legs found in both larvae and adult thrips, which tends to improve adhesion to surfaces and the ability of thrips to walk upside down or vertically (Heming, 1971; Kirk, 1996). Thrips are therefore sometimes referred to as the fringe wings, or bladder-footed insects (Ananthakrishnan, 1979). They possess asymmetrical punching and sucking mouthparts with only the left mandible developed, two well-developed maxillae, a labrum and a labium (Moritz, 1997).

Thrips feed on a wide range of food sources (Kirk, 1995). The economically important species are phytophagous, feeding on epidermal and subepidermal plant parts (Lewis, 1973; Ananthakrishnan, 1993; Kirk, 1996). Some other species of thrips are sporophagous and mycophagous (Terry, 1997) or omnivorous (Terry, 1997). Thrips can serve as vectors of bacteria, yeast and fungi (Lewis, 1973) and viruses (Ullman *et al.*, 1992; Tsuda *et al.*, 1996; van de Wetering *et al.*, 1996). Thrips' ability to transmit viruses in the genus *Tospovirus*

(Bunyaviridae) is considered as one of the most significant negative effects of thrips on plants (Reitz *et al.*, 2011). There are thrips species that serve beneficial purposes, for example some species of the genera *Frankliniella* and *Thrips* are actively involved in crop pollination (Kirk, 1997; Terry, 2002) and other species serve as predators of pests (Ananthakrishnan, 1979; Kirk, 1997). A few predaceous species feed on mites, whiteflies or coccids while others feed on other species of thrips, which make them potential biocontrol agents (Ananthakrishnan, 1979). It would therefore be wrong to assume that all thrips are pests.

The thrips (Thysanoptera) are divided into two sub-orders: Terebrantia and Tubulifera. The sub-order Terebrantia has a life cycle with two non-feeding stages (propupa and pupa) while the Tubulifera sub-order has an additional non-feeding pupal instar (Lewis, 1997b). Most adult female Terebrantia possess four distinguishing saw-like ovipositor valves and they insert their eggs in plant tissues (Mound & Walker, 1982). Tubulifera has only one family, Phlaeothripidae, while Terebrantia has seven families (Uzelothripidae, Merothripidae, Adiheterothripidae, Aeolothripidae, Thripidae, Fauriellidae, and Heterothripidae) (Mound *et al.*, 1980; Mound, 1997), though Bhatti (2006) proposed the reclassification of the sub-order Terebrantia into 28 families in 10 superfamilies. Phlaeothripidae and Thripidae include about 93% of all known thrips species, with the largest being Phlaeothripidae with about 3100 species already described (Mound, 1997). Thripidae, the largest known family, in the sub-order Terebrantia, has about 1700 species identified (Mound & Teulon, 1995; Mound, 1997). Though about 6,000 species of thrips have been identified throughout the world (Ananthakrishnan, 1979; Mound, 2007) out of an estimated 8000 existing species (Lewis, 1997b), only 100 are economically important pests (Mound, 1997). In Britain, only three families of thrips have been recorded, Thripidae, Aeolothripidae and Phlaeothripidae (Kirk,

1996). *Frankliniella occidentalis* (Pergande) of the family Thripidae is the most economically important species in this region (Kirk, 1996).

The western flower thrips, *F. occidentalis* is a major agricultural and horticultural pest of economic importance in most parts of the world (Kirk, 2002; Kirk & Terry, 2003). It likely originated from western North America from where it has become an international pest (Mound, 1997; Kirk, 2002; Kirk & Terry, 2003). This insect can be found across very wide topography, from sea coast sand to as high as about 2700 m (Bryan & Smith, 1956; Waterhouse & Norris, 1989). It was accidentally introduced into Europe and has become a major greenhouse pest (van Lenteren & Loomans, 1995). The first report of *F. occidentalis* in Britain was in 1986 (Kirk, 1996; McDonald *et al.*, 1997).

Frankliniella occidentalis like some other thrips species are highly variable in morphology (Bryan & Smith, 1956; Kirk & Terry, 2003), resistance to insecticides (Martin & Workman, 1994), transmission of tospoviruses (van de Wetering *et al.*, 1999), biotype variation on cucumber (de Kogel *et al.*, 1997), colour (Bryan & Smith, 1956) and host plant utilisation (Baez *et al.*, 2011). Molecular evidence has clearly revealed that *F. occidentalis* is a complex of two cryptic species (the "lupin" and "glasshouse" strains) (Rugman-Jones *et al.*, 2010). The New Zealand "lupin" strain, usually found in tree lupins (*Lupinus arboreus* Sims), was not problematic to crops grown in New Zealand, and was less resistant to insecticides compared with the "glasshouse" strains (Martin & Workman, 1994).

1.2 Pest status

The pest *F. occidentalis* is a polyphagous insect (Lewis, 1997a) and an opportunist, with high vagility i.e ability to spread freely and migrate, polyvoltinism and short lifecycle (Mound & Teulon, 1995). It infests virtually all crops and vegetables. In greenhouses located in the USA, Canada and Europe, 219 species from 59 genera of vegetable and ornamental

crops have been reported susceptible to its damage (Brødsgaard, 1989). The damage it causes ranges from activities such as feeding and ovipositing on plants (Childers & Achor, 1995; Childers, 1997) to transmission of tospoviruses (Sakimura, 1960; Wijkamp *et al.*, 1995; Ullman *et al.*, 1997; Riley *et al.*, 2011). *Frankliniella occidentalis* is a vector of *Tomato spotted wilt virus*, *Impatiens necrotic spot virus*, *Chrysanthemum stem necrosis virus*, *Groundnut ringspot virus* and *Tomato chlorotic spot virus* (Pappu *et al.*, 2009)**.** The injury caused by *F. occidentalis* also predispose plants to attack by other secondary infections such as fungi. It is also a major problem in the ornamental plant industries because virtually all floricultural crops are susceptible to its attack (Immaraju *et al.*, 1992; Robb & Parrella, 1995). Many thrips are attracted to flowers possibly because of the scent (Kirk, 1985b) or the colour (Kirk, 1984).

Adult *F. occidentalis* feed on leaves, fruits, flowers, pollen and vegetables reducing yield and market value of produce (Childers, 1997; Kirk, 1997). The eggs laid by females on fruits and flowers also cause reduction in yield and market value (Funderburk, 2009). In particular, crops like cucumber, chrysanthemum and strawberry grown in greenhouses are susceptible to its attack through transmission of viruses and feeding (Jacobson, 1997; Sampson & Kirk, 2013).

F. occidentalis causes significant economic losses in tree fruits. In apple, for example, it causes fruit damage because of oviposition (Reitz *et al.*, 2011). Oviposition often results in spotting in fruits which may affect the market value of the produce because the female thrips insert eggs under the plant epidermis resulting in plant physiological damage (Reitz, 2009) as observed in some varieties of apple (Madsen & Jack, 1966).

According to Kirk (2002), up-to-date information on the cost of damage done by *F. occidentalis* is difficult to get partly because it is not often published and also due to the

complications associated with the presence of other similar thrips species. Damage caused by feeding activities of thrips can be confused with those caused by other pests and diseases (Steiner & Goodwin, 2005) and such damage is sometimes not immediately apparent thus leading to wrong diagnosis and subsequently misuse of pesticide (Reitz, 2009). In Florida, records of Nuessly & Nagata (1995) estimated that losses due to *F. occidentalis* and *Thrips palmi* exceeded $10 million in 1993. Eradication measures in Finland between 1987-1990 costs the government FIM 1.6 million (Rautapää, 1992). Also, an estimate of about £8 million loss per annum has been attributed to *F. occidentalis* in UK strawberry (Sampson & Kirk, 2013).

The extent of damage by *F. occidentalis* has prompted the need for control. Farmers generally have low tolerance for this pest because it damages the flowers and the buds (Cloyd & Sadof, 1998). In addition, the pest is difficult to control since eggs are oviposited in plant tissues and adults feed in hidden places within flowers and young plant parts (Robb & Parrella, 1995). The use of insecticides is therefore the primary approach to control (Bielza, 2008). Insecticide resistance has become a common problem in the management of *F. occidentalis* reviewed by (Jensen, 2000; Bielza, 2008; Gao *et al.*, 2012). With recent legislation on sustainable use of pesticides by the EU (DIRECTIVE 2009/128/EC), Integrated Pest Management involving minimal use of chemical and the use of other control methods such as host plant resistance, biological control, use of semiochemical and other advances in biotechnology, with emphasis on compatibility may be the way forward (Cloyd, 2009; Mouden *et al.*, 2017).

1.3 Life stages of *F. occidentalis*

The life cycles of thrips species in the sub-order Terebrantia (which includes *F. occidentalis*) are similar and they include the egg, two voracious and active larval stages, the non-feeding

propupal and pupal stages, and a winged adult stage (Lewis, 1973, 1997b) Figure 1.1 . Reproduction in *F. occidentalis* is haplodiploid, with the unfertilised haploid eggs resulting in male offspring while the fertilised eggs result in females (Lewis, 1997b; Moritz, 1997). Development is temperature-dependent (Gaum *et al.*, 1994; Ishida *et al.*, 2003) and the optimum temperature is between 25-30ºC (McDonald *et al.*, 1998). The life cycle from egg to adult on five different leaves was between 9-13 days at 27 ºC (Zhang *et al.*, 2007) and about 12 days at 25 ºC when reared on bean pods (MacDonald, 2002). At a lower temperature of about 10 ºC development takes over 100 days while no development occurs at 35 ºC (McDonald *et al.*, 1998). Development is rapid and many generations are possible in a cropping season (Reitz, 2009).

Eggs are laid into leaves, petioles, flower bracts, petals, and developing fruits with the aid of a saw-like ovipositor (Reitz, 2009). Oviposition starts 72 hours after emergence and continues throughout the life of the adult; an average of 0.66 to 1.63 eggs are laid per day at 26.6°C (Bryan & Smith, 1956). An average of 4.9 eggs has been reported per day at 23°C, with a total average of 75.6 eggs in a female entire lifetime (Kumm, 2002). Oviposition occurs mainly during the day (Kirk *et al.*, 1999) and it is also diet dependent, for example addition of pollen increases oviposition rate (Trichilo & Leigh, 1988; Kirk, 1997) and fecundity was increased when *F. occidentalis* was fed with cucumber leaves compared with tomato leaves (Li *et al.*, 2015a). Females feed on the flowering part of the plant, which contains the nutrients needed for proper development and to enhance reproductive ability (Higgins & Myers, 1992). Photoperiod also influences the oviposition rate. The number of

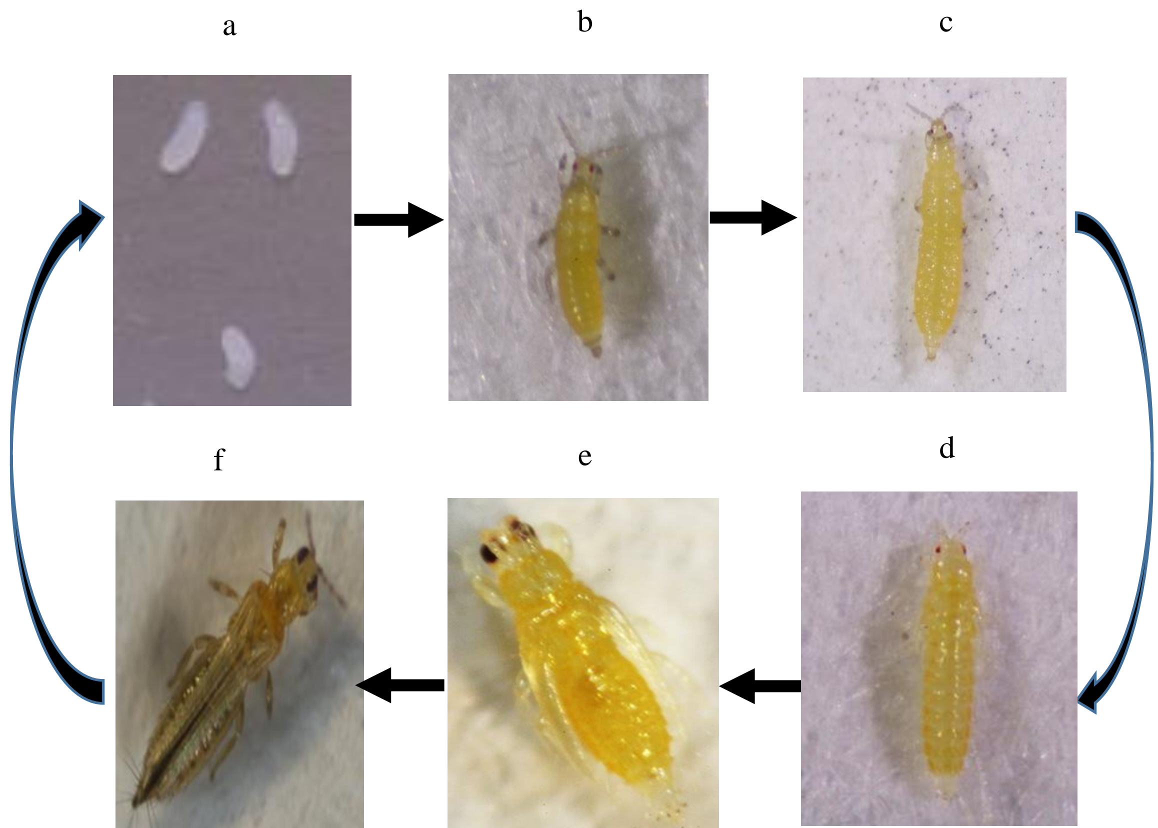

Figure 0.1 Life cycle of *F. occidentalis* from egg to adult. (a) eggs, (b) first instar larva, (c) second instar larva, (d) propupa, (e) pupa, (f) adult female.

eggs laid in 24 hours increased with increasing photophase from 24 hours darkness to 24 hours full light (Whittaker & Kirk, 2004). Eggs are vulnerable to desiccation and mortality may therefore be high at the egg stage (Waterhouse & Norris, 1989). Terebrantian eggs therefore usually need the support of the environment and surrounding plant tissues a few hours after they are laid (Moritz, 1997), which may explain why they lay their eggs in plant tissues. The deposition of eggs in confined places may be a strategy to protect them from attack by predators and other external factors. Also, eggs laid in confined places are difficult to detect and are not susceptible to fumigation (MacDonald, 1993; Janmaat *et al.*, 2002; Simpson *et al.*, 2007). Eggs of *F. occidentalis* usually hatch at about the fourth day after oviposition. They have a characteristic smooth kidney shape and pale white colour (Figure 1.1).

The first instar larva emerges from the plant surface with the head first, and the subsequent expansion and contraction of the undulating abdomen helps the larva move to the plant surface with the aid of the forelegs (Bryan & Smith, 1956). The first larval stage lasts for 2-3 days (Robb *et al.*, 1988). The second-instar larvae move quickly after emerging and seek crevices where they can hide or feed (Waterhouse & Norris, 1989) and this stage takes between 2-4 days (Robb *et al.*, 1988). The larval developmental period depends on temperature, at 10 °C the larval stages lasted for 59 days while at 30 °C they lasted for about 6 days (McDonald *et al.*, 1998). The body parts of the two larval stages, just like adults, are divided into the head, thorax and abdomen. They feed and share a similar ecological niche and host food with adults (Kirk, 1997; Hansen *et al.*, 2003). The two stages are similar but the first instar larva is half the size of the second (Gaum *et al.*, 1994; Reitz, 2008) (Figure 1.1).

At the end of the second larval instar, the insects may drop to pupate in the soil (Bennison, 2002), but many may also remain on host plants, especially in a situation where the hosts

have complex floral architecture (Broadbent *et al.*, 2003). Pupation behaviour can therefore be influenced by plant type (Buitenhuis & Shipp, 2008). In some instances, the second larval stage may also travel to the base of the plant and enter the growing medium to pupate (Thoeming *et al.*, 2003). Reduced relative humidity (less than about 81%) has been observed to induce dropping of the second larval stage (Steiner *et al.*, 2011). The second instar larva develops first into the first pupal instar termed the propupal stage and then the pupal stage. The survival of the pupal stage in the soil or growing medium is influenced by factors such as the pH and the pupation depth (Varatharajan & Daniel, 1984). The two non-feeding stages (the propupa and the pupa) are very sluggish but move when disturbed. Propupa and pupa developmental durations are about 1-2 days and 2-3 days respectively at the optimum temperature of 25-30°C (McDonald *et al.*, 1998). Propupae can be differentiated from pupae by the presence of short wing pads and an erect antenna. At the pupal stage, the pads become longer and the erect antennae become rested behind the head (Figure 1.1).

Both male and female adults are winged but the females are larger than the males, with a wider abdomen and they are usually darker in colour compared with the males. In the laboratory condition, as observed at Keele University, female *F. occidentalis* are more frequent than males. In field populations, females often predominate in most species of thrips (Lewis, 1973). Depending on temperature and other factors, females may live up to 40 to 90 days while males, after emergence, have about half this life expectancy (Bryan & Smith, 1956). The newly-emerged adult female is white to light yellow and is relatively inactive for the first day but later becomes active (Waterhouse & Norris, 1989).

1.4 Modes of reproduction in insects

Most insects reproduce sexually (Thornhill & Alcock, 1983; Gullan & Cranston, 2005) but there are over a thousand species of insects without any form of mating and females of such species lay eggs that develop into females by obligate parthenogenesis (Normark, 2014). In

parthenogenetic reproduction, the egg cells develop into a new individual without fertilization which is common in insects of the order Hymenoptera and in some aphids (Suomalainen, 1962; Gullan & Cranston, 2005). Parthenogenesis can occur naturally or artificially (prompted by some stimulus), it can be obligate or facultative (when eggs develop whether or not they are fertilised) and it can be grouped based on impaternate (fatherless) nature of their offspring (Whiting, 1945). There are three different forms of impaternate parthenogenesis among insects (1) thelytoky: unfertilised eggs develop into female progeny, (2) haploid parthenogenesis (arrhenotoky): unfertilised eggs develop into males (though fertilised eggs develop into females) (Mittwoch, 1978) and (3) deuterotoky: the production of males and females from unfertilized eggs (Hoy, 2003). Thelytoky can be complete (the only means of reproduction) in some species of insects such as stick insects (White, 1977), while in other insect species, it may be alternated with sexual reproduction (cyclical thelytoky) such as in the aphid *Pemphigus bursarius* (Linnaeus, 1758) (Thornhill & Alcock, 1983). Arrhenotoky is most common in the order Hymenoptera but can also be found among the Hemiptera while the three forms of reproduction are found in the Thysanoptera (Gullan & Cranston, 2005). Other forms of reproduction include gynogenesis (a form of reproduction in which sperm is needed by females only to trigger egg development) and hermaphroditism (offspring derived from sperm and egg produced meiotically by the same individual) such as in coccid bugs (Thornhill & Alcock, 1983; Gullan & Cranston, 2005).

1.5 Parthenogenesis in Thysanoptera

Reproduction in thrips can be sexual, parthenogenetic or even both (Bryan & Smith, 1956; Ananthakrishnan, 1990). The population of thrips in the field are often bisexual (Lewis, 1973), but previous collections of bisexual species of thrips have shown deviation in sex ratio (Whiting, 1945), with more females than males collected while males of some species remain unknown (Stannard, 1968). Differences in sex ratio has often been thought to suggest

arrhenotoky (Hamilton, 1967) thus signifying arrhenotoky as a major form of reproduction in thrips. Sex ratio may however be misleading if adult sampling is not done accurately in the field especially in species where only the females hibernate or where there are differences in longevity and temperature responses of both sexes (Lewis, 1973) or even differential mortality of the larval stage.

Arrhenotoky, thelytoky and deuterotoky have been reported in thrips (Lewis, 1973; Moritz, 1997). Arrhenotoky can be obligate or facultative. While obligate arrhenotoky is common in some thrips species such as *F. occidentalis*, facultative arrhenotoky occurs in species such as *Echinothrips americanus* (Morgan, 1913) (Karadjova & Krumov, 2003)*, Caliothrips indicus* and *Chirothrips mexicanus* (Ananthakrishnan, 1993). In species of both Tubulifera and Terebrantia where males are rare or unknown, thelytokous parthenogenesis is the major form of reproduction (Whiting, 1945; Lewis, 1973). Arrhenotoky and thelytoky, the most common form of reproduction in thrips, are often found among the phytoghagous species of thrips (Ananthakrishnan, 1993) and deuterotoky may be scarce in the order Thysanoptera (Moritz, 1997). In *Thrips tabaci*, the three forms of reproduction have been reported (Zawirska, 1976; Chatzivassiliou *et al.*, 2002; Toda & Murai, 2007; Nault, 2007; Kobayashi & Hasegawa, 2013; Li *et al.*, 2015c).

Arrhenotoky in thrips is generally reported as haplodiploid since fertilized eggs produce diploid females, whereas unfertilized eggs produce haploid males. Haploid male and diploid females have been confirmed in *Franklinothrips orizabensis*, *Frankliniella occidentalis* and *Frankliniella fusca* using flow cytometry genome size estimates while polyploidy was observed in some thelytokous *Thrips tabaci* (Jacobson *et al.*, 2013).

In *F. occidentalis*, arrhenotoky (haplodiploidy) is the only known mode of reproduction. Virgin females produce only male offspring while mated females produce both male and

female offspring. Virgin female *F. occidentalis* were however observed to produce a few female progenies when developmental stages were subjected to different temperatures, though further testing was not done to confirm if these females could reproduce (Kumm, 2002; Kumm & Moritz, 2009).

1.6 Mating behaviour in thrips

Mating behaviour in insects can simply be defined as all the immediate actions that surround insemination (the transfer of a male's sperm to a female) from formation of a pair through courtship and copulation to the final breakup of the mating pair (Matthews & Matthews, 2010). The mating behaviour of the sub-order Tubulifera has been largely studied (Crespi, 1986a-b, 1988a-c). Some of the studied tubuliferans are mycophagous and gall-forming species, they live in colonies with characteristic sex-limited polymorphism or wing dimorphism (Crespi, 1986b, 1988b, c, 1992). Mating success in mycophagous thrips species largely depends on male-male competition. During male-male competition, thrips fight by stabbing or flipping the opponent to protect or guard territory or oviposition sites where females can be found, while other males sneak or challenge the guarding males. In *Hoplothrips pedicularius* for example, males meet at the oviposition site and fight in defence of the oviposition site, usually (about 80% of times) dominant males secure mating (Crespi, 1986b). Fighting is usually done with the enlarged forelegs or the abdomen. Success in fighting and the tactic employed by males was associated with the size of the males in *Elaphrothrips tuberculatus* (Crespi, 1988a) with larger males winning in most fights and having more mating successes (Crespi, 1986a). Fighting can be lethal in species like *Hoplothrips karnyi* (Crespi, 1988b).

Due to the diverse lifestyles in terebrantians, the mating system may vary between species as influenced by the environment (Terry, 1997). Studies of the mating system in this sub-

order have concentrated on description of the sequence of activities before and during copulation (Russell, 1912; Lewis, 1973; Kirk, 1985a; Terry & Schneider, 1993). There have also been some reports of aggregation and fighting among males (Terry & Gardner, 1990; Terry & Dyreson, 1996). The sequence of activities leading to copulation includes: a male approaching a female, mounting her, bending the abdomen beneath her and copulating with her (Russell, 1912; Lewis, 1973; Kirk, 1985a; Terry & Schneider, 1993; Terry, 1997; Milne *et al.*, 2007; Rafter & Walter, 2013). During copulation, male antennation of a female's head and stroking the dorsal surface of the female thorax down to the dorsal surface of the abdomen with one of the mid-legs are two important common behaviours typically observed among terebrantians (Terry & Schneider, 1993; Milne *et al.*, 2007; Rafter & Walter, 2013; Krueger *et al.*, 2017). A recent publication has also described in some detail for the first time the internal processes involved during copulation in *E. americanus* (Krueger *et al.*, 2017).

Males of some species of thrips in the genera *Thrips* and *Frankliniella* aggregate on flowers and such aggregation eventually results in mating. Male *Thrips major* Uzel and *Thrips fuscipennis* aggregate in flowers of *Calystegia sepium* (L.) while some patrol on the open part of the flowers without any obvious attempt to claim territory but to intercept and mate with females landing within the aggregation around the corolla of the flower (Kirk, 1985a). Aggregation behaviour has also been reported in *F. occidentalis* (Terry & Gardner, 1990; Terry & Dyreson, 1996), *Frankliniella schultzei* Trybom (Milne *et al.*, 2002), *Parabaliothrips newmani* (Gillespie *et al.*, 2002) and *Megalurothrips sjostedti* (Niassy *et al.*, 2016). In some species including *F. occidentalis* where aggregation has been reported, more than one male can attempt to mate with a single female during aggregation (Kirk, 1985a; Milne *et al.*, 2002), however, a female *F. occidentalis* observed in the field copulated with the first male she encountered within an aggregation and then rejected advances by other

males before moving away from the aggregation (Terry & Gardner, 1990; Terry & Dyreson, 1996).

In a laboratory bioassay, virgin female *F. occidentalis* were observed to only accept copulation 15 days after the first copulation when tested at 1, 5 and 15 days though this was based on a small sample of eight and some of the males used were not virgins (Terry & Schneider, 1993). However, reluctance in females to copulate after initial copulation suggests possible competition for virgin females among males. In thrips of the sub-order Terebrantia, studies have shown a very low female copulation frequency after the first mating within a test period, including: *E. americanus* (Li *et al.*, 2014; Krueger *et al.*, 2015a) and *F. schultzei* (Milne *et al.*, 2007). In *T. tabaci* where multiple copulation was reported, the frequency of mating was less than 3 when copulation was tried every 2 days between 2-30 days test period (Li *et al.*, 2015b).

Fighting among male *F. occidentalis* can be brief, aggressive or escalated (Terry & Dyreson, 1996). When interaction is not aggressive, males simply part after meeting or with a slight abdominal flicking (Terry & Dyreson, 1996) but when aggressive interaction occurs, usually at the mating sites, it is characterised by flipping the abdomen, grabbing and flicking the abdomen of the male opponent (Terry & Gardner, 1990; Terry, 1995; Terry & Dyreson, 1996). Males involved in escalated fights chase other males away from a particular area (Terry & Dyreson, 1996) and such escalated fights may last for up to 20 minutes (Terry & Dyreson, 1996). Terry (1997) suggested that a male involved in an escalated fight may be clearing a small area where females can land since these fencing interactions occurred when females were not present (Terry & Gardner, 1990). Such behaviour could involve pheromone-marked areas where females are more likely to land within the aggregation (personal communication, W. D. J. Kirk) (Terry & Dyreson, 1996). Males vary in size and those that possess larger legs and wider abdomens appear to fight better (Terry & Dyreson,

1996). Escalated fighting is density dependent (Terry, 1995). In a laboratory experiment, during a 10 minute observation period, fighting peaked at an intermediate density of 8-10 males in a 9 mm diameter arena (Olaniran, 2013). Male-male interaction leading to abdominal flicking was also observed in *F. schultzei* (Milne *et al.*, 2002).

1.7 Pheromone identification in thrips

Pheromones are chemicals secreted externally by an organism to send information to members of the same species and are used extensively by insects to communicate with each other (Welter *et al.*, 2005). The first identified pheromone in thrips was the alarm pheromone observed in *F. occidentalis*. The second instar larvae produce an anal droplet containing an alarm pheromone consisting of decyl acetate and dodecyl acetate (Figure 1.2 and 1.3), produced as a response to predator attack (Teerling *et al.*, 1993b; Teerling, 1995**)**. The alarm pheromone increases alertness and vigilance in thrips larvae (de Bruijn *et al.*, 2006), and it decreases landing and increases take off in *F. occidentalis* (MacDonald *et al.*, 2002). The larvae produce the alarm pheromone in a volume and ratio that changes with age of the larvae and could therefore be exploited by predators with preference for a specific larval age (MacDonald *et al.*, 2003).

Aggreration pheromone has been identified in *F. occidentalis*. Two compounds, (*R*)-lavandulyl acetate and neryl (*S*)-2-methylbutanoate (Figure 1.4 and 1.5 respectively) collected by solid-phase microextraction (SPME) and gas chromatography/mass spectrometry (GC/MS) in the headspace volatiles of adult male *F. occidentalis* (Hamilton *et al.*, 2005) were absent in the females (Kirk & Hamilton, 2004). Neryl (*S*)-2-methylbutanoate by itself attracted both sexes of *F. occidentalis* while (*R*)-lavandulyl acetate did not, instead it reduced trap catch in the field (Hamilton *et al.*, 2005); thus neryl (*S*)-2-methylbutanoate

was identified as the aggregation pheromone in *F. occidentalis* (Hamilton *et al.*, 2005). This was the first identified aggregation pheromone in the order Thysanoptera. The role of (*R*)-

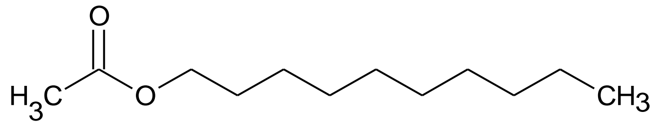

Figure 0.2 Molecular structure of decyl acetate

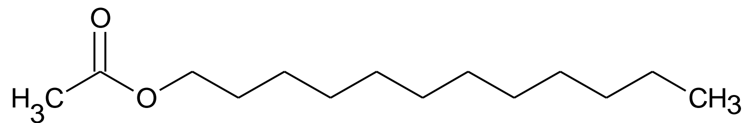

Figure 0.3 Molecular structure of dodecyl acetate

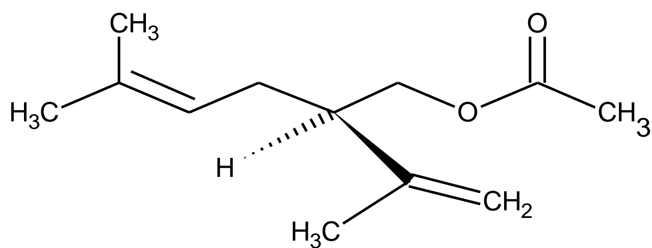

Figure 0.4 Molecular structure of (*R*)- lavandulyl acetate

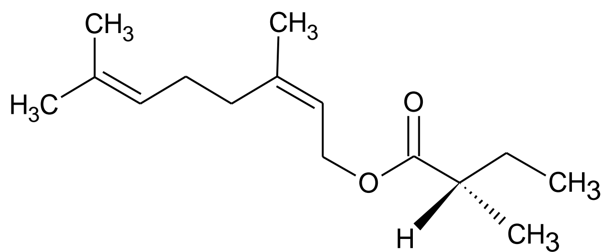

Figure 0.5 Molecular structure of neryl (*S*)-2-methylbutanoate

lavandulyl acetate however still remains unknown. Apart from the evidence that (*R*)-lavandulyl acetate reduced trap catch in the field (Hamilton *et al.*, 2005; Sampson, 2014), it has also been shown in a laboratory bioassay to alter activity levels of male and female *F. occidentalis* (Olaniran, 2013). Exposure of thrips to (*R*)-lavandulyl acetate, reduced the activity level in female *F. occidentalis,* but increased it in males (Olaniran, 2013). Olaniran (2013) therefore suggested that (*R*)-lavandulyl acetate may play a role in male fighting or that an adult male *F. occidentalis* may be using the compound as an adult female arrestant during mating.

Similarly, the same chemical compound identified in male *F. occidentalis* (neryl (*S*)-2-methylbutanoate and (*R*)-lavandulyl acetate) has been identified as an aggregation pheromone in male *F. intonsa* (Zhang *et al.*, 2011; Zhu *et al.*, 2012). Differences in ratio between the two compounds has been claimed to play a role in interspecies recognition between *F. occidentalis* and *F. intonsa* (Zhu *et al.*, 2012). However, while both neryl (*S*)-2-methylbutanoate and (*R*)-lavandulyl acetate were claimed to be part of the aggregation pheromone in *F. intonsa* (tested only with male- and female-produced odour and not synthetic compounds)*,* (*R*)-lavandulyl acetate has not been observed to be part of the aggregation pheromone of *F. occidentalis* in both field and laboratory experiments (Hamilton *et al.*, 2005; Olaniran, 2013; Sampson, 2014).

In *T. palmi*, the aggregation pheromone was identified as a monoterpene pentenoate ester, (*R*)-lavandulyl 3-methyl-3-butenoate, collected from the headspace volatiles of males (Akella *et al.*, 2014). This identified male-produced compound is similar to those observed in *F. occidentalis* and *F. intonsa* though it is only one compound. The absence of a second compound may be associated with differences in mating behaviour such as aggressive

behaviour observed in *F. occidentalis* (and *F. intonsa*) but not in *T. palmi* (Kirk & Hamilton, 2009). The rate at which (*R*)-lavandulyl 3-methyl-3-butenoate increased trap catch in both female and male *T. palmi* (62% females, 33% males) (Akella *et al.*, 2014) is similar to the proportion of increase detected with traps bated with neryl (*S*)-2-methylbutanoate for *F. occidentalis* (54% females, 45% males) (Hamilton *et al.*, 2005) suggesting that it can be of potential use in pest management.

The only contact pheromone presently identified in the order Thysanoptera, is 7-methyltricosane ($C_{24}H_{50}$) (Figure 1.6). It was extracted from the cuticle of male *F. occidentalis* though traces were also found in females (Olaniran *et al.*, 2013). This compound, which is present in on adult males (198 pg male^{-1}), was also extracted in almost equal amount on a substrate exposed to male thrips for about 5 h (Olaniran *et al.*, 2013). Considering the metabolic cost associated with production of pheromone, it has been suggested that 7-methyltricosane may play an important role in mate recognition, mediating male-male fighting or may be involved in mating behaviour (Olaniran *et al.*, 2013).

Two dibasic esters (dimethyl adipate and dimethyl glutarate) were extracted from the head and thorax region of male *E. americanus.* Dimethyl adipate acted as an antiaphrodisic pheromone in *E. americanus* and was extracted on from males and mated females but not on from virgin females (Krueger *et al.*, 2016).

Adult males of most species of thrips contain sternal pore plates, that are possibly associated with pheromone production (Bode, 1978; El-Ghariani & Kirk, 2008; Mound, 2009; Krueger *et al.*, 2015b). These pore plates are found in more than 60% of the genus of the family Thripidae (Mound, 2009), where all the male produced aggregation pheromones have been identified. However, the source of biosynthesis of the only identified antiaphrodisiac pheromone cannot be the sternal gland found on the abdomen since the pheromone was

observed on the head and thorax region of adult males (Krueger *et al.*, 2016). Sex pheromone has not been found in thrips yet.

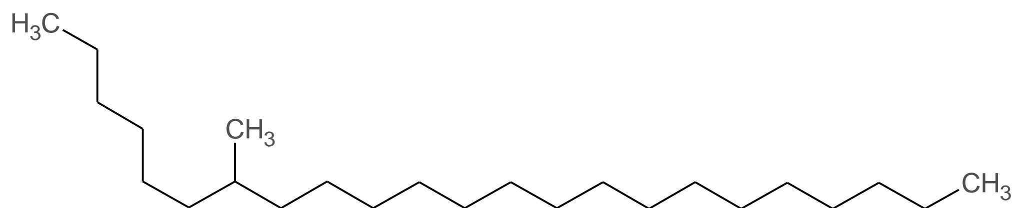

Figure 0.6 Molecular structure of 7- methyltricosane

1.8 Use of pheromones in integrated pest management of thrips

Due to the dangers associated with the misuse of insecticides in pest management, such as potential health, environmental and ecological problems, there is an increasing interest in the use of alternative potentially safe means of pest management. Furthermore, evolution of insecticides means that new solutions are urgently needed. Where it is difficult to eliminate completely the use of chemicals, the best approach is to combine chemical use with other management strategies such as biological and cultural control methods. Pest management can incorporates programmes like the use of pheromones and sterile insect techniques (Boake *et al.*, 1996). However, to effectively use these methods, a good understanding of the thrips mating system is important (Carde & Minks, 1995).

The alarm pheromone, dodecyl acetate, has been suggested for use in the integrated pest management of *F. occidentalis* (MacDonald, 2002). The addition of this alarm pheromone, enhanced the efficacy of insecticides (fipronil and maldison) against *F. occidentalis*, resulting in significant increase in larval mortality and is therefore a potential synergist in insecticide control (Cook *et al.*, 2002). The use of alarm pheromone in pest management may also be utilised as a synergist in biological control since predators, which are potential

biological control agents, such as *Amblyseius cucumeris* and *Orius tristicolor* can use the alarm pheromone as a kairomone for finding prey (Teerling *et al.*, 1993a).

The aggregation pheromone of *F. occidentalis*, neryl (*S*)–2–methylbutanoate attracts both sexes (Hamilton & Kirk, 2003) and was patented for monitoring/controlling *F. occidentalis* (Hamilton & Kirk, 2003). It is available commercially as Thripline ams, (Syngenta Bioline, Clacton, UK) and it has been used as a lure to augment the effectiveness of sticky traps in the field (Hamilton *et al.*, 2005; Gómez *et al.*, 2006; Sampson, 2014). Thripline-ams, increased the effectiveness of traps in catching *F. occidentalis* by up to 300% in orchard in western Australia when compared with unbaited traps (Broughton & Harrison, 2012). Application of synthetic aggregation pheromone (neryl (S)-2-methylbutanoate) to blue sticky roller traps approximately doubled the trap catch of *F. occidentalis* in semi-protected strawberry (Sampson & Kirk, 2013). A similar increase in trap catch was also observed in tomatoes and sweet pepper planted in a greenhouse (Gómez *et al.*, 2006). The advantage of using pheromone-baited mass trapping in *F. occidentalis* pest management is that it is species-specific thus reducing its impact on other non-target species and is also considered environmentally safe (Sampson & Kirk, 2013). The potential use of aggregation pheromone for pest management in thrips has been reviewed (Kirk, 2017).

1.9 Aims of study

Studies of reproduction and mating behaviour play an important role in answering questions about the biology and relationships within insect species. Such studies may also help in developing safer methods for the control of problematic insects such as in the use of sex pheromone baited traps in insect pest control.

Frankliniella occidentalis is polyphagous and is an important pest of many crops throughout the world (Kirk, 2002; Kirk & Terry, 2003). Therefore, research is needed to improve our

understanding of this insect in order to develop an effective integrated management system for it. Previous study has analysed the copulation behaviour of *F. occidentalis* but this was before the identification of any pheromone in adult thrips. To properly understand the copulation processes in *F. occidentalis*, this study investigated and analysed the activities involved in the pre-copulation, copulation and post-copulation of adult male and female *F. occidentalis* and the possible role of mating pheromone. It also investigated the copulation strategies and variation associated with copulation in male and female *F. occidentalis*.

The hypothesis tested include:

1. Pheromones are involved during recognition and copulation in *F. occidentalis*
2. Female *F. occidentalis* copulates multiple times.
3. Male and female *F. occidentalis* make choice based on copulation status

The specific objectives were to:

1. determine the sequences of activities and behaviour during the pre-copulation, copulation and post-copulation phases in virgin male and virgin female *F. occidentalis*.
2. identify behaviours that may predict pheromone involvement during copulation in *F. occidentalis*
3. identify the possibility of multiple copulation in female *F. occidentalis*
4. study possible male and female choice based on copulation status
5. attempt to identify the pheromones that may be involved in male assessment of female copulation status and species recognition by males.

Chapter 2

General methods

2.1 Introduction

General methods are those that were common to more than one of the experimental chapters. Details of some of the equipment used during the research are also given. These methods are presented here to avoid repeating the same method in different chapters; instead references were made to this chapter when relevant. The methods described in this chapter include thrips mass rearing; rearing of virgin and known-age male and female *F. occidentalis*, methods of collecting thrips from the culture, microscopy and video recording of thrips.

2.2 Rearing of *F. occidentalis*

Insect rearing is fundamental to any insect-related experiments done in the laboratory. Rearing sufficient numbers of thrips in the laboratory can be achieved using different methods and the method used depends on the purpose of the research (Loomans & Murai, 1997). Thrips are reared in the laboratory for several reasons including the study of their life history (Gaum *et al.*, 1994; Zhang *et al.*, 2007), potential control with natural enemies (Blaeser *et al.*, 2004), pesticide toxicity (Morse *et al.*, 1986) and insecticide resistance (Thalavaisundaram *et al.*, 2008), virus transmission related studies (Ullman *et al.*, 1992), behavioural and pheromone studies (Terry & Schneider, 1993; Kirk & Hamilton, 2004;

Milne *et al.*, 2007; Rafter & Walter, 2013). Rearing has been successfully done on whole plants such as chrysanthemum, portions of the plant (leaf discs, fruits and pods) and on artificial diets such as honey, sugar solutions, pollen and leaf powder (Loomans & Murai, 1997).

There are three important factors to consider for proper rearing of thrips in the laboratory

1. The quality of the food that can both provide the required nutrient and can be utilised as an adequate oviposition site by the thrips.
2. The cage used must provide adequate ventilation so as to prevent condensation but must also be thrips-proof in order to prevent thrips escape. Therefore, in designing the ventilation vent, the small size of thrips (some less than 2 mm) must be considered.
3. The rearing relative humidity must be high (between 60-90%) and the temperature should be optimal for thrips development. A temperature between 24 and 27°C has been successfully used in Keele University.

Mass rearing of mixed age and mixed sex thrips is better achieved when rearing is done on a whole plant, because it can last longer and the desired number of thrips can easily be generated and this is often called a stock culture. Stock culture involves rearing of mixed-sex and mixed-age thrips and insects from the stock culture can be used for any bioassay that is not age-specific. This method of rearing on whole plants (using flowering pot chrysanthemums) has been adopted for maintaining populations of *F. occidentalis* in Keele University laboratory continuously for over a decade (Kirk & Hamilton, 2004; Olaniran *et al.*, 2013). Flowering pot chrysanthemum is suitable for rearing because thrips do well on this plant and the plants are available in most supermarkets.

When rearing is for the purpose of getting individual, known-age, or synchronised cohorts of larvae and adult thrips, rearing can be done on plant parts such as fresh beans (*Phaseolus vulgaris* L.) (Bryan & Smith, 1956; MacDonald, 2002; Kirk & Hamilton, 2004; Koschier *et al.*, 2007; Li *et al.*, 2011), leaves and leaf discs (McDonald *et al.*, 1997; Egger *et al.*, 2014; Egger & Koschier, 2014). Tea pollen (*Camellia sinensis*) and pine pollen (*Pinus thunbergii*) have been used for egg production and rearing of larvae respectively (Murai & Loomans, 2001), however the problem with the use of pollen is the difficulty of collecting a sufficiently large amount (Murai & Loomans, 2001). To rear thrips of the same age, the time allowed for oviposition on the substrate is often reduced, therefore, pollen grains can be added to increase fecundity and hence aid the number of eggs laid within that period (Teulon, 1992).

2.3 Conditions in the rearing room

The rearing room was a temperature-controlled room dedicated to thrips culture and bioassay. The room had only one door for both entrance and exit and did not have a window. It was therefore very easy to monitor the temperature and humidity of the rearing room, which was necessary because any slight change in temperature could affect thrips development. To control the temperature, there was an in-built heating system in the room. The heater had a thermostat regulator (Allen-Martin, UK) attached to the wall of the room so as to regulate the temperature as required. To monitor the temperature in the rearing room, thermo-hygrometers, DRT 880 (Digitron, Torquay, Devon, UK) were used, they were placed in the cages and the bioassay tables. The temperature of the rearing rooms was maintained at 25 ± 2°C.

2.4 Maintenance of a stock culture in rearing cages

The strain of *F. occidentalis* used for this work has been maintained as a mixed-age and mixed-sex colony in Keele University from a culture collected from Horticulture Research

International, Warwick University in 1997 (MacDonald, 2002). The colony is maintained on pot chrysanthemum, placed in three different rearing cages (see 7.4.1) and up to six cages when there is high demand for thrips. Each of the cages contains four pots of chrysanthemum with each pot in turn a week older than the other. One new chrysanthemum pot plant, purchased from commercial stores, was added every week to each of the cages while the oldest was discarded every week. Usually, at the end of the fourth week, the oldest plant having dried out will cause all surviving thrips to move to the newer plants. Plants were rotated in the cages in order of their ages in such a way that the oldest plant was always in the same position and could easily be identified and removed from the cage.

2.5 The rearing cage

As mentioned in the introduction (2.2), rearing cages must provide adequate ventilation, temperature, humidity and must be thrips proof. The cages (height 600 mm × width 430 mm × depth 430 mm) used at Keele University were specially constructed with Perspex (Rubberfast Ltd., Fenton, UK) (Figures 2.1 and 2.2). The four sides of each of the cages were made with transparent Perspex while the top was covered with a UVA-transmissible plastic sheet so as to mimic a more natural daylight necessary for proper thrips development. The transparent Perspex in the front wall of each cage (370 × 540 mm) was removable, providing access through which flowers were replaced and thrips could be collected. The front panel had two vents (84 × 84 mm) integrated so as to maintain constant relative humidity while the back panel had a 12 V DC, 0.8 W rotating fan (80 × 80 mm), (Papst-Motoren, St. Georgen, Germany) attached to it. The fans provided air circulation in the cages. The constant air circulation and ventilation in the cages helped to prevent condensation that could kill thrips (thrips can drown in the condensed water) and also to prevent microbial growth on the plants placed in the cages. The base of the cage was not

Figure 2.1 Front view of the rearing cages containing pot chrysanthemum used for mass rearing of *F. occidentalis*

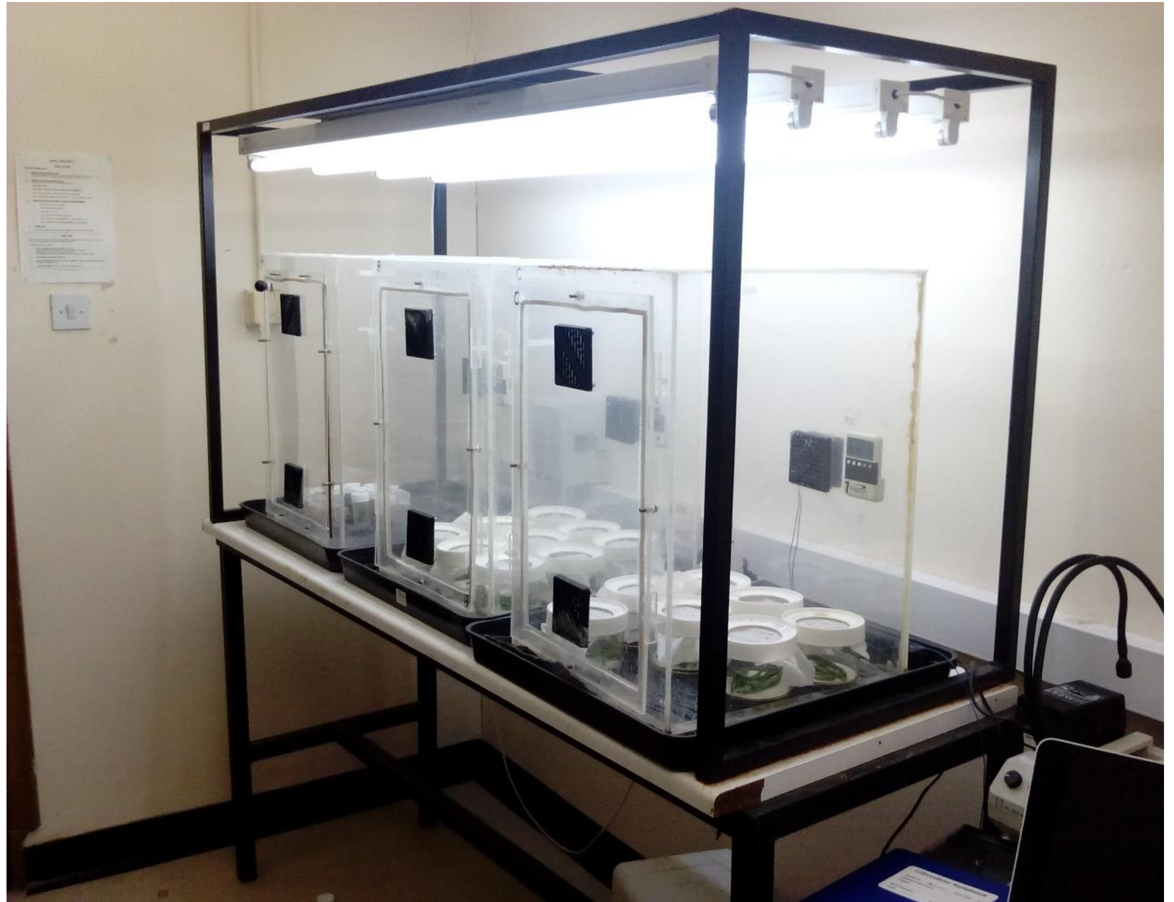

Figure 2.2 Oviposition and rearing pots arranged in three cages and maintained at 65±15% RH, 26±2ºC and 18:6 L:D

covered but was placed directly on a double layer of capillary matting (Vattex Black, Berrycroft Stores Ltd., UK). The capillary matting was cut to a considerably larger size than the area of the base of the cage (about 540 x 540 mm) and was placed as matting in a 540 × 540 mm black plastic tray (Figure 2.1). The essence of this arrangement was to allow for easy watering without having to open the cages. The watering could be done from the matting extension; the mat absorbed the water through into the cages so that plants had access to it. Constant relative humidity and temperature are vital in thrips culturing and this arrangement also helped to maintain the required temperature and humidity. Watering also helped to keep the humidity constantly high. Watering was usually done once every two days to keep the matting wet but not waterlogged. The relative humidity in the rearing cage was maintained at 65±20 % while the temperature was typically maintained at 25±2°C because any higher temperature than this was not conducive for the chrysanthemum plant survival.

2.5.1 Lighting

Rearing was under a fluorescent lighting regime automated using a switch socket**.** Four fluorescent tubes (58 W Sylvania Activa 172 professional, 1500 m length and 26 mm diameter, 240 V AC; Sylvania Lighting International, West Yorkshire, UK in 4 strips) mounted horizontally on top of the rearing cages and the bioassay table at a height of about 78 cm above the bench provided illumination for the rearing and bioassay work. A photoperiod of L16:D8 was used because this simulates the lighting situation in the field during the summer when the population of thrips builds up. Except when bioassay recording involves switching off the light manually during experiments, the light was automated, switching on at 05:00 and off at 21:00 GMT. This lighting regime has previously been

adopted by other authors (Terry & Schneider, 1993; de Vries *et al.*, 2004; O'Leary, 2005) and is suitable for rearing thrips in the laboratory.

2.6 Rearing known-age virgin adult males and females

In some experiments, it was important to know the copulation status and age of male and female *F. occidentalis*, therefore another strategy was employed in rearing to meet these requirements. The technique used for known-age rearing by MacDonald (2002) was modified and used in this study. Rearing of known-age virgin males and females involved two important steps: The first step involved rearing from egg to larval or pupal stages while the second step involved isolation of individual larvae or pupae and rearing them individually to adult stage in order to ensure their virginity at adulthood.

2.6.1 The culture pots used for the first-step rearing (oviposition and rearing pots)

The culture pots used for oviposition and rearing were clear plastic with a screw-on lid (500 ml, height 7.6 cm, diam. 11.3 cm, Nalgene Company, USA). The lid had a large hole cut at the centre (diam. 8 cm). The hole provided aeration and prevented condensation in the pot. Four filter paper discs (QL 100, 90mm diameter, Qualitative, Fisher Scientific, UK) were arranged on the base of the pots. The filter paper discs served to provide potential pupation sites for the second instar larvae in the rearing pot. The filter papers were also added to the oviposition pots for uniformity. To prevent thrips from escaping through the holes in the lid of the pots, the pots were covered with a piece of double layered Kimtech wipe (Kimtech Science, Kimberly-Clark Professional, UK) which allowed air and light to filter through but prevented thrips from escaping.

2.6.2 First step (rearing from egg to larval or pupal stage)

This stage involved oviposition and rearing of a cohort from eggs to larvae, in order to have similar age. Approximately 100-150 mixed-age and mixed-sex adult *F. occidentalis* were

collected from the stock culture maintained at Keele University (described in 2.4 above) by gently shaking the head of the chrysanthemum flower directly into a white plastic collection bowl. A modified standard aspirator (E713 'Pocket Pooter', Watkins & Doncaster, Kent, UK) was then used to aspirate thrips from the plastic dish and introduce them into the oviposition pot containing four bean pods. The bean pods were regularly purchased from supermarkets. Usually, the origins of these beans were either from Kenya, Egypt or France. The quality of the bean pods was ascertained when purchased from the supermarket by checking the expiry date, after which the bean pods were washed in a soft detergent solution (Teepol) and rinsed in water so as to get rid of any insecticide residue that might be on the bean pods and to also kill any insect eggs that might have been laid on the pods. The bean pods were kept in the refrigerator until needed. Usually, beans were not used after the best before date so as to maintain the quality of the bean pods. Pine pollen (usually *Pinus sylvestris*) was scattered around the bean pods in the pots. The inclusion of pollen in the diet of *F. occidentalis* was to increase egg production (Kirk, 1985c; Trichilo & Leigh, 1988). The pine pollen used for the experiment had been stored in the freezer for some years. Pine pollen can remain viable for many years in a freezer (W. Kirk, pers. comm., 2015).

The adult thrips introduced into the oviposition pots were allowed to lay eggs on the pods for about 24 hours after which the bean pods were gently removed from the oviposition pot and transferred into the rearing pots and the date was recorded. Any adult thrips holding onto the pods were dislodged from the pods by striking the pods against the wall of the oviposition pot. Four new pods were introduced into the oviposition pot to replace the ones removed and a similar process continued until the number of eggs laid by the thrips reduced significantly (usually after the fourth or fifth time) after which the culture was discarded for fresh adults. Pollen scattered around the subsequent bean pods after the first one was reduced to about half to allow for the pollen that was already in the pots. At about 7-8 days, second-instar

larvae were already developing and the pods were drying out, therefore three new pods were added into the rearing pots to maintain the quality of the food (Table 2.1). Occasionally, when virgin females were available in abundance, they were used for rearing virgin males using a similar rearing procedure as described above, since virgin females usually lay only male eggs.

Both the rearing and oviposition pots were placed in a similar cage to those used for stock culture maintenance (Figure 2.2). When the fans on the rearing cage (7.4.1) were on, the relative humidity level reduced in the cage to about 50%, therefore the fan was off throughout the period of the rearing to maintain a high humidity (Figure 2.2). The relative humidity in the rearing cage was maintained at about 65±20 % but could be up to 94% when the light was off and temperature dropped slightly at night. The temperature was maintained at 26±2 ºC. The matting was watered regularly to maintain high humidity. The humidity in the cage was monitored with a thermo-hygrometer, DRT 880 (Digitron, Torquay, Devon, UK) placed close to the rearing pots and the whole arrangement was placed under the lighting system with photoperiod of L16:D8, as for the stock culture on pot chrysanthemum.

2.6.3 Second step (rearing from larvae or pupae to adult stage)

At this stage, individual immature thrips were picked and isolated in order to ascertain their mating status. While all females used were picked strictly at the larval stage, males were picked at larval, pro-pupal and pupal stages. This was to avoid possible copulation of females at the pupal stage. Male pupae were generally smaller and thinner than females. At about 9-11 days after the introduction of pods with eggs laid into the oviposition pots, second-instar larvae and male propupae or pupae were picked from the pods (Table 2.1), layers of filter papers, the bottom of the pots and in-between the double layered Kimtech wipe (used to cover the lid of the pots) using a slightly moistened paint brush, trimmed to just a few

Table 2.1 Developmental stages (egg to pupa) of *F. occidentalis* reared on bean pods at 65±20% RH and 26±2ºC

Days from start	Action	Time from oviposition (days)	Time from hatching (days)	Developmental stage
0	Six bean pods introduced into the oviposition pot containing usually about 150 mixed-age and mixed-sex adult thrips			
1		0-1		Egg
2	Bean pods in the oviposition pot cleaned of adult thrips and transferred into the rearing pot	1-2		Egg
3		1-3		Egg
4		1-4	0-1	Newly hatched larvae I on pods
5		4-5	1-2	Mature larvae I on pods
6		5-6	2-3	Larvae II on pods
7 -9	New pods introduced into the rearing pots	5-7	3-4	Larvae II on pods with a few on the filter papers
9-11	Larvae and male prepupae or pupae were removed and were also removed and placed individually in the modified microcentrifuge tube containing small portions of pods and pollen	8-10	6-7	Most larvae II on the filter papers and pods some within the double layered ply cover. Some prepupae or pupae also present
12	Check for adult	10-12	8-9	Larvae, propupae and pupae or adult
13	Check for adult	11-13	9-10	Propupae, pupae or adults
14	Check for adult	12-14	10-11	Usually pupae or adults
15	Check for adult	13-15	11-12	Fewer new pupae remaining and adults

bristles. This was done very carefully to prevent injury and they were introduced into a separate modified microcentrifuge tube (1.5 ml, Life Sciences International, or Starlab, UK) so as to ensure their virginity as adults. A hole of about 4 mm diameter was melted into the lid of the microcentrifuge tube to provide aeration. A pod was cut diagonally into about 25 pieces using a pair of scissors. They were cut this way to prevent as far as possible immature thrips from hiding inside the small portions of pods. A little pollen was scattered on these small pod portions and was evenly mixed with the portions of pods. Each of the small portions was placed into the modified microcentrifuge tube containing the isolated immature insect or vice versa. In order to prevent thrips escape via the melted hole, a piece of precision wipe paper about 2 cm by 2 cm (Kimwipes Lite, Kimberly-Clark, UK) was folded and placed over the mouth of the tube before closing the lid. All the tubes containing the isolated insects were placed in a plastic pot without lid and put inside the cage. The propupae and pupae were checked for adult emergence daily while the larvae were left for about 2-3 days after isolation before doing a daily check for adult emergence. The microcentrifuge tubes were sufficiently transparent to confirm emergence of adults and their sexes using the naked eye without having to bring out the insects, but when in doubt, they were further confirmed under the dissecting microscope. The small portion of pod was replaced after the adult had emerged and newly emerged adults were placed in a container and labelled with the appropriate date of emergence and the sex to avoid confusion. Newly emerged adult thrips were also placed inside the cage until they were needed for the experiment.

2.7 Collection and handling of thrips

Adult *F. occidentalis* are very delicate and small and are often very active; collection has to be done very carefully. Collection from the stock culture was done by gently shaking the head of the flower directly into a white plastic collection bowl. Thrips were aspirated from the plastic dish using a modified standard aspirator (E713 ‘Pocket Pooter’, Watkins &

Doncaster, Kent, UK). The aspirator was modified by connecting it to a blue graduated pipette tip (101 – 1000 µl) (StarLab, Blakelands, Milton Keynes, UK) trimmed at the tip to provide an opening of about 1.5 mm diameter which made sucking up of thrips very easy. A piece of bridal veil material (fine cotton mesh) was tied to the base of the suction tube (John Lewis, Cambridge, UK) to prevent inhalation of thrips during suction. Thrips were then collected into a soda glass aspirator vessel (length 50 x 25 mm diam.) (Scientific Glass Laboratories Limited, Hanley, UK). When picking larvae from the rearing pots, the larvae were sometimes tapped onto a plain sheet of paper to make them visible and a slightly moistened brush trimmed to just a few bristles often damped on the paper was used to pick up individual thrips.

2.8 Cleaning of bioassay apparatus

The materials used for the experiment were washed in a solution of Teepol and were subsequently rinsed in tap water. Glassware was also rinsed with distilled water, dried with acetone (99+ % purity, Fisher Scientific Supplies, Loughborough, UK) and left in the laboratory oven overnight at about 180°C but materials made from rubber or plastic were not placed in the oven.

2.9 Arena technique for copulation behaviour study

The technique used for pollen-feeding by Kirk (1987) and adopted by Olaniran & Kirk (2012) was modified to carry out this study. A piece of toughened dental modelling wax (e.g. Anutex, www.kemdent.co.uk) was cut into small pieces (30 mm long, 20 mm wide, and 1.5 mm thick) and a 5mm diameter hole removed from the centre with the aid of a cork borer (C70-410 No. 5 Orme Scientific, Manchester, UK) (Figure 2.3). This hole served as the arena for the observation of thrips copulation behaviour. The piece of 'arena wax' was sealed onto the middle of a microscope slide (76 mm long, 26 mm wide Fisherbrand, UK)

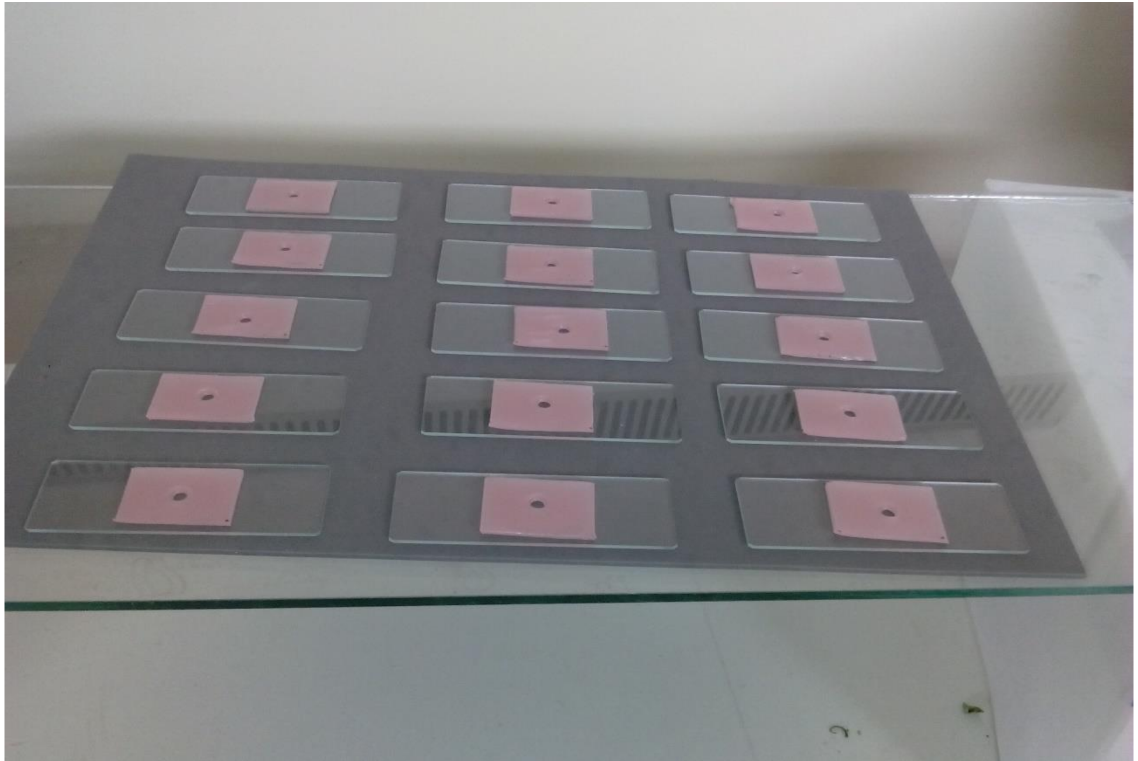

Figure 2.3 Fifteen mating arenas prepared from pieces of modelling wax sealed onto the middle of a microscope slide made ready for copulation bioassays

by a gentle warming of the slide on a flame for about 3 seconds in order to soften the wax. The wax was then pressed against the slide to secure a firm seal and thus prevent thrips escape. The slide served as the floor of the arena. During observation, the arena was covered with a glass cover slip (usually about 19 mm diameter, 0.09-0.13 mm thick, Fisherbrand, UK) gently pressed into the wax to form a transparent roof.

2.10 Video recording

The viewing was done under a camera mounted on a dissecting microscope (Wild M5A Heerbrugg) set at 60x magnification or a Zeiss Stemi 508. The camera body (Canon EOS 70D) had an adjustable LCD monitor which displayed the image from the arena. The microscope was focused to obtain a sharp image on the LCD of the camera before filming began. The camera displayed the recording time on the LCD which was used to monitor the 10 minute duration for each of the observations. The microscope illuminator (Schott, KL 1600 LED) was a fibre-optics cold-light source set to give a light that was sharp enough to display the image on the camera. It had two flexible light guides that projected at an angle of about 45° each from the arena and raised at about 40 mm length away from the arena. Only one of the flexible light guides was used at a time. The arena was placed under the microscope and viewed directly on the LED of the video camera. The video was stored on a 16, 32 or 64 GB SD memory card. After filming, the data were copied from the memory card to a hard disc and the video analysis used VLC media player (VideoLAN Team, Paris).

2.11 Statistical analysis

All analyses used *t*-test and one-way ANOVA or their non-parametric equivalents, the Mann-Whitney test or the Kruskal-Wallis test (Minitab 17, Minitab Inc., USA). When the non-parametric Kruskal-Wallis test was done, multiple comparisons were compared using multiple comparison Holm’s adjusted modification of Bonferroni procedure *P*-values as

suggested by Wright (1992) after multiple Mann-Whitney tests. This was carried out with computer programme, WINPEPI (http://www.brixtonhealth.com/pepi4windows.html). The chi-square test was used for differences in the proportion of individuals. Chi-square was used with exact probability in order to allow for small sample size using IBM SPSS Statistics 21 (IBM Corp., New York).

Chapter 3

Copulation behaviour of virgin western flower thrips

3.1 Introduction

3.1.1 Sex recognition and copulation duration in insects

Sex recognition is vital during courtship and copulation behaviour in insects. Sex recognition in insects may involve visual, tactile, auditory or acoustic and chemical senses. Chemical attractants such as pheromones are employed in attraction of the opposite sex over long or short distances and these attractants play a vital role in mate recognition in insects (Engelmann, 1970; Johansson & Jones, 2007). Apart from using chemical cues from a distance, cuticular hydrocarbons found on the surface of an insect's body also play a role in recognition and as sex attractants in some insects (Singer, 1998). Acoustic sex recognition involving the use of sound has been studied in important insect orders such as in the order Orthoptera (von Helversen & von Helversen, 1997; Robinson & Hall, 2002) and Diptera (Gibson & Russell, 2006; Cator *et al.*, 2009; Warren *et al.*, 2009). Olfactory and visual cues were shown in males to jointly influence short-distance orientation towards a black-coloured rod treated with female extract in *Anoplophora malasiaca* (Fukaya *et al.*, 2004). The mechanism of sex recognition has not been clearly demonstrated in the order Thysanoptera,

but there is evidence that it may involve the use of chemical and visual cues (Kirk, 1985a; Milne *et al.*, 2007) and this is discussed further in 3.2. Sex recognition and successful courtship is usually followed by copulation, though strangely, in many species of insects, courtship also occurs during and after copulation (Eberhard, 1994).

Copulation in insects may sometimes be very brief, between 8-42 s in *Hoplothrips karnyi* (Crespi, 1988b) and can sometime be very complex lasting for hours and even days in some species (Cordero, 1990; Carroll, 1991; Schöfl & Taborsky, 2002). Long copulation duration suggests that copulation in these species goes beyond just the transfer of sperm; especially because male and female insects could benefit from brief copulation. Brief copulation will afford both sexes more opportunity to invest in other important activities (Thornhill & Alcock, 1983). Prolonged copulations may however be serving other important purposes in males such as mate guarding (Schöfl & Taborsky, 2002), displacement of sperm of rivals (Córdoba-Aguilar *et al.*, 2003), transfer of additional materials such as seminal fluid, spermatophores and mating plugs during copulation (Thornhill & Alcock, 1983).

3.1.2 Sex recognition and initial antenna contact in thrips

Antennae appear to play an important role in insect copulation. Lewis (1973), stated that there are sense cones on the antenna of thrips that help sexes find each other. Antennal signalling between a male and a female occurs at different times in different species of thrips. In most species of thrips, copulation does not begin until a male makes antennal contact with the female, for example in *Frankliniella occidentalis* a male approached and contacted the female head, thorax or abdomen with his antennae before proceeding to mount the female (Terry & Gardner, 1990). Similarly, in *H. karnyi*, prior to the male mounting the female's back, inserting his aedeagus and copulating, the male made antennal contact with the female (Crespi, 1988b). However, antennal contact in *Scirtothrips aurantii* does not directly lead to

mounting, rather after contact males and females S. aurantii engaged in a rhythmic antennal bout (movement of the two antennae by the pair without making contact even though they were close) and this behaviour continued until the male eventually tapped the female's antennae with his antennae *(Rafter & Walter, 2013)*. In a laboratory study of copulation behaviour in S. aurantii, this rhythmic antennal movement before contact was associated with successful intromission (Rafter & Walter, 2013). Antennal contact or visual cues may therefore play a role in sex recognition and courtship behaviour in thrips.

In some species such as *F. schultzei* (Milne *et al.*, 2007), sex recognition seemed to occur before antennal contact. A laboratory study of precopulatory behaviour showed that a virgin female *F. schultzei responded to a virgin male's presence by increasing her movement prior to contact. She also turned around and vibrated her wings. The female approached the male first, while the male increased his activity, groomed his abdomen with the legs and flicked the abdomen* (Milne *et al.*, 2007). *Male Thrips major* and *Thrips fuscipennis* also appear to detect a female just a few millimetres away from each other (Kirk, 1985a).

Aggregation observed in male thrips and the production of aggregation and contact pheromones in male thrips may also suggest that long and short range cues are involved in species and sex recognition in Thysanoptera (Kirk, 1985a; Milne *et al.*, 2002, 2007; Hamilton & Kirk, 2003; de Kogel & van Deventer, 2003; Kirk & Hamilton, 2004; Hamilton *et al.*, 2005; Olaniran *et al.*, 2013).

3.1.3 Copulation in thrips

Copulation in thrips occurs between an adult male and adult female, although an uncommon form of copulation has been reported between an adult male and a female prepupa in *Limothrips denticornis* (Bournier, 1956a; Lewis, 1973). Copulation usually takes place 2-3 days after pupal eclosion (Lewis, 1973), but males of some thrips species can copulate 6

hours after eclosion (Terry, 1997). The delay in female copulation may be to allow the newly emerged female to feed and mature the reproductive organs for copulation. In *Caliothrips* (*Heliothrips*) *fasciatus* for example, copulation does not usually take place until the emerged adults have fed for 1-2 days (Bailey, 1937).

Males generally copulate multiple times during their adult lifetime (Stannard, 1968; Lewis, 1973), but in females, frequency of mating varies among different species (Terry, 1997). Copulation typically follows the same trend in both Tubulifera and Terebrantia, starting with a male climbing a female, twisting his abdomen around the end of the female's abdomen, attempting to encounter the female vagina and insertion of the aedeagus (Russell, 1912; Lewis, 1973; Crespi, 1986b, 1988b; Ananthakrishnan, 1990; Terry, 1997). However, in *Hoplothrips pedicularius* and *Hoplothrips karnyi* (Tubulifera), after mounting a female, a male turned back, and held the female's abdomen with its fore-legs temporarily before copulation began (Crespi, 1986b, 1988b). This behaviour called 'reverse climb' was considered a way of assessing a female's reproductive condition (whether she has oviposited or not) (Crespi, 1988b). Males of these species have enlarged legs that they use to fight and defend territories around the female oviposition site, and assessment of female oviposition condition may therefore help in male choice of a female since these guarding males were observed to mate more frequently with females that had not oviposited (Crespi, 1988b).

Stannard (1968) suggested that certain appendages and setae may be involved in attaching a male and a female together in position during copulation though there was no evidence to support this. Pelikan (1951) however suggested that sternal glands in male *Pezothrips* (*Taeniothrips*) *dianthi* do not serve as a means of attachment to the female, rather it may be effective as an osmeterium since male sternites do not make contact with the female body during copulation.

Copulation durations in thrips are generally longer in Terebrantia than in Tubulifera (Lewis, 1973) (Table 3.1). This may not be entirely correct in all cases for example, a long copulation of up to 6 hours was observed in *Suocerathrips linguis* (Tubulifera) (Kumm, 2002). However, a short copulation duration of as short as 62 s was observed in the field for *F. schultzei* (Terebrantia) (Milne *et al.*, 2007). Copulation duration of between 1-15 minutes appears typical of the sub-order Terebrantia (see Table 3.1). In *F occidentalis* copulation duration ranges between 2.9-4.1 minutes (Terry & Gardner, 1990; Terry & Schneider, 1993; Terry & Dyreson, 1996; Kumm, 2002).

Table 3.1 Copulation duration of insects of the order Thysanoptera. F=field, L=laboratory

Species	**Copulation duration**	**References**
Tubulifera		
Hoplothrips pedicularius, (F)	7-70 s	Crespi (1986b)
Hoplothrips karnyi (F)	8-42 s	Crespi (1988b)
Ponticulothrips diospyrosi (F)	164 s	Tsuchida & Ohguchi (1998)
Suocerathrips linguis (L)	6 h	Kumm (2002)
Terebrantia		
Caliothrips fasciatus (F)	3-10 min	Russell (1912)
Echinothrips americanus	5.7-16.5 min	(Krueger *et al.*, 2017)
Rhipiphorothrips cruentatus	3-15 min	Ananthakrishnan (1990)
Retithrips syriacus	1-7 minutes	Ananthakrishnan (1990)
Thrips major and *Thrips fuscipennis* (F)	Just over 6 min	Kirk (1985a)
Frankliniella occidentalis (L)	241s and 210 s	Terry & Schneider (1993) and Kumm (2002) respectively
Frankliniella occidentalis (F)	189 s and 176 s	Terry & Gardner (1990) and Terry & Dyreson (1996) respectively
Frankliniella schultzei (L)	145-219 s	Milne *et al.* (2007)
Frankliniella schultzei (F)	62 s and 126 s	Milne *et al*. (2002)
Thrips tabaci (arrhenotokous) (L)	183.3 s	Li *et al.* (2015b)
Thrips tabaci (arrhenotokous) (L) male and thelytokous female)	223.2 s (mostly unsuccessful)	Li *et al.* (2015b)
Scirtothrips aurantii (L)	712 s	Rafter & Walter (2013)
Scolothrips sexmaculatus	8.5-18.3 min (11.9 min average)	Gilstrap & Oatman (1976)
Apterothrips apteris	15 min-over 1 hour (35 min average)	Strauss & Karban (1995)

3.1.4 Antennation and stroking during copulation

Antennation and stroking are two important behaviours that have been observed during copulation (Terry & Schneider, 1993; Milne *et al.*, 2007; Rafter & Walter, 2013). These behaviours usually occur after a male climbs on a female. Antennation after climbing is different from the initial antennal contact before a male climbs a female (see 3.1.1). The reason for these two behaviours is not clear, although in *F. schultzei, a male antennated the female antennae and tapped his front leg on the dorsal part of the abdomen (stroking) until the female stood still* (Milne *et al.*, 2007). This suggests that this behaviour (at least in this species) may be associated with calming the female during copulation. Pelikan (1951) has suggested that a volatile lipoid substance released during copulation in *Pezothrips* (*Taeniothrips*) *dianthi* prevented females from running around as copulation proceeded. This volatile liquid may therefore be applied on the female during antennation or stroking. In a laboratory study of the copulation behaviour in *S. aurantii, a male mounted a female and both antennated each other until the male introduced the aedeagus and then the female stopped antennation while the male continued occasional antennation and he also stroked the female with his metathoracic legs* (Rafter & Walter, 2013). Similarly, in *F. occidentalis*, the male palpated the base of the female antennae with his own antennae and stroked the thorax and abdomen of the female with one of his mid legs during copulation (Terry & Schneider, 1993). This was followed by a relatively calm period of both the male and the female before the couple finally detached (Terry & Schneider, 1993). Although these two behaviours have been largely associated with calming females during copulation, this has not been experimentally confirmed.

Another possible reason for antennation and stroking may be that a male was applying or detection of an antiaphrodisiac pheromone to mark the female during copulation. Male antiaphrodisiac pheromone (dimethyl adipate) has been identified in *Echinothrips*

americanus (Krueger *et al.*, 2016), a species in which antennation and stroking have been reported (Krueger *et al.*, 2017). This compound was found on mated females and males but not on virgin females (Krueger *et al.*, 2016), which implied that this compound was applied by the male during copulation possibly through stroking or antennation. Evidence for an antiaphrodisiac pheromone has also been found in *F. occidentalis* (Chapter 5).

3.1.5 Aims of study

Previous study on the copulation behaviour of *F. occidentalis* (Terry & Schneider, 1993) was before the identification of pheromones in adult *F. occidentalis*. The identification of pheromones in male *F. occidentalis* (Hamilton & Kirk, 2003; Kirk & Hamilton, 2004; Hamilton *et al.*, 2005; Olaniran *et al.*, 2013) has therefore brought to light the important functions pheromones may be playing in the mating and copulation processes in *F. occidentalis*. There is therefore a need to further understand copulation in virgin male and female *F. occidentalis* with the aim of identifying the fundamental roles pheromones may be playing immediately before, during and after copulation. Therefore, the aim of this chapter was to

1. study the sequence of behaviour during pre-copulation, copulation and post-copulation phases in *F. occidentalis* and
2. to identify behaviours that may indicate pheromone use during copulation.

Hypothesis:

1. Activities during the pre-copulation period influences copulation success.
2. Antennation and stroking pays a role in detection and/or application of pheromone during or before copulation.

3.2 Materials and methods

3.2.1 Rearing of adult 3-5 days old virgin males and females

To study the copulation behaviour of virgin male and female *F. occidentalis*, the age and virginity status of adult males and females must be established. To do this, known-age virgin adult males and females were reared using the procedure described in Chapter 2. Newly emerged adult thrips isolated at the larval stage in the modified microcentrifuge tubes were fed individually with small portion of pod with pollen. The date of emergence was written on the pot used for collecting all the tubes containing individual insects that emerged the same day and the sex at emergence was also distinguished to avoid confusion. In all the experiments, 3-5 day old male and female adults were used.

3.2.2 Video recording

An individual adult virgin female was first placed carefully within the arena (see Chapter 2), and covered with a glass cover slip to prevent her from escaping. The glass cover slip was then carefully opened and a virgin male was introduced into the same arena within 5 minutes of introducing the female and the arena was immediately re-covered to prevent the pair from escaping. The arena with the thrips was placed on the stage of the camera- mounted dissecting microscope (see Chapter 2) and recorded for approximately 10 minutes. However, if the pair were still copulating after 10 minutes, they were left until they finished copulation. All experiments were performed at a temperature of 25±1 °C.

3.2.3 Observations

The experiment was carried out between 08:00 and 13:00 h for consistency in behaviour over several days. This was to avoid changes in behaviour that may be associated with time of day. What happened before and during the copulation process was recorded and saved on a SD memory card. The video was later played back to identify and measure the behaviours

displayed in the pre-copulation, copulation and post copulation phases. The timing involved between activities was also measured (Table 3.2). Copulation behaviour was carefully analysed for paired virgin male and female *F. occidentalis* using the descriptions in Table 3.2. Behaviours such as grooming and wing combing before copulation were not considered in this study, as they would not be relevant because they occurred randomly. In addition, grooming and wing-combing at the initial stage by a male and a female in the arena may be due to the moisture on the insect's surface as a result of the moist brush used in the transfer of these insects into the arena. Grooming and wing combing were however measured after copulation. In some of the pairs, some behaviours could not be measured because of difficulty in viewing them under the microscope (e.g., those that copulated on the roof of the arena). Such behaviours were not included in the analysis.

Table 3.2 Definition of terminologies used at pre-copulation and copulation phases

Category	What to measure/Definition
Pre-copulation	
Time to first contact	The time between the introduction of male and female thrips in the arena and time they made the first contact
First contact to end of climbing	Time between male-female first contact and male climbing a female. Male end of climbing is when most of the male is on the back of the female head, thorax or abdomen and is facing the same direction as the female. This was before the male bent his abdomen beneath that of the female.
Initial antenna contact	A male often made contact with a female using his antennae before climbing the female
Bending abdomen	This is a process by which a male curled his abdomen under that of the female.
Orientation at first contact	The relative position of adult male and female during the first contact
Copulation	
Duration of copulation	The time between when a male curled its abdomen around and beneath the female's abdomen tip and the final separation or detachment of the tip of the male's abdomen from that of the female.
Antennation during copulation	Duration of continuous antenna palpation by the male on the female's head or thorax. The male often palpates the antennae, head or thorax of the female with his own antennae during copulation.
Stroking	The duration of strokes on the female body (dorsal part of the thorax and abdomen) with the male's midleg during copulation .
Female reduced activity	Reduced activity in the female was measured as a phase during the copulation process. Reduced activity started when the female stops moving the leg for at least 4 s as copulation begins. During the reduced activity process, the female may take some few steps but these must not result in dragging the male for more than 4 s. Continuous rapid grooming for more than 4 s was also considered as a behaviour that interrupts the reduced activity process.

3.3 Statistical analysis

Pearson's chi-squared was used to test frequencies of categorical variables using IBM SPSS Statistics 21 (IBM Corp., New York). Associations between copulation success and some of the behaviours measured such as pause before first contact and approach at first contact were tested using chi-squared. Two means were separated using non-parametric Mann-Whitney tests in Minitab 17 (Minitab Inc., 2017). One-way ANOVA was also used to compare differences in time to first contacts Minitab 17 (Minitab Inc., 2017)

3.4 Results

3.4.1 General description of behaviour of virgin male and female

The copulation behaviour followed a general pattern outlined in Figures 3.1, 3.2 and 3.3. The behaviours were categorised into pre-copulation, copulation and post-copulation phases.

3.4.2 Description of behaviour during the pre-copulation phase

A total of 78 pairs of virgin males and females were used for this experiment. Once a virgin male and a virgin female were placed in the arena, they either temporarily stayed in one place, groomed or walked around until they located each other in the arena. Usually, a male approached a female (at least 59% of those that copulated and 79% of those that did not copulate, but made contact) and at about a distance less than 2 mm to the female (Figure 3.4), he either paused for a few seconds (up to 8 s in some cases) before proceeding to touch the female (51% of those that copulated and 50% of those that made contact but did not copulate) or proceeded to contact the female without prior pausing. Some males also paused for a few seconds after making contact (15% of those that copulated and 33% of those that did not copulate, but made contact). Pausing before first contact or after first contact was not associated with copulation success ($\chi^2_{(1)} = 0.01$, $P = 1.00$ and $\chi^2_{(1)} = 2.04$, $P = 0.20$),

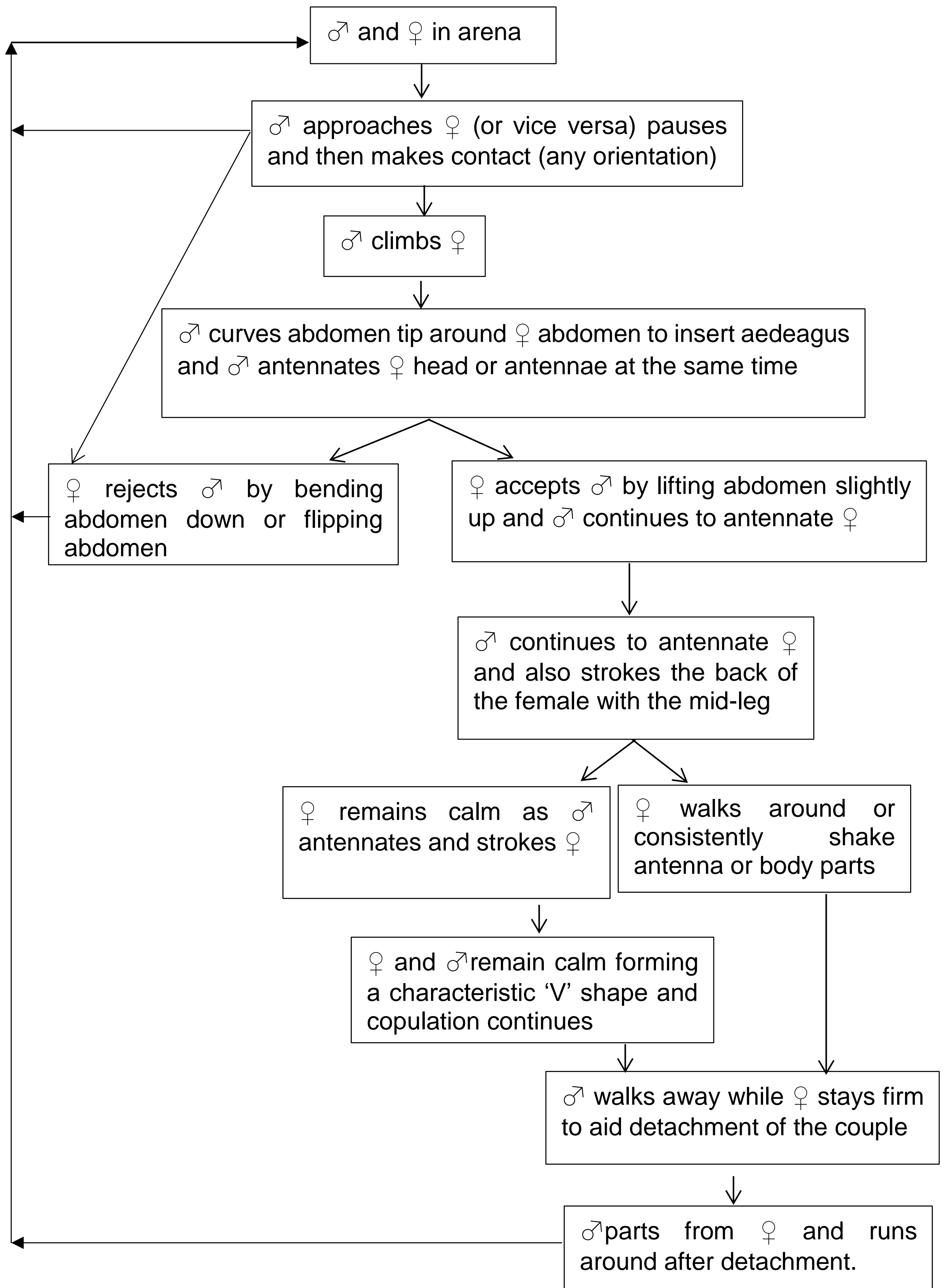

Figure 3.1 Copulation ethogram of adult virgin male and female *F. occidentalis*. This ethogram was produced from observation of pairs of virgin male and virgin female *F. occidentalis* used in this study

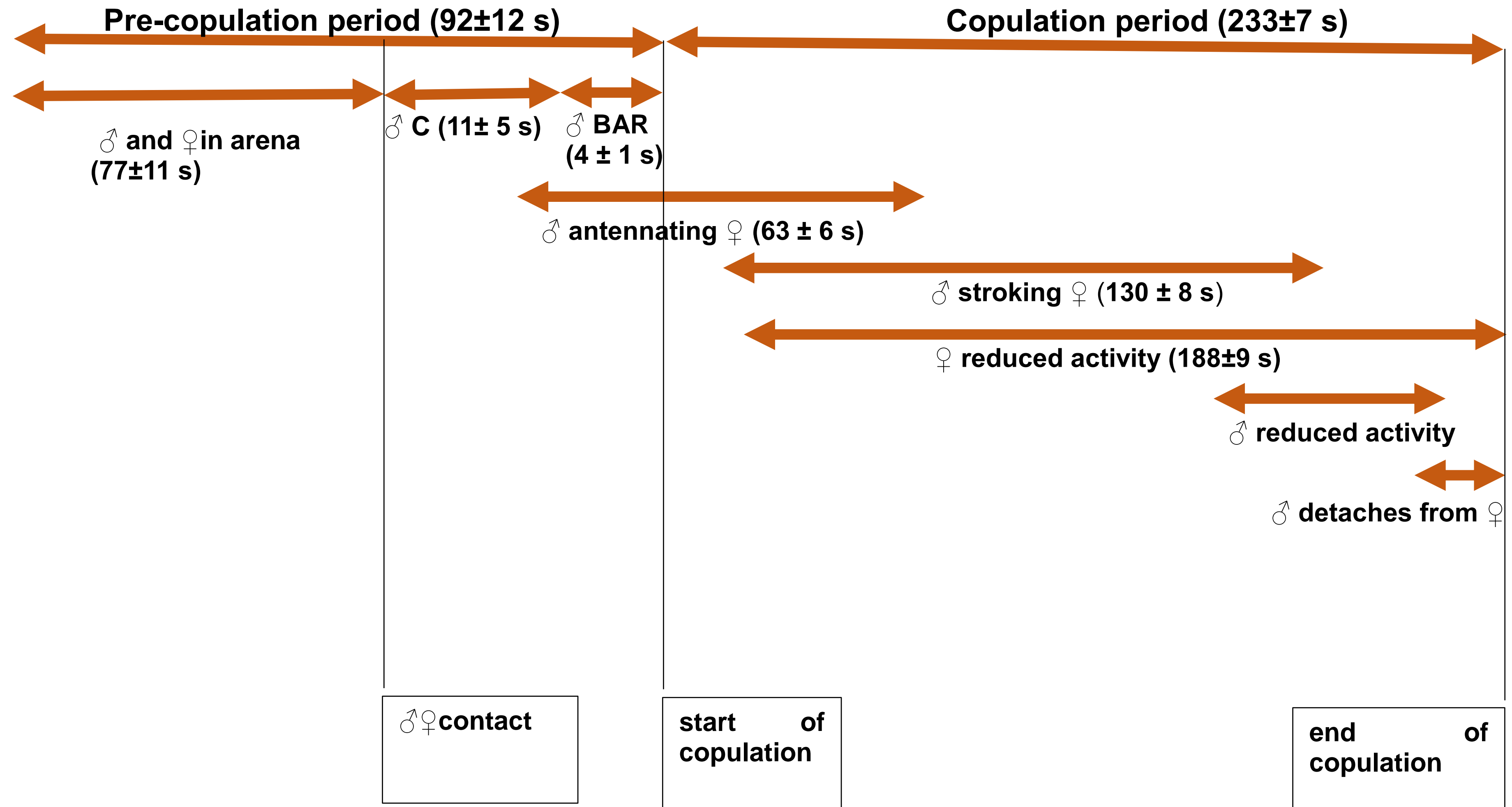

Figure 3.2 Sequence of copulation behaviour in virgin male and female *F. occidentalis*. This stylized sequence was produced from observation of 65 pairs that copulated successfully. c =climbing on female; A=Abdomen BAR = bend abdomen round female abdomen. Specific behaviours in a few of the samples could not be observed due to position during copulation in the arena or difficulty of viewing. The numbers are mean ± standard error

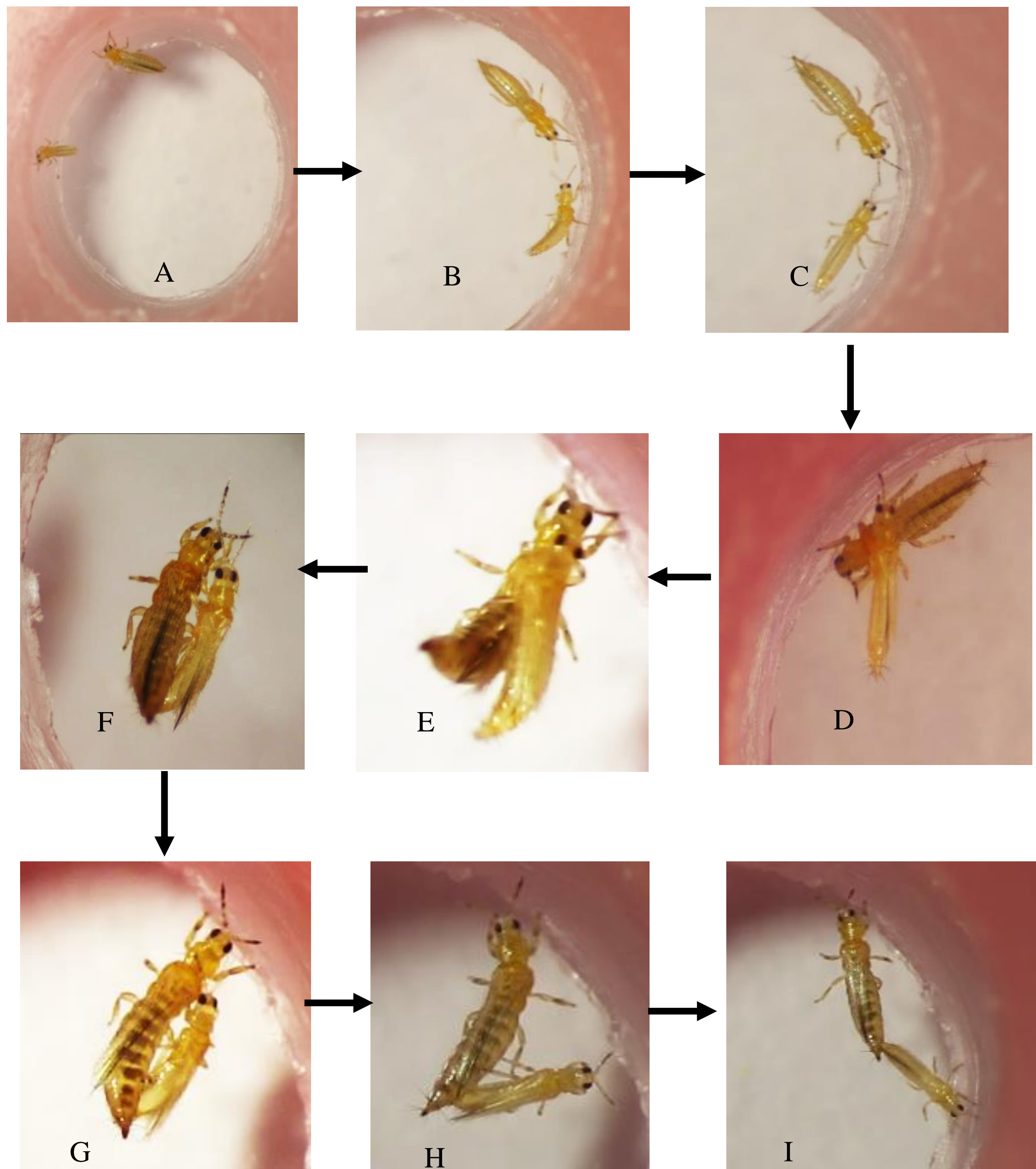

Figure 3.3 Photos of sequence of copulation behaviour in virgin male and female *F. occidentalis*. (A) male and female in the arena, (B) male and female approach each other head to head, (C) a male and a female make antenna to antenna contact, (D) male climbs female, (E) male mounted female and begins to bend abdomen beneath female abdomen while female lifted abdomen tip, (F) male secured attachment and continued anttenation of female antenna, (G) male antennating the female antenna and stroking the female at the same time, (H) male and female in a characteristic 'V' shape position, (I) male pulling himself away from the female while the female was still thus concluding copulation.

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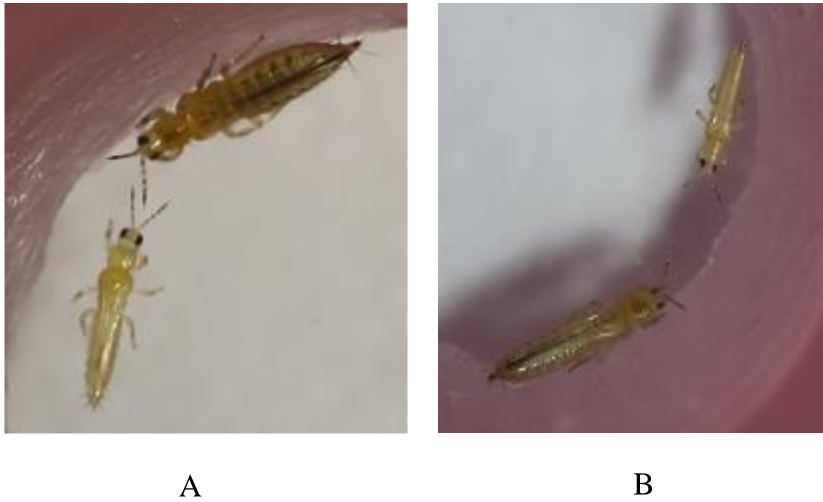


Figure 3.4 Male staying in a position without contact (pause before contact) (A) at a distance less than 0.2 mm before contact, (B) at a distance less than 1 mm before contact

respectively. It generally took longer time to make first contact when compared to other sequence of activities that took place at the pre-copulation phase (Table 3.3, and 3.4).

Significantly more males approached females prior to first contact ($\chi^2_{(1)}$ =10.90, *P*=0.001) (Tables 3.5) and the sex that approached the other prior to first contact was not associated with acceptance or rejection by females ($\chi^2_{(1)}$ =0.42, *P*=0.67). However, there was an overall difference in time to first contact when comparison was made between when a male approached a female vs when a female approached a male vs when they both approached each other (one-way ANOVA, $F_{2, 61} = 4.1$, *P*=0.02) (Figure 3.5). Time to first contact was longer time (P<0.05) when females approached males than when males approached females (Figure 3.4). The orientation at first contact was either male head to male head or male head to female thorax or abdomen (Table 3.4, 3.5 and Figure 3.6). On a few occasions, it was the female that contacted the male first.

After the male contacted the female, the female typically stayed still or reduced activity while the male climbed her first with the fore legs, from the head, thorax, abdomen or even the tip of the female abdomen and then proceeded to the top of the female's thorax or the dorsal surface of the abdomen. He then manoeuvred himself until he laid parallel on top of the female with the head parallel to the female head and his abdomen parallel to the female abdomen (Figure 3.3 (E)).

As the male climbed on the female, he also curled his abdomen under the posterior part of the female abdomen to insert the aedeagus into the female vagina (this process was called abdomen bending). During this process, the male's fore-legs were usually on the pronotum of the female, while one of the mid-legs was between the pronotum and the abdomen of the female and the other hanging free or touching the side of the abdomen. One of the hind legs also rested on the abdomen and the other on the floor of the arena. This arrangement made

Table 3.3 Duration of activities during precopulatory and copulatory phases in virgin male and female *F. occidentalis*. The activities during the copulation phase do not add up to total copulation duration because some of them overlapped. Specific duration of some of the behaviours in a few pairs could not be observed due to the position of the insect in the arena or difficulty of viewing before or during copulation

Activity	**Duration (s) (mean ±SE)**	**Range**	**N**
PRE-COPULATION			
Total Pre-copulatory phase	92±12	2-406	63
Time to first contact	77±11	0-401	63
First contact to end of climb	11±5	0-319	63
End of climb to start of copulation	4±1	0-60	64
COPULATION			
Total copulation phase	233±7	75-492	65
Male antennation during copulation	63±6	13-201	64
Male stroking female	130±8	11-251	57
Female reduced activity period	188±9	0-397	64

Table 3.4 Duration and percentages of occurrence of activities during pre-copulatorion phase in virgin male and female *F. occidentalis* that contacted but did not copulate during the 10 minutes duration. Two of the pairs made contact but did not climb the female.

CATEGORY	Mean ±SE	Range	Percentage	N
PRE-COPULATION				
Time to first contact (s)	41±15	0-116		7
First contact to end of climb (s)	16±13	1-67		5
First contact orientations				7
Head to head			0%	0
Male head to female body (abdomen or thorax)			72%	5
Female head to male body (abdomen or thorax)			14%	1
Undetermined			14%	1
Approach before first contact				7
Male			79%	
Female			14%	
Both			0%	
Undetermined			14%	

Table 3.5 Percentage of activity occurrence during pre-copulation, copulation and post-copulation phases in copulated virgin male and female *F. occidentalis.* Grooming and wing combing were observed for 30 seconds after copulation.

Activities	**Percentage**	**n**
PRE-COPULATION		
Orientations at first contact		65
Head to head	49%	32
Male head to female body (abdomen or thorax)	39%	25
Female head to male body (abdomen or thorax)	6%	4
Undetermined	6%	4
Approach before first contact		65
Male approaches female	59%	
Female approaches male	18%	
Male and female approach each other (both)	9%	
Undetermined	14%	
COPULATION		
Initiator of final detachment after copulation		65
Male	77%	
Female	8%	
Both	15%	
POST-COPULATION		
Male self-grooming	97%	58
Female self-grooming	82%	61
Male wing comb	5%	62
Female wing comb	10%	62

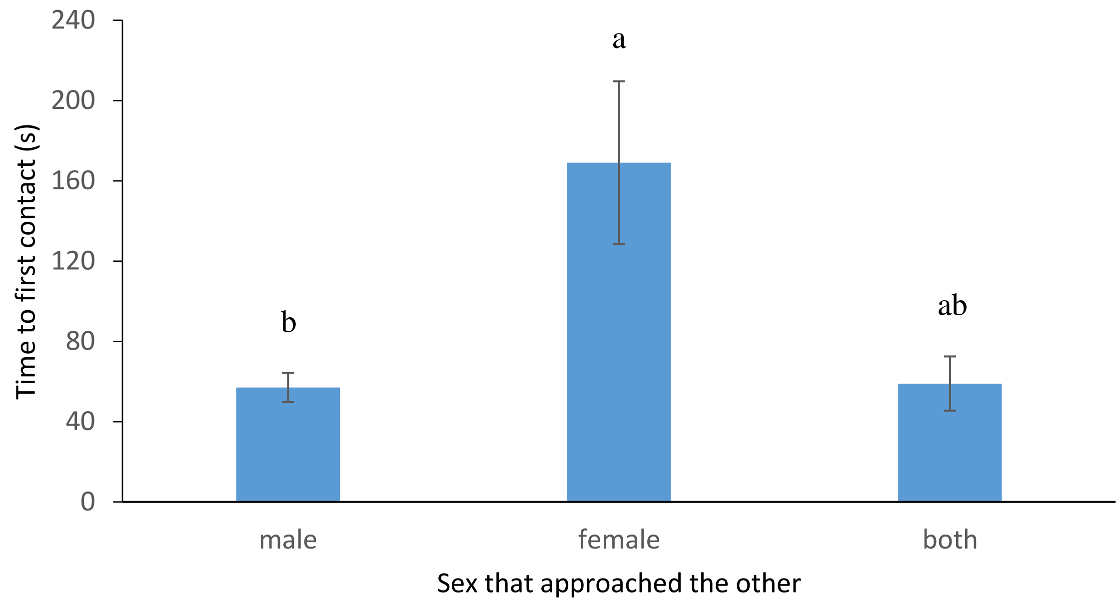
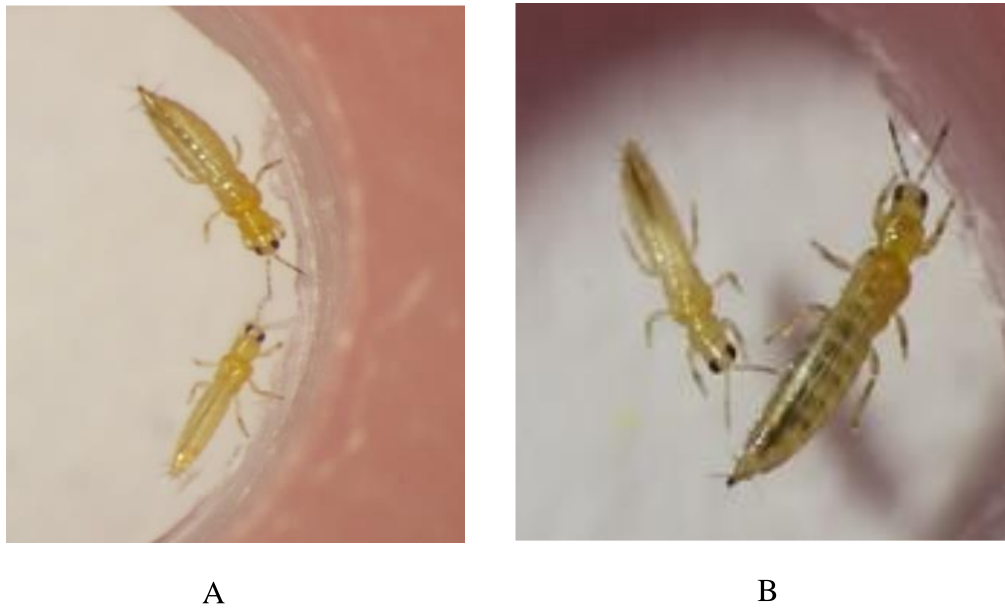


Figure 3.5 Time to first contact among males that approached females, females that approached males and when both approached each other. Data were transformed before analysis using Log_{10} (x+1). Bars with the same letter are not significantly different from each other. (one-way ANOVA, $F_{2,61} = 4.06$, $P = 0.02$)



A B

Figure 3.6 Orientations at contact: (A) antenna to antenna contact, (B) male antenna to female abdomen.

male abdomen bending possible while at the same time, the male palpated the antennae or head (and sometimes thorax) of the female with his own antennae (antennation). Among those that copulated, the male either bent his abdomen to the right (35%) or to the left (65%) of the female's abdomen. The male bent his abdomen more from the left side than the right side during copulation ($\chi^2_{(1)} = 5.55$, $P = 0.03$). This may however have to do with the male approach position before mounting, male approached more from the left than right ($\chi^2_{(1)} = 5.12$, $P = 0.04$), possibly due to the bias in the position of the light source (which is from the left) or the side from which the male was introduced into the arena containing the female (usually from the right). In a situation where approach was head to head, males bent the abdomen more to the left (64%) than the right (36%), but there was no significant difference between the two positions ($\chi^2_{(1)} = 1.96$, $P = 0.23$). This generally suggests that males bent the abdomen more to the side they approach the females from. The duration of copulation and of reduced activity (period when the female remains relatively calm without dragging the male along continuously during copulation) were not significantly different regardless of the sides the male bent his abdomen from ($W = 1338.5$, P=0.52 and $W = 814.0$, $P = 0.36$ respectively).

3.4.3 Description of behaviour during the copulation and post-copulation phases

After climbing and bending the abdomen, antennation continued and the male moved the abdomen around the lower tip of the female's abdomen until he secured attachment (presumably inserted the aedeagus into the vagina). How a male secured attachment with the female vagina was not visible unless copulation occurred on the roof of the arena. When copulation occurred on the roof of the arena, the pair were upside down and viewing could be observed from above (Figure 3.7). The aedeagus was assumed to have been inserted into the female vagina when the male stopped moving the abdomen tip and it appeared secured in position. In about 4 s after securing attachment, and as male antennation continued, the

Figure 3.7 A male and a female copulating on the roof of the arena. Attachment of the pair could only be seen under this condition but it was difficult to view stroking whenever this happened.

male thrips used one of the mid-legs (the one on top of the female) to stroke or rub the dorsal surface of the female thorax down to the dorsal surface of the abdomen (Figure 3.3 (F) and (G)). The male sometimes used the other mid-leg to stroke empty space or the side of the female where the leg was hanging, in what looked like a by-product of using the other leg. The stroking typically did not start until the male had secured attachment. Usually, as the male antennated and stroked the female, and copulation continued, a receptive female often reduced her activity and become relatively calm with just occasional activities such as movement of the antennae.

In this research, reduced activity in the female was measured as a phase during the copulation process. It started when the female stopped moving the legs for at least 4 **s** after copulation began. A few of the females dragged the male along during copulation at an angle (80-90°) between the two abdomens.

Antennation began just after climbing and continued during copulation for a duration of 63 s (mean) or 51 s (median) (Table 3.3), while stroking lasted for about 130 s (mean) and 127 s (median) (Table 3.3). Thus the male continued to stroke the female (usually not as rapidly as when the male started) after it had stopped the antennation process. At the point when the male stopped stroking, both pairs remained motionless and sometimes took a characteristic "V" shaped position. Before final detachment, the male sometimes briefly palpated the female antennae with his own antennae. To finally separate, the male walked away and pulled out of the female (77 %) while the female remained still. After separation, male and female groomed themselves and wing combed (Table 3.5)

Copulation duration was measured as the time between when a male curled his abdomen around and beneath the female's abdomen and when the pair finally separated or detached the tip of the male's abdomen from that of the female. The average copulation duration was

233 s (Table 3.3). The minimum copulation duration was 75 s while the maximum was 492 s (Fig 3.8). The pair that copulated for 75 s re-copulated with the same female for 56 s almost immediately after the first copulation. Another male copulated again with a female for 586 s after an initial copulation duration of 153 s. The latter female appeared unusually long and did not reduce activity during the copulation process. These two pairs had the shortest initial copulation durations observed in this research.

3.4.4 Acceptance and rejection

The total number of copulation videos analysed in order to study virgin male and virgin female copulation behaviour was 78. Out of the 78 pairs used, 65 copulated, 7 made contact but did not copulate while 6 did not make any contact in 10 minutes observation period.

A receptive virgin female, typically stayed in one position within the arena and lifted the tip of her abdomen as the male mounted her while the male bent the tip of his abdomen below the tip of the female abdomen (Figure 3.3(E) and 3.3(F)) and inserted his aedeagus. Some virgin females also bent low as the males climbed on them.

An unreceptive female rejected the male by flipping her abdomen to dislodge the male climbing her and prevented him from attaching himself to her. In a few cases, females responded by bending the tip of the abdomen downward, which seemed to prevent the male from bending the tip of his abdomen beneath that of the female to secure attachment. This behaviour was observed when males made climbing contact with the female from the setae at the tip of her abdomen.

Out of the seven pairs that contacted but did not copulate in 10 minutes, two made just a single contact in 10 minutes. This contact appeared sudden, that is contact happened in a position where one of the pair appeared unaware before the other made the contact. Of these

two pairs, one male flew and landed on the female's back in a position where the female

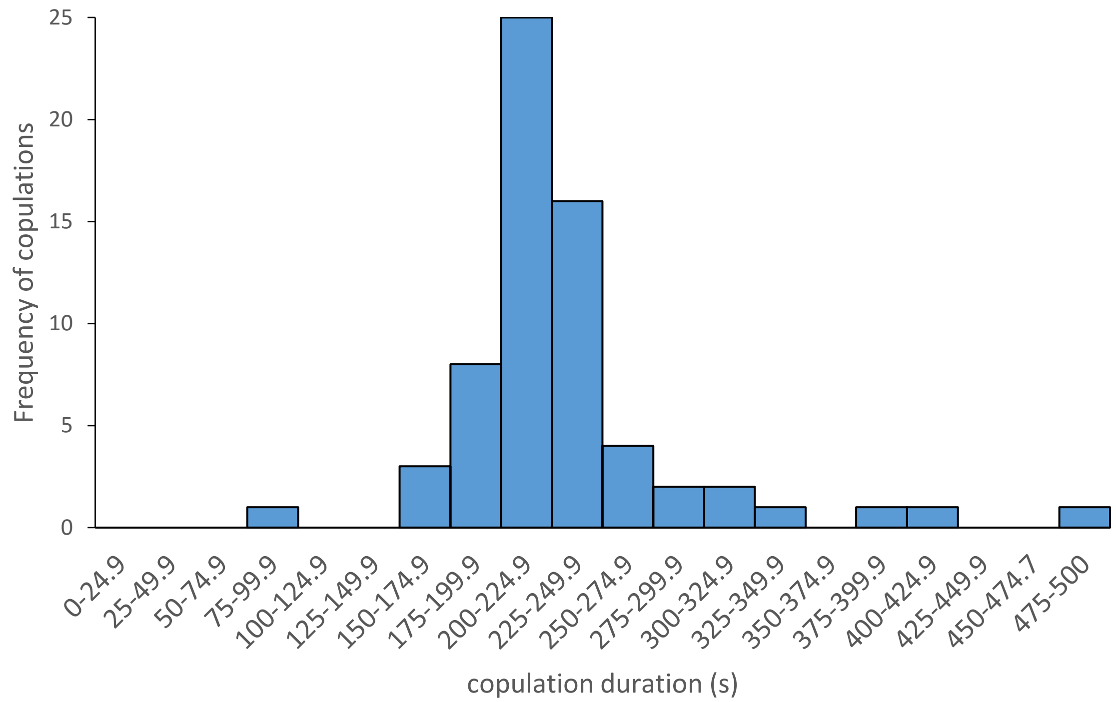


Figure 3.8 Histogram of copulation duration of adult virgin male and virgin female *F. occidentalis*. n = 65.

female appeared not to have previously seen the male approaching her while in the other case, the female made the first contact by running towards the male and contacting the male from his abdomen tip. This male then remained in the same position without movement for a long time. These two cases were not the usual form of approach.

In the remaining five pairs where contact did not result in copulation, the males in each pair successfully climbed on the female (Table 3.4). I observed that if a male could not combine antennation of the female head with inserting the aedeagus (two behaviours that happened almost at the same time and at opposite ends of the insects) for some minutes, the female flipped her abdomen to dislodge the male. Usually, a male continued to attempt antennation of the female even when it was difficult to secure attachment. One male, for example, appeared too short (compared to the female paired with him). He successfully climbed and antennated the female and even bent his abdomen below that of the female but could not secure attachment of the aedeagus while at the same time antennating the female. The female eventually flipped its abdomen to dislodge him. Therefore, a female may lift the abdomen indicating acceptance, but if the male could not secure attachment of the aedeagus to the vagina of the female (for up to 3 minutes of failed attempts on one occasion), the female began to flip its abdomen and dislodge the male. In two other cases, the male approached and started climbing the female from the posterior end. The female rejected them by bending the tip of its abdomen down preventing the male from inserting the aedeagus and after minutes of unsuccessful attachment of the aedeagus to the vagina, the female flipped its abdomen to dislodge the male.

3.4.5 Did pre-copulation activities influence copulation success?

1. In order to identify if duration of activities before first contact determined copulation success, time to first contact and first contact to climbing were compared between pairs that

copulated and those that did not. The median time to first contact for those that copulated and those that did not were 46 s and 11 s respectively while the median time from first contact to climbing for those that copulated and those that did not copulate but made contact were 3 s and 2 s respectively. There were no significant differences between time to first contact and time from first contact to climbing in pairs that copulated and those that did not (W = 2286.0, P=0.34) and (W = 2182.5 P=0.84, n=63) respectively (Figure 3.9). Time to first contact between male and female thrips seemed random and did not appear to reflect readiness to mate.

2. It was possible that acceptance by a female may be associated with initial head to head contact between a male and a female because this was common among pairs. I therefore hypothesised that a female might reject a male based on the orientation at first contact. Head to head orientation occurred when a male and a female made the first contact (usually facing each other) with the male antennae contacting the female's head or antennae. Head of male to female abdomen/thorax orientation occurred when a male made the first contact by touching the female's thorax or abdomen or the setae at posterior end of the female's abdomen with his antennae. Associations between orientation at first contact (male head to female head and male head to female abdomen) and copulation success (acceptance or rejection) were therefore compared. The result showed a significant association between orientation and copulation success ($\chi^2_{(1)} = 5.80$, $P = 0.02$). None of the females that rejected a male had a head to head first contact) (Tables 3.4).

3.4.6 Why does a male antennate and stroke a female during copulation?

1. I tested the hypothesis that continuous antennation or stroking might be associated with continuous calming in virgin female thrips during copulation. There was however no relationship between the duration of antennation and the duration of reduced activity in

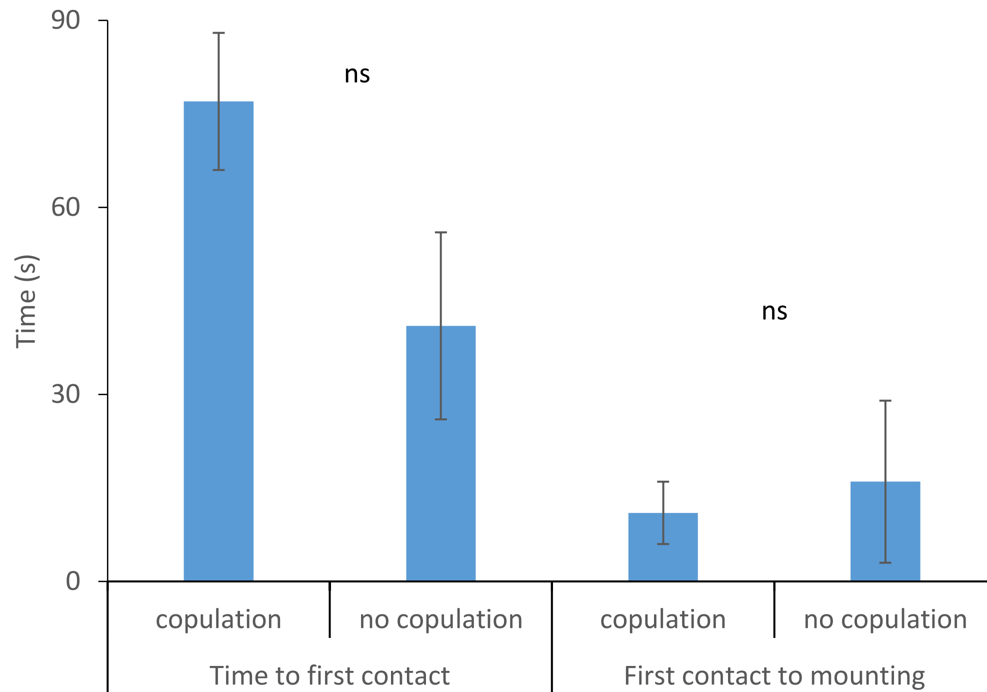


Figure 3.9 Time to first contact and first contact to climbing between those that copulated and those that did not. Data were analysed using a non-parametric Mann-Whitney test. Time to first contact: W = 2286.0, P=0.34, n=63 (copulated), 7 (no copulation). Time from first contact to mounting: W = 2182.5 P=0.84, n=63 (copulated), 5 (no copulation). Error bars are values of lower and higher confidence intervals and ns = not significant.

females during copulation (r = 0.08, $P = 0.52$) or stroking and the duration of reduced activity in females during copulation (r = 0.18, $P = 0.17$).

2. Could the male antennate or stroke the female to provide a physical signal to prepare the female for copulation and to allow for easy insertion of the male aedeagus? This speculation may be correct for antennation behaviour since the male began antennation just as he started climbing on the female while at the same time bent his abdomen below that of the female. These two activities happened at the anterior and posterior ends of the pair. If antennation is not important to securing attachment, a male could have simply focused on bending the abdomen and securing attachment, rather than attempting to antennate the head of the female at the same time. A proportionately short male also antennated the female even when his small length appeared to make securing attachment with the female difficult (Figure 3.10) This behaviour therefore suggested that antennation was important to successful penetration by the male. The reason a male stroked a female cannot be to prepare a female for copulation or aedeagus attachment because a male often started stroking a female after aedeagus attachment had been secured.

3. Another possible reason why a male antennated or stroked a female could be to apply or detect an antiaphrodisiac pheromone on the female. Evidence for an antiaphrodisiac pheromone in *F. occidentalis* will be discussed in Chapter 6.

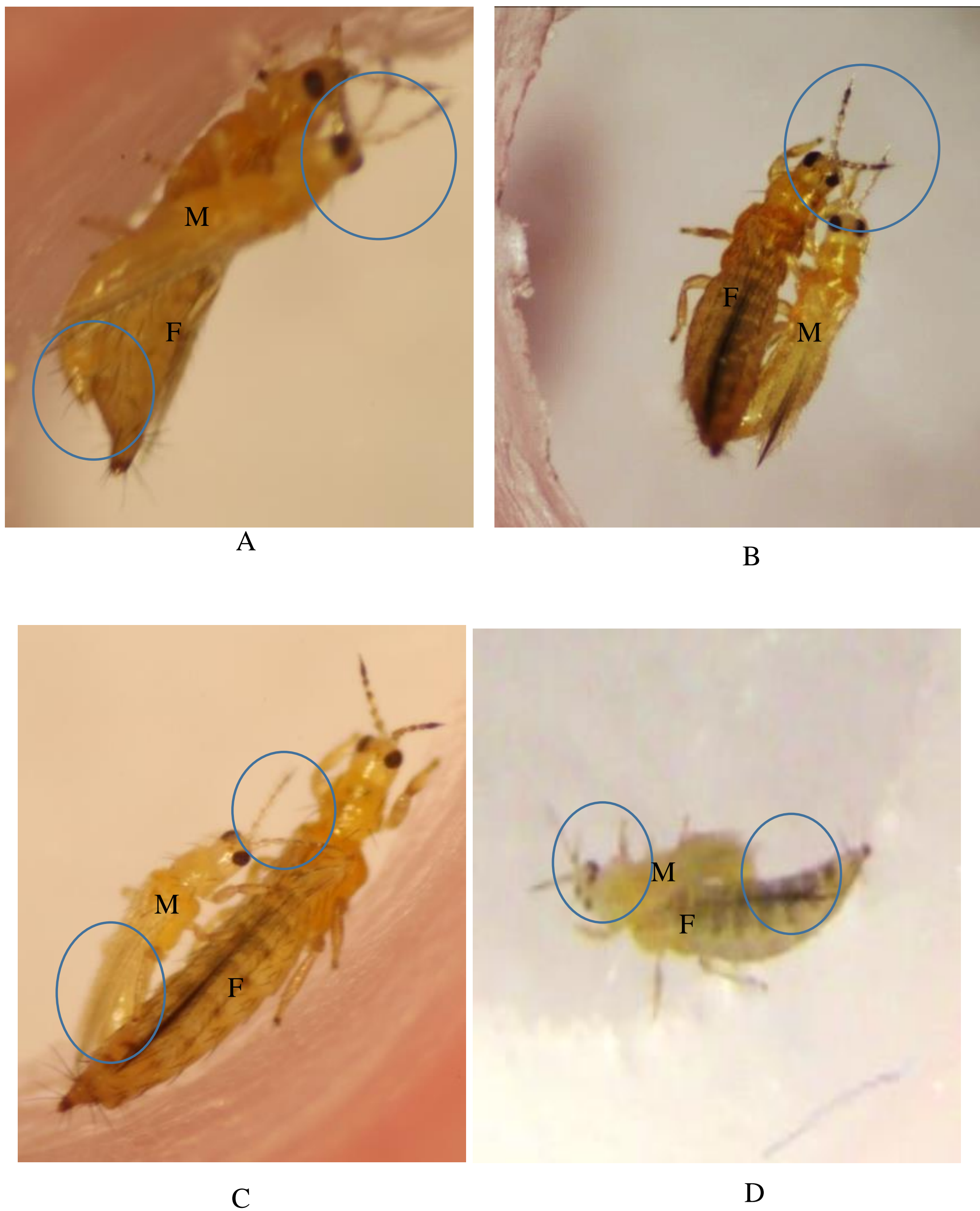


Figure 3.10 The males (M) in (A) and (B) above antennate and bend abdomen beneath the tip of the female (F) abdomen at the same time while the male in (C) bent abdomen but could not antennate the head of the female and (D) antennates the head of the female but could not reach the tip of her abdomen to secure attachment.

3.5 Discussion

Virgin females were receptive to virgin males usually at first or second contact. A receptive female would stay in position as the male approached her, and the male then climbed her without her flipping the abdomen to dislodge the male. She also lifted the tip of her abdomen up while the male bent the tip of his abdomen beneath that of the female. Out of 72 pairs of virgin male and virgin female observed in 10 minutes, 83% copulated. In a similar experiment, Terry and Schneider (1993) observed a similar non-flipping behaviour in a receptive female. However, a behaviour Terry and Schneider ((1993) called ‘duck’ or ‘squat’ in which a receptive female bent her legs, lowered her abdomen and thorax, was observed only a few times in this study. I have shown that a female lifting her abdomen tip to allow the male to bend his abdomen tip under was most common behaviour observed in receptive females.

The observed non-contact (8 % of all pairs tested) between a virgin male and a virgin female left for about 10 minutes may be due to chance, especially because similar percentages of occasional non-contact were observed in subsequent experiments (Chapter 4). Sometimes, either of the sexes just remained in the same position grooming or not actively moving around thus making it less likely for the other sex to locate or run into it. *Frankliniella occidentalis* are known to be sometimes arbitrarily inactive during the day.

The reasons why some of the virgin females rejected the virgin males introduced to them after making contact (about 9 %) may be due to the nature of the contact. For example, when one of the sexes jumped on the other or when one of the pairs quickly approached the other from a position where it was impossible for the other to see it, such contact could be taken for a predator attack. Another important reason for rejection by virgin females might be because the male could not successfully insert the aedeagus to the vagina of the virgin female

after a series of attempts. A male thrips usually combined antennation of the female (which happened at the anterior part) with bending his abdomen beneath that of the female at the posterior end; therefore, securing aedeagus attachment to the vagina may be difficult if a male is paired with an excessively long female**.** Even when copulation occurred, in a pair where a female appeared excessively longer than the male, the female was observed to drag the male along without a calm phase throughout the copulation period presumably to detach herself from the male. As in some other insects, female *F. occidentalis* are generally bigger than males, and male *F. occidentalis* also vary in sizes of abdomen and leg (Terry and Dyreson, 1996) The likelihood for lack of copulation due to mismatch in size observed in this research may therefore suggest the possibility for assortative mating in *F. occidentalis* (Terry and Schneider, 1993). The fact that more virgin females (28%) rejected males in the experiment by Terry and Schneider (1993) compared to 9% in this experiment, may also be due to the increased possibility of pairing a short male with a long female. Such mismatch in size accounted for over 36 % of observed rejections in their experiment (Terry and Schneider,1993).

There were no differences in time to first contact or time from first contact to end of climbing between those that copulated and those that did not, suggesting that pre-copulation duration was not an indication of readiness to copulate. In *Thrips tabaci*,a species of similar small size as *F. occidentalis*, no difference in pre-copulation duration was observed when arrhenotokous or thelytokous females were copulated with arrhenotokous males (Li *et al*., 2015b). It is likely that pre-copulation duration between a male and a female is mostly random in most thrips species.

A virgin male sometimes paused for a few seconds (up to 13 s in a pair) after approaching and locating a female and before contact and occasionally after contact. This behaviour suggests that a visual cue was involved in detecting a female a few millimetres before

contact. Similar observation by Kirk (1985a), in a study of the mating behaviour of *Thrips major* and *Thrips fuscipennis*, suggested that males of these two species could detect females from a few millimetres distance. A possibility of a visual cue being involved in species and sex recognition in *F. occidentalis* is therefore an option. Since no sex or aggregation pheromone has been identified in female *F. occidentalis* unlike males where aggregation and contact pheromones have been identified (Hamilton & Kirk, 2003; Kirk & Hamilton, 2004; Hamilton *et al.*, 2005; Olaniran *et al.*, 2013), there is no evidence that a male is attracted to the female via any pheromone cue. Another possible reason for such pause by a male before contacting the female could be to avoid predation, but this is not a likely reason because the pause distance between the pair was sometimes as close as 1 mm and can last as long as 10 s in some pairs. Also, the pause was often followed by a swift contact and climbing on the female. This will likely not be the manner of behaviour if they were trying to avoid a predator. Therefore, the likely first step in sex or species recognition in male *F. occidentalis* may be visual.

After making contact with a female, a virgin male often climbed on the female, and contact before climbing could be very brief (< 1 s), but could be longer sometimes. It is therefore possible that a male further confirmed the sex of the female after contact. The fact that a male *F. occidentalis* when introduced to a thelytokous female *T. tabaci* in most of the cases made contact with his antennae before moving away after such contact (Chapter 7) suggests that first contact between a virgin male and a female *F. occidentalis* was needed for species or sex recognition. When male *F. occidentalis* meet each other, they simply part after meeting or with a slight abdominal flicking or flipping the abdomen, grabbing and flicking the abdomen of the male opponent (Terry & Gardner, 1990; Terry & Dyreson, 1996; Olaniran, 2013). These fighting behaviours were not observed when males made contact with the virgin females, rather a male proceeded to climb the female after contact. Thus a

male could recognise a virgin female at least after he has made contact with her. Lewis (1973), stated that there are sense cones on the antennae of thrips that help sexes find each other. Similarly, extract from the female body has been shown to be involved in sex recognition of *S. aurantii* (Rafter & Walter, 2013).

After contact and climbing on the female, a male bent his abdomen beneath that of the female in order to insert the aedeagus. Males were observed to bend the abdomen more to the side from which they approached the female. In this research, males bent the abdomen more to the left than the right which may be due to the bias resulting from the position from which the male approached the female. In a similar experiment, Terry and Schneider (1993) did not find any significant different between the number of males that bent the abdomen to the right or to the left.

As a virgin male climbed on a virgin female, he was observed to also antennate the female head at the same time. This behaviour was observed in all pairs tested. Even when a female attempted dislodging a male (after the male had climbed on the female) by flipping her abdomen, the male continued the antennation. The reason for this is not so clear, but since a male began antennation just as he climbed on the female and at the same time bent his abdomen to insert aedeagus, combining these two activities suggests that antennation may be important in female receptivity or successful insertion of the aedeagus into the vagina of a female. Similar antennation behaviour was observed in *F. schultzei* (Milne *et al.*, 2007) and *S. aurantii* (Rafter & Walter, 2013). Antennation continued for some time even after a male had secured aedeagus attachment and the female had remained relatively calm. This is slightly different from what was observed in *F. schultzei,* where *antennation* continued until the female stood still. A male *F. schultzei* only curved his abdomen beneath that of the female after the female stood still (Milne *et al.*, 2007). Antennation may therefore help in sustaining attachment at least for the first few minutes after penetration.

There was no correlation between duration of antennation and that of female reduced activity in *F. occidentalis.* This may not be a sufficient reason to suggest that the purpose of antennation was not to calm the female, since such behaviour may be a programmed one. A positive correlation observed between duration of antennation and stroking suggests that these two behaviours were programmed. Terry and Schneider (1993) observed that any male that could not palpate the female because the male was too short were rejected by virgin females, and they suggested that a close-range cue was involved in calming females. The reason for antennation may also be to detect pheromone on the female.

Stroking of the female dorsal abdominal surface by male mid leg occurred about 4 s after the male had bent the abdomen and secured attachment to the female, therefore the role cannot be to calm the female prior to attachment of the aedeagus. It may however play a role in continuous calming of a female after copulation had started. Stroking behaviour was also observed in some other species in the sub-order Terebrantia such as in *S. aurantii* (Rafter & Walter, 2013), *F. schultzei* (Milne *et al.*, 2007) and *E. americanus* (Krueger *et al.*, 2017). In *F. schultzei, the* male *tapped the female with his leg after climbing until the female became stationary* before bending his abdomen to insert the aedeagus (Milne *et al.*, 2007), suggesting that stroking in this species was associated with calming. The possibility of stroking a female to calm her or to apply a 'calming pheromone' could not be ruled out in *F. occidentalis* since stroking continues substantially during the copulation process. Pelikan (1951) suggested that male *P. dianthi* produced a secretion from the sternal glands which helped to calm a female during copulation. It is possible that sperm transfer took place after stroking when a male and female *F. occidentalis* maintained a calm position (this is further discussed in Chapter 4). Stroking may therefore play important role in helping the couple form a bond.

Another possible role of stroking behaviour may be that the male was applying an antiaphrodisiac pheromone to mark the female during copulation. Male antiaphrodisiac

pheromone dimethyl ester of hexanedioic acid (dimethyl adipate) has been identified in male *Echinothrips americanus* (Krueger *et al.*, 2016), a species in which stroking occurs (Krueger *et al.*, 2017). Evidence of production of an antiaphrodisiac pheromone has also been identified in western flower thrips (Chapter 6).

Copulation duration (233±7 s) in *F. occidentalis* observed in this study was very consistent in most of the replicates and so there was a very low standard error, which suggests that during copulation a consistent and programmed series of events was taking place. The duration of copulation was also similar to the durations that have previously been reported. Durations in the laboratory were 241 s (Terry and Schneider, 1993), 210 s and in the field, 189 s (Terry & Gardner, 1990) and 176s (Terry & Dyreson, 1996). Copulation duration in *F. occidentalis* is within the range typical of the sub-order Terebrantia (between 2 and 15 minutes). To finally separate, the male walked away and pulled out of the female while the female remained still. I have therefore shown that males appear to initiate copulation in most of the cases.

Short copulation (below 20 minutes) is of benefit to insects, since it is less energetic and will give the male an opportunity to copulate with other females thereby increasing the chances of fertilizing more females. This is especially true in species where females become non-receptive after first copulation. *Frankliniella occidentalis* copulates once and some reject further copulations (Chapter 4) (Terry and Schneider, 1993), therefore a short copulation duration may favour this species. Long copulation of 6 hours occurs in *Suocerathrips linguis* (Kumm, 2002). Unlike *F. occidentalis*, it is not known if female *Suocerathrips linguis* copulates only once, but multiple copulation has been reported in some species in the sub-order Tubulifera and all these species had short copulation duration (Crespi, 1986b, 1988a).

Chapter 4

Multiple copulation in males and females

4.1 Introduction

Copulation in insects serves two purposes, one is the transfer of sperm and the second is the transfer of nutrients (usually present in the seminal fluids and the spermatophore) that are of benefit to females (Thornhill & Alcock, 1983; Arnqvist & Nilsson, 2000). These two purposes appear to influence the frequency of copulation and receptivity of females (Arnqvist & Nilsson, 2000).

4.1.1 The costs and benefits of multiple copulations in female insect

A male's success in maximising reproduction capacity depends on how many females his sperm can successfully fertilise (Parker, 1984). In females however, life span, rate of egg production and fertility influences the number of offspring production throughout life (Arnqvist & Nilsson, 2000). Female reproductive success is therefore achieved by maximising the number of viable eggs that they can produce and this can often be achieved with one or a few copulations with males (Arnqvist & Nilsson, 2000) since they can store sperm in the spermatheca. If a female can achieve these benefits just by copulating once, it will be unnecessary to engage in multiple copulation considering the cost associated with

multiple mating such as energy cost committed to periods of courtship and copulation and exposure to predation (Thornhill & Alcock, 1983; Franklin *et al.*, 2012). However, despite these costs, females of most insect species are rarely monogamous (Snook, 2014). The benefits females achieve by copulating several times seem to outweigh their negative effects (Arnqvist & Nilsson, 2000). A female will therefore copulate multiple times if it will increase her lifespan and reproductive success (Walker, 1980).

One obvious reason why a female will be receptive to more males after initial copulation is for sperm replenishment. This takes place in a situation where a single sperm donation by a male may not be sufficient for female fertility throughout her lifetime (Walker, 1980; Thornhill & Alcock, 1983). It also serves as an insurance strategy against possible male infertility (Snook, 2014). When several species of female *Drosophila* were dissected at intervals, the sperm stored contents were observed to diminish with time (Patterson, 1954). Also in a New Zealand seed bug, *Nysius huttoni*, females copulated multiple times just for sperm replenishment without any evidence of other material or genetic benefits to the female (Wang & Davis, 2006). However, in many insect species, a male insect transfers more sperm than needed by the female for egg fertilization almost throughout their lifetime (Thornhill & Alcock, 1983), for example in mealworm beetle, *Tenebrio molitor*, females copulate more frequently than is required for sperm replenishment suggesting that such copulations are not just for sperm replenishment but for other material benefits (Drnevich *et al.*, 2001).

Reynolds (1996) in a review hypothesised the potential benefits of multiple copulation by female insects and these include:

1. direct benefits such as reproduction or ovulation stimulation, sperm replenishment, nuptial gifts and other food benefits, parental care from more than one male, mate retention, prevention of male harassment and for assessment of mates.

Direct material benefits to females include enhanced longevity in females such as in the beetle *Callososbruchus maculatus* (Fox, 1993), contribution to egg production, fecundity, oviposition and egg viability in females (Boggs & Gilbert, 1979; Hou & Sheng, 1999; Singh & Mishra, 2010). In some lepidopterans, multiple copulations lead to increased female fitness. For example, males of the arctiid moth *Utetheisa ornatrix* transfer spermatophore and a nuptial gift of pyrrolizidine alkaloids to females during copulation. Multiple copulations give these females more access to nuptial gifts from males through the increased number of ejaculates received from more than one male and thus resulting in increased general female fitness (Lamunyon, 2000). In this species, a female may receive up to 13 spermatophores.

2. genetic benefits such as sperm competition, choice of good male sperm, avoidance of inbreeding and genetic defects from sperm of low quality and for selfish reasons such as preventing other males from having access to females. Genetic benefits of multiple copulations include perceived inferior genetic disadvantage from the former partner, genetic diversity, genetic compatibility through avoidance of inbreeding and increased heterozygosity. These genetic benefits have been reviewed (Walker, 1980; Thornhill & Alcock, 1983; Jennions & Petrie, 2000; Simmons, 2001; Snook, 2014). Genetic benefits of multiple copulations is especially true if copulation is with different males.

Multiple mating in female insects to avoid the cost associated with male harassment or sexual attempts (Parker, 1984; Rowe, 1992) was termed 'convenience polyandry' by Thornhill & Alcock (1983).

4.1.2 Copulation frequency in thrips

Multiple copulations are not uncommon in male thrips (Lewis, 1973; Terry & Schneider, 1993; Terry, 1997). Female thrips however vary in their frequency of copulation. Multiple

copulations occur in some species while others copulate only once. In the sub-order Tubulifera, multiple copulation has been reported in *Hoplothrips karnyi* (Crespi, 1988b) and *Elaphrothrips tuberculatus* (Crespi, 1988a) where females copulate multiple times with the same or different males and in *Dunnatothrips aneurae* females mate multiple times with different males (Gilbert & Simpson, 2013). Two microsatellite loci genotypes of female soldiers have shown evidence of multiple copulation in female *Oncothrips habrus* (Chapman & Crespi, 1998). Evidence of single copulation has also been identified in *Oncothrips tepperi* (Chapman & Crespi, 1998) and some gall-forming thrips (Varadarasan & Ananthakrishnan, 1982). In these gall-forming thrips, males mature before females. They then copulate with females after which the females reject further copulations from other males (Varadarasan & Ananthakrishnan, 1982).

In Terebrantia, there seems to be restriction in the frequency of copulation in already studied species. Adult male *E. americanus* has been observed to stay close and guard a female pupa and copulate with her immediately after emergence (Krueger *et al.*, 2016), a behaviour that clearly suggests strong competition for virgin females. Most female *E. americanus* (19 out of 20) do not copulate again for 30 days whether they were paired with mated or virgin males (Li *et al.*, 2014). Other recent studies have confirmed the low female re-copulating frequency in *E. americanus* (Krueger *et al.*, 2015a, 2017). Female *F. schultzei* paired with a male did not copulate more than once when the pairs were left for about 60 minutes (Milne *et al.*, 2007). The 60 minutes test period suggested a reluctance to copulate again after an initial copulation but it did not test if female *F. schultzei* only copulate once throughout their lifetime, this may require further studies. However, *Thrips tabaci* females copulated multiple times when tested with both the same and different males in a 30 days test period, this seems to be the only species in the sub-order Terebrantia where multiple copulation was reported (Li *et al.*, 2015b). However, even in this species, the accumulated copulation frequency was

less than 3 times when tried at 2, 4, 6, 8, 10, 15, 20, 25 and 30 days (Li *et al.*, 2015b). This also suggest a restriction in the frequency of copulation in this species.

Female *F. occidentalis* have been widely cited to reject copulations from males for several days after initial copulation (Terry & Dyreson, 1996; Terry, 1997; Olaniran *et al.*, 2013; Li *et al.*, 2014, 2015b). Most of these authors referred to the research carried out by Terry & Schneider (1993). However, a careful look at the publication revealed that observation and interpretation by Terry & Schneider (1993) was too general and was based on few samples of eight females, and the males used for the multiple copulation experiment were not all virgin. Terry and Dyreson (1996) has observed more than one copulation in female *F. occidentalis* in a field study. These females copulated again especially with a challenger male that had remained on the back of the female during the first copulation (Terry and Dyreson, 1996). I have also observed two males competing for a virgin female in a 5 mm diameter arena in the laboratory (Figure 4.1).

4.1.3 Do copulated females respond to aggregation pheromone?

Female *F. occidentalis* will likely respond to male pheromone if she is willing to copulate and will not respond to the male pheromone if she is unwilling to copulate. More female than male *F. occidentalis* were attracted to aggregation pheromones (neryl (S)-2-methylbutanoate) used in pepper and semi-protected strawberry crop fields (Sampson, 2014). This observation of increase in female *F. occidentalis* catch by an aggregation pheromone (neryl (S)-2-methylbutanoate) baited trap has been observed in other crops in the field (Hamilton *et al.*, 2005). The copulation status (virgin or copulated) of the females caught was not reported and will require further investigation. However, since most field populations of female *F. occidentalis* are expected to have copulated, one would rather expect a low number of virgin females in the field.

Figure 4.1 Two males competing for copulation with a virgin female within the arena in the laboratory. One of the males (M1) copulated with the female while the other challenged the copulating male. M1=copulating male or M= challenging male, F=female.

Laboratory study of response to aggregation pheromone has clearly demonstrated that most females picked at random from a mixed culture maintained in Keele University actually responded positively to synthetic aggregation pheromones (Hamilton & Kirk, 2003; Dublon, 2009; Olaniran, 2013). I have demonstrated in this thesis that most of the females (over 90 %) from the culture are likely to have copulated at least once (Chapter 5). This observation therefore suggests that both virgin and copulated female *F. occidentalis* were attracted to the pheromone produced by male *F. occidentalis*. This implies that female *F. occidentalis* may copulate again with males while species like *E. americanus,* that has been clearly demonstrated to only copulate once (Li *et al.*, 2014), may not be attracted to aggregations by males if they occur. However, such aggregation has not been observed in this species (Krueger *et al.*, 2015b).

4.1.4 The sperm bundle and formation of the 'spermatophore' in thrips

The sperm and seminal fluid in male insects are transferred to the female either in bundles or as individuals and can be transported directly into the spermatheca or through small cases called spermatodoses or with the aid of a spermatophore produced by a male (Pascini & Martins, 2016). The spermatheca in adult female *Frankliniella* is visible by light microscopy. It is spherical and contains yellow, orange or brown pigment granules (Heming, 1970a; Dallai *et al.*, 1996). In *F. occidentalis*, the spermatheca is connected to the vagina (Dallai *et al.*, 1996; Kumm, 2002). There is a strong indication that fertilisation of the eggs takes place in the vagina of the female in *F. occidentalis* since the vagina is connected to the spermatheca duct through the muscle-inserted vagina diverticulum (Dallai *et al.*, 1996). This arrangement forms a valve that may help in the control of spermazoan passage through the duct into the vagina from the spermatheca (Dallai *et al.*, 1996).

The spermatheca in inseminated females of most Thripidae including *F. occidentalis* contains a ball of twisted spermatozoa surrounded by a capsule that opens into a tube within the spermatheca duct (Heming, 1995). This was similar to what was described as a spermatophore tube in *F. fusca* (Heming, 1970a). The spermatophore is strictly defined as any kind of container which encloses the semen during its transfer from the male to the female (Perker, 1970). The secretions from the male accessory glands in insects help in formation of a spermatophore as reviewed by Leopold (1976).

Heming (1970a) believed that the precipitate observed in the spermathecal lumen of the inseminated female was produced in the male and transferred to the female during copulation. Heming (1970b) suggested that in male *F. fusca,* the spermatophore is not formed until the materials needed for its formation have moved through the extraphallic part of the ejaculatory duct and that the spermatophore subsequently formed is presumably passed through the spermathecal duct, while the material remaining in the *ductus conjunctus* forms an extension called the spermatophore tube (Heming, 1970b). The spermatophore tube according to Heming (1970a) opens into the spermatheca duct just before the spermathecal duct opens into the dorsal side of the vagina. The spermatophore in the spermatheca is always attached to the spermathecal duct (Heming, 1995).

In a later review, Heming (1995), while confirming observation of a spermatophore by Bournier (1956b), claimed that since the single ball of spermatozoa found within the spermatheca in most Thripidae is always attached to the spermathecal duct, and it is always intact, these species likely copulate successfully only once. However, recent observation in *E. americanus* has shown that the coagulation of the sperm bundle takes place within the spermatheca of the female (Krueger *et al.*, 2017). After insertion of the aedeagus, the male *E. americanus* gradually transferred the accessory materials near the spermatheca duct after which the spermatozoa was transferred (Krueger *et al.*, 2017). This confirmed the

observation by Bode (1975) and suggestion by Heming, (1995) that the sperm bundle was formed in the body of the female, possibly by the materials produced by the male.

4.2 Aims of study

The widely cited claim that female *F. occidentalis* copulate once and that samples of copulated females consisted of a single sperm bundle is contrary to the observation that already copulated females were attracted to aggregation pheromone. Therefore, re-investigating the frequency of copulation in female *F. occidentalis* is relevant. The aim of this chapter is to:

1. investigate the frequency of copulation in female *F. occidentalis* and
2. identify if subsequent female copulations by virgin males lead to successful transfer of sperm. The possible reasons for multiple copulation in *F. occidentalis* are also discussed.

Hypothesis tested:

Female *F. occidentalis* copulate more than once

Multiple copulations stimulate egg production

Sperm transfer occur in subsequent copulations

4.3 Materials and methods

4.3.1 Rearing of adult 1-5 days old virgin males and virgin females

To study multiple copulations in female *F. occidentalis*, the age and virginity status of adult females and males must be established. To do this, the procedure for rearing known-age virgin adult *F. occidentalis* described in Chapter 2 was used.

Newly emerged adult thrips isolated at the larval stage (and pupa stage for thrips reared from field-collected thrips) into a modified microcentrifuge tubes (described in Chapter 2) were

fed individually with small portion of pod (about 5 mm long) with pollen. The date of emergence was written on the pots. Tubes containing individual insects that emerged the same day were placed in pots and the date of and sex at emergence were noted to avoid confusion.

4.3.2 Bioassay to test for multiple copulation

1. Will a female copulate multiple times in immediate succession?

To test this hypothesis, adult virgin male and female thrips (2-5 days old) were used. A virgin female was first placed carefully within the arena (described in Chapter 2) and covered with a glass cover slip to prevent her from escaping (see Chapter 2). The glass cover slip was carefully opened and a virgin male (2-5 days old) was introduced into the same arena within about 5 minutes. The male was placed as close as possible to the female and the arena was covered immediately to prevent the pair from escaping. After copulation, the male was removed and another virgin male of similar age was introduced to the same female within the same arena, this process continued until four virgin males had been introduced to the same female in succession. This experiment was carried out in five replicates.

2. Will a copulated female copulate multiple times over days?

In order to test if female thrips that had copulated once will copulate again after two days, 45 female thrips (3-5 days old) that had copulated once (these were some of the females used for Chapter 3 experiments) were kept individually for two days in the modified microcentrifuge tubes described in Chapter 2 and were fed individually with a small portion of bean pod with pine pollen. In order to control for age difference and to test if a two day age difference can influence the copulation response of virgin females, virgin females (3-5 days old) reared as discussed in Chapter 2 were individually introduced into an arena at a similar period when the experiment in Chapter 3 was carried out. Each was exposed to the

microscope light source for 10 min, but no male was introduced into the arena. They were kept individually for two days in the modified microcentrifuge tubes described in Chapter 2 and were fed individually with a small portion of pod with pollen. Two days later, each of these females both virgin and copulated (now 5-7 days old) was placed one after the other carefully within the arena and covered with a glass cover slip to prevent her from escaping (see Chapter 2). The glass slip was carefully opened and a virgin male (3-5 days old) was introduced into the same arena within 5 minutes and the arena was covered immediately to prevent the pair from escaping. The arena containing the thrips was placed on the stage of the camera mounted on a dissecting microscope (see Chapter 2) and recorded for approximately 10 minutes. They were however left for over 10 minutes if the pair were still copulating until copulation was concluded.

Using the same procedure described above, 20 of the already copulated females that copulated again from the experiment above were kept for a further 3 days (now 8-10 days old) and were introduced individually to a virgin male (3-5 days old) to test if they would copulate again. All experiments were performed at a temperature of 25±1°C.

3. Will a single copulated female copulate again after two days if field samples were used for breeding?

In order to test if the repeated copulation and reduction in copulation duration observed after initial copulation (results from the above experiment) were due to inbreeding that might have been associated with laboratory colonies of thrips used for this research, we used thrips samples sourced from semi-protected strawberry in the United Kingdom. The test was necessary because the laboratory colonies used for the above experiments have been maintained at Keele University for about 20 years.

Samples collected were allowed to lay eggs on bean pods with pine pollen using the same procedure described in Chapter 2. A total of 17 adult virgin males and females (3-5 days old) reared from this field sample copulated (as described for the laboratory colony sourced experiment above) and 12 of the females that copulated were kept for two days in the modified microcentrifuge tubes described in Chapter 2 and were fed individually with a small portion of pod with pollen. After two days, 12 of the females that copulated initially were individually introduced into an arena and a virgin male (3-5 days old) was introduced to her using the same method described above for the laboratory-colony sourced experiment.

4.3.3 Observations

The first experiment to investigate multiple copulation in immediate succession was was video recorded and saved on a SD memory card. The durations of copulations were measured by playing back the video using VLC media player (VideoLAN, Paris). The second experiment to investigate multiple copulation over days was carried out between 08:00 and 13:00 h for consistency in timing and behaviour over several days. This was to avoid changes in behaviour that may be associated with time of day. What happened before and during the copulation process was recorded and saved on a SD memory card. The video was later played back to identify and measure the behaviours displayed in the pre-copulation, copulation and post-copulation phases. In some of the pairs, some behaviours could not be measured because of difficulty of viewing under the microscope (e.g. for those that copulated on the roof of the arena, it was difficult to see the stroking) during copulation. Such behaviours were not included in the analysis.

4.3.4 Does multiple copulation stimulate egg production by females?

Female insects may copulate multiple times to receive material benefits from males. This material benefit may include protein from male accessory materials that she can use for egg

production. To test if copulation or multiple copulations stimulate egg production, 50 virgin females (4-6 days old) were used for the experiment. The test was carried out by modifying the design used by Kirk (1985c). The design consisted of an oviposition tube (30 mm x 13 mm diam.) placed in a humidity tank (Figure 4.2). The oviposition tube was prepared using a 50 mm x 13 mm polythene tube (5 ml tube with a lid snapped on the tube, Agar Scientific Ltd., Essex, UK). Each of the tubes was cut into 30 mm length. A hole (about 3 mm diam.) was drilled 20 mm from the top (Figure 4.2b)). The hole was made thrips proof by using a Kimwipes Lite Precision Wipes (Kimberly-Clark Ltd., Kent, UK) sealed in place with a clear adhesive tape (Figure 4.2b). The adhesive tape had a hole of about 3 mm diameter melted into it, using a hot metal rod. The hole allowed for ventilation and prevented condensation, while the whole arrangement kept the thrips from escaping. The top of the oviposition tube was covered with a stretched Parafilm membrane (American National Can ™, Greenwich, USA) with Parafilm sealed around the tube. An adhesive tape (18 mm wide) was rolled round the top edge of the tube to create a reservoir of about 10 mm deep above the stretched Parafilm (Figure 4.2a). A silicone rubber 'O'-ring was rolled round the adhesive tape overlapping the Parafilm in order to prevent leakage of water from in between the Parafilm and the adhesive tape (Figure 4.2a).

In order to test if copulation stimulates oviposition in *F. occidentalis,* 30 virgin female thrips were copulated individually with individual males collected from the culture maintained in the laboratory. The males were collected by tapping the flower head into a collection bowl and often covered until needed to prevent them from escaping. Males were carefully picked using a moist brush. The remaining 20 thrips were left as virgins. From the inception of the experiment, 20 out of the 30 copulated females were designated to be used for comparing the number of eggs laid with that of the virgin females. Each of the tubes was

placed in a humidity chamber (Figure 4.2c). The humidity chamber was a square glass tank (300 mm x 257 mm x 87 mm) containing a plastic grid with wooden

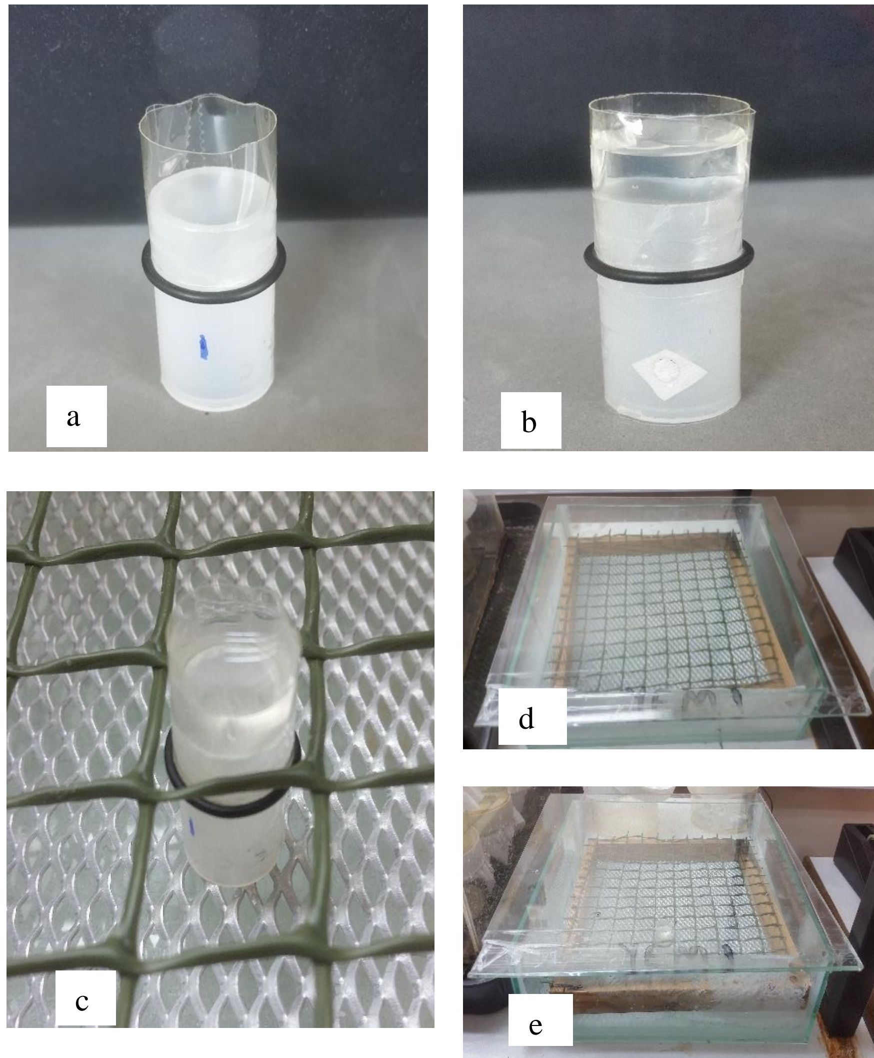


Figure 4.2 Materials used for oviposition experiments (a) the oviposition tube (b) the oviposition tube with reservoir containing deionised water (c) oviposition tube supported by the plastic grid (d) The humidity tank (e) The humidity tank containing one oviposition tube.

edge (280 mm x 225 mm) rested on a zinc mesh (293 x 239 mm). The mesh was placed on four square solid platforms (19 mm high), at the four edges of the tank within the humidity chamber. The humidity chamber contained potassium tartrate saturated solution (about 10 mm deep). This solution helps to maintain the humidity at about 65-70 % humidity and 26 ±1 ºC. Each of the thrips was placed carefully in the oviposition tube from the base and the base was closed immediately with a snap-on lid. The oviposition tubes containing the thrips were arranged individually within the supporting grid in the humidity chamber (example Figure 4.2c). About 1 ml of deionised water was added to each of the oviposition tubes in the upper reservoir part formed using the adhesive tape (Figure 4.2b). The humidity chamber was covered with a plate of glass (350 mm x 300 mm) which helped to maintain the humidity in the tank.

The insects were not fed on pollen or any other food source throughout the experiment in order to allow the insects to become protein starved. This was on the assumption that if the reason for multiple copulation in female was to receive more protein from males, then those that were later copulated multiple times should lay more eggs when compared with those that copulated once. The insects therefore survived only on water in the reservoir throughout the period of the experiment.

After 2 days, the number of eggs laid were counted under a dissecting microscope. The number of eggs laid by virgin females were compared with those laid by 20 copulated females already designated for this experiment. Two individuals from the virgin females and three from the copulated females were excluded from the analysis because of leakage of the oviposition tube.

To test if multiple copulations influence the number of eggs laid, 30 tubes with copulated females were used. Because of the problem caused by leakage of some of the oviposition

tubes and escape of some of the thrips, not all the 30 copulated females were available for the subsequent experiment. Twelve of the copulated females were randomly selected and copulated with at least one virgin male (5-8 days old). Seven re-copulated with two males in succession while five re-copulated with one male, the remaining 13 females were not introduced to a male to copulate again. All the insects were individually placed in an oviposition tube, deionised water was added to the reservoir and the oviposition tube was placed in the humidity chamber as described above. The number of eggs laid were counted the following day.

4.3.5 At what time does sperm transfer occur during copulation?

Results from the above experiment (4.3.2) showed that female *F. occidentalis* copulate more than once but the subsequent copulations were always very short. Therefore, we investigated if these subsequent short copulations led to transfer of sperm. To do this, we set up an experiment to find out the time when a male thrips transfer his sperm to the female during copulation. Copulations between males and virgin females were interrupted at different times. The experiment was done in 2 phases. For the first experiment, five different ranges of times were used: (1) No copulation (left as virgin females) (2) Interrupted at 20-30 s (3) Interrupted at 45-55 s (4) Interrupted at 80-91 s (5) Allowed full copulation. Virgin females (4-5 days old) were used for the experiment. The order of the five treatments was randomised between block and replicated seven times.

A female was put in the arena made with dental wax and a microscope slide (described in Chapter 2) and covered with a glass cover slip. Males from the laboratory culture were used for the experiment. Males and females were tapped from the flowers used for rearing in the laboratory into a collection bowl. Individual males were picked up using a fine trimmed brush, introduced into the arena containing the female and covered with a glass cover slip.

The stop watch was started immediately, and the pair was viewed under the dissecting microscope. At about the appropriate time to interrupt copulation the arena was carefully carried after the cover slip had been removed. At the specified time interval, the copulating pair was tapped into a petri dish that was placed in a bowl containing ice. Almost immediately after landing on the already cold petri dish, the timing was stopped. At this point, the couple became anaesthetic (completely motionless). The assumption here was that all internal activities also stopped once they became completely motionless. A fine trimmed brush was then carefully used to separate the couple (usually within less than 2 minutes after the female was dropped into the cold petri dish) and the female was kept in a modified centrifuge tube (described in Chapter 2). Pollen grains and a bean pod section (about 1-1.5 cm long) were added as food source and oviposition site. After about 6-7 days, all the females were re-copulated (including the virgin females and those that copulated full time) with virgin males (2-4 days old) and the complete copulation duration was measured. The eggs laid by these females before the subsequent copulations were also reared unto adult to determine the sex of the majority of the offspring. *Frankliniella occidentalis* practices arrhenotokous mode of reproduction, virgin female *F. occidentalis* lays unfertilized eggs that result in males while fertilised females lay eggs that results in both males and females. Therefore, if the eggs laid were males then it suggests that sperm was not transferred to the spermatheca of the female.

Another follow up experiment was done because the result of the experiment above suggested that male sperm did not fertilise the female when copulation was between 0 and 91 s because they produced offspring that were males. The aim was to test if fertilisation will occur in females when copulation process was interrupted after it has been ongoing for more than 91 s. In this experiment, virgin females (5-8 days old) were allowed to copulate individually with males for a longer time than in the previous experiment. The copulations

were interrupted at 88-91 s, 148-151 s, 202-214 s. The experiment was carried out as discussed above but the re-copulation was carried out 13 days after the first copulation. To prevent the females from copulating with any male that could have emerged earlier, the females were removed before the 13th day into another modified centrifuge tube and small portions of pods and pollen were added.

4.3.6 Statistical analysis

Minitab 17 (Minitab Inc., USA) was used for performing one-way ANOVA analysis. The copulation durations were compared using one-way ANOVA. Residuals were tested for normality using the Anderson-Darling normality test and Lavene's test was also carried out to test for equal variances. Mean were separated using Tukey. When the residuals were not normally distributed, the Kruskal-Wallis test was used and mean separated using Holm's adjusted *P* values. Chi square was used to test for differences in proportions using IBM SPSS Statistics 21 (IBM Corp., New York).

4.4 Results

4.4.1 Will a female copulate multiple time in immediate succession?

When four virgin males were introduced to a female in immediate successions, they copulated with the female (100% repeat copulation). The duration of subsequent copulations of males with the same female after the first copulation of a virgin male and a virgin female were however significantly shorter than the first copulation duration (One-way ANOVA, $F_{(3,15)} = 782$, $P = <0.0004$). The mean duration for the first copulation was 201s while for the subsequent copulations, the mean copulation durations ranged from 39-47 s (Figure 4.3). The fourth copulation of one of the females lasted for 466 s. This was unusually long and was considered an outlier. This male was tried with another virgin

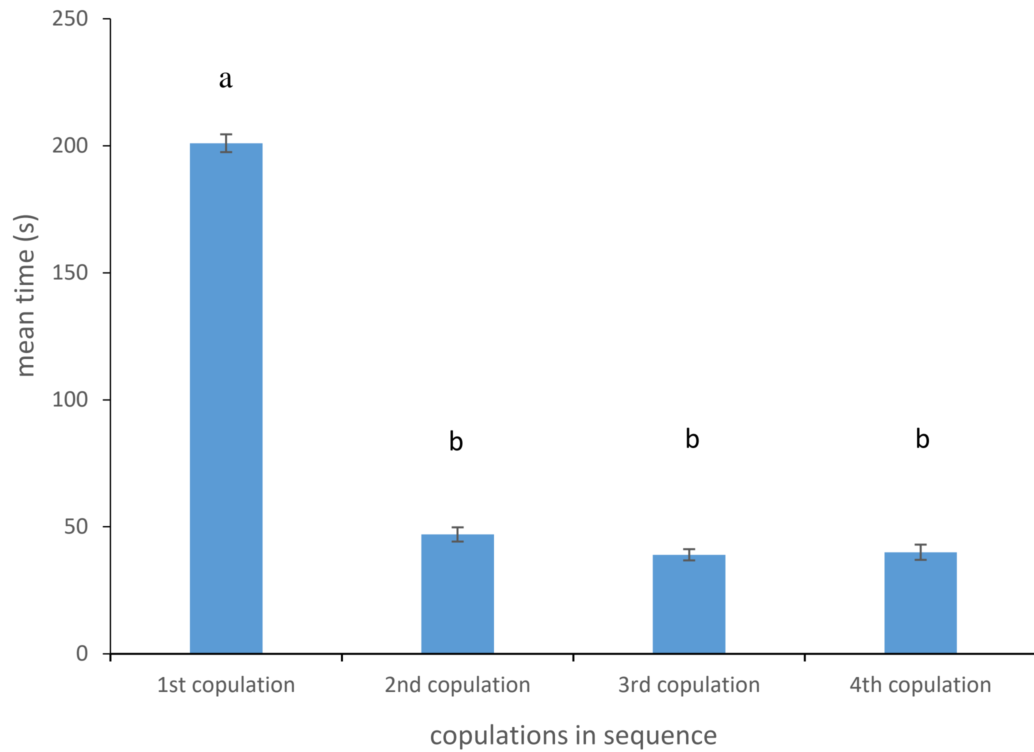


Figure 4.3 The duration of copulation of females ± SE, when 4 virgin males were introduced to the same female in immediate succession. Virgin males and virgin females were 2-5 days old. (One-way ANOVA, $F_{(3,15)} = 782$, $P < 0.0004$). Means with the same letter are not significantly different from one another. n=5.

female but could not copulate. The male was probably wounded resulting in this excessively long copulation. However, when this extreme value was included in a non-parametric analysis, it did not affect the result. The duration of subsequent copulations of males with the same female after the first copulation of a virgin male and a virgin female were also significantly shorter than the first copulation duration (Kruskal-Wallis Test, $H = 9.9$, df = 3, $P = 0.02$).

4.4.2 Will a female copulate multiple times over days?

When females were left for 2 days after first copulation as virgin, the proportion that copulated again was 64 % (Table 4.1**).** About a quarter of the 36 % of females that did not copulate again did not make any contact with the male in 10 minutes. There was a significant difference between the proportion that copulated and those that did not copulate during the first and second copulation trials ($\chi^2_{(1)}=5.7$, $P=0.03$). The number of replicates for 3rd copulation (already copulated twice) sets was low when compared with the 1st and 2nd copulation sets, so the chi square analyses were not reported. The median copulation duration also decreased significantly after the first copulation (Kruskal-Wallis Test, $H = 77.2$, df = 2, $P < 0.001$), but the copulation duration of those that had copulated once previously was not significantly different ($P > 0.05$) from the subsequent copulation three days later (Figure 4.4). The range of copulation durations for subsequent 2nd and 3rd copulations were 6 -154 s and 10-101 s respectively (Figure 4.5). In pairs where copulation did not take place and there was contact, the females were observed to either reject the males or the male just climbed over the female without bending his abdomen beneath that of the female.

Pre-copulation activities were similar between first copulation and subsequent copulations, suggesting that most of the actions during the pre-copulation periods were random and not specific to the copulation status of the female (Tables 4.2 and 4.3). There were no significant

Table 4.1 The number of females that copulated when virgin females (3-5 days old) were introduced to virgin males (3-5 days old) and re-copulated after two and five days. Only the females that copulated the first time were tested again for second copulations and only those that copulated the first and second times were tested for the third copulations. There was a significant difference in the proportion of females that copulated between the 1st copulation trial (virgins) and the 2nd copulation trial (non-virgin) ($\chi^2_{(1)} = 5.7$, P=0.03).

Mating status	1st copulation trial (3-5 days old)	2nd copulation trial (5-7 days old)	3rd copulation trial (8-10 days old)
Copulated	65 (83%)	29 (64%)	15 (75%)
Did not copulate	13 (17%)	16 (36%)	5 (25%)

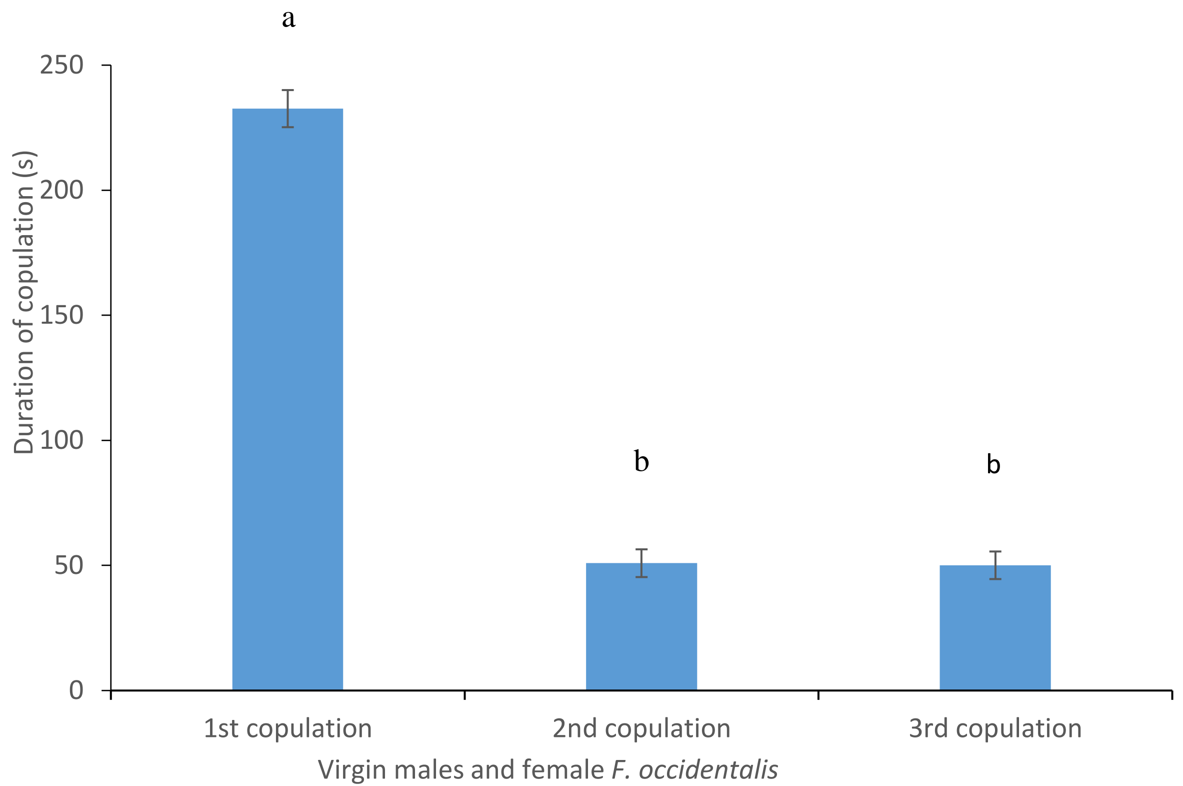


Figure 4.4 The mean duration (± SE) of 1st copulation (virgin male and female, 3-5 days old), 2st copulation (Virgin male, 3-5 days old and copulated female 5-7 days old (these were females that copulated at first introduction)) and 3rd copulation (Virgin male, 3-5 days old and copulated females 8-10 days old (these were females that copulated at first and second introductions)). Kruskal-Wallis Test, H = 77.24, df = 2, P <0.001. Mean with the same letter are not significantly different from one another.

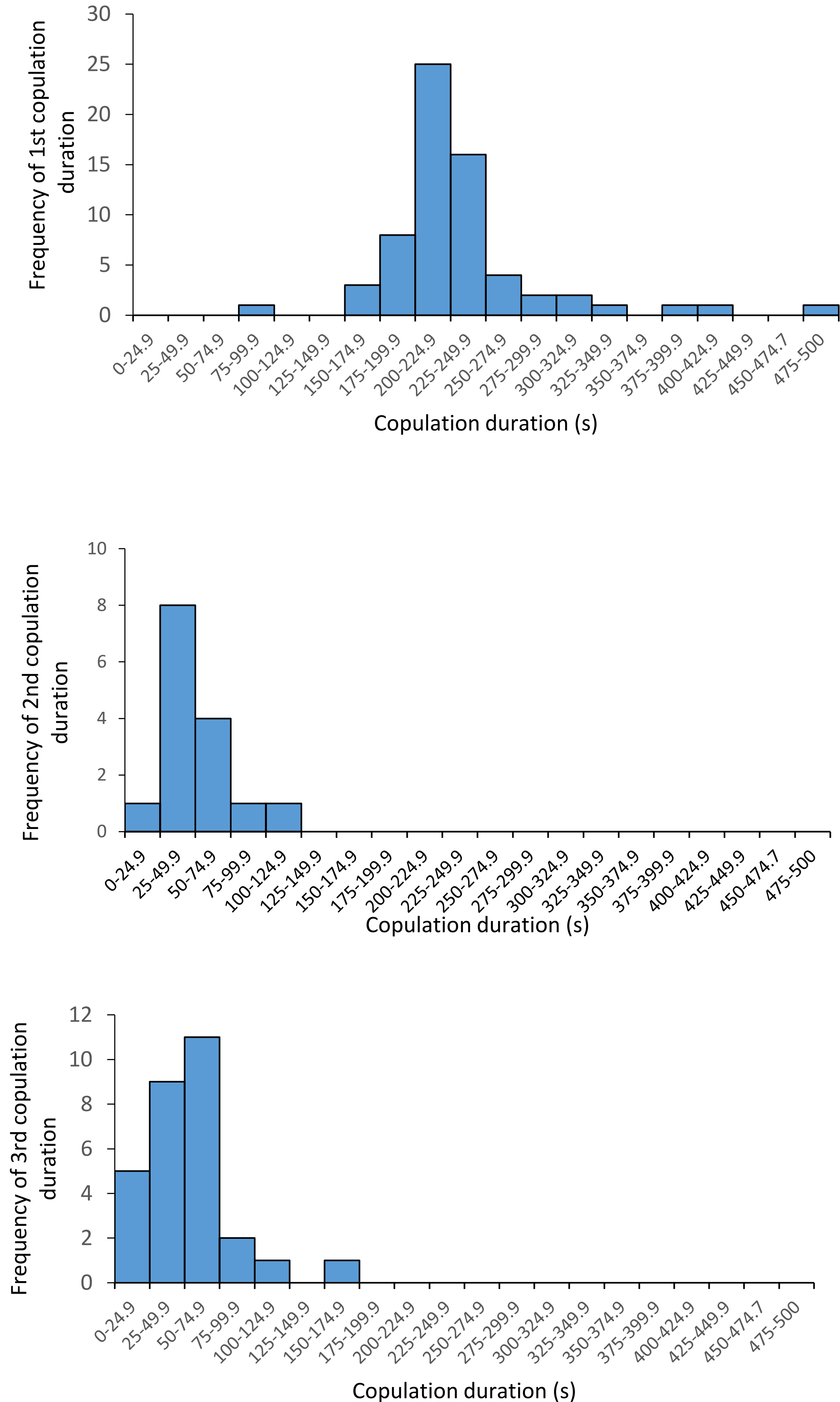


Figure 4.5 The histogram of copulation durations for 1st , 2nd and 3rd copulations1st copulation (virgin male and female, 3-5 days old), 2nd copulation (virgin male, 3-5 days old and copulated female 5-7 days old, these were females that copulated at first introduction) and 3rd copulation (virgin male, 3-5 days old and copulated females 8-10 days old, these were females that copulated at first and second introductions).

Table 4.2 Duration of activities during pre-copulation and copulation phases in virgin male and female *F. occidentalis* that has previously copulated once. The activities during the copulation phase did not add up to total copulation duration because some of them overlapped.

Activity	**Mean duration ±SE (s)**	**Range**	**N**
PRE-COPULATION			
Total Pre-copulation phase	114±29	4-556	27
Time to first contact	92±28	0-543	27
First contact to start of climb	18±11	1-287	27
Start of climb to end of climb mating	4±2	0-63	29
COPULATION			
Total copulation phase	51±6	6-154	29
Male antennation during copulation	26±6	5-156	27
Male stroking female	34±6	0-136	22

Table 4.3 Duration of activities during pre-copulation and copulation phases in virgin male and female *F. occidentalis* that has previously copulated twice. The activities during the copulation phase did not add up to total copulation duration because some of them overlapped.

Activity	**Mean duration ±SE (s)**	**Range**	**N**
PRE-COPULATION			
Total Pre-copulation phase	96±24	16-355	14
Time to first contact	89±24	0-349	14
First contact to mounting	5±2	0-27	14
Mounting to mating	3±1	0-16	15
COPULATION			
Total copulation phase	50±6	10-101	15
Male antennation during copulation	23±6	8-76	15
Male stroking female	33±7	0-90	11

differences in time to first contact between virgin male with virgin female and virgin male with once copulated female (2 days later) and virgin male with twice copulated female (2 and 5 days later) (Kruskal-Wallis Test, $H = 2.16$, df = 2, $P = 0.34$).

However, at the copulation phase, in addition to the general reduction in the copulation duration after first copulation, there were differences in the relative duration of some of the activities (Figure 4.6). A male used an average of about 50 % of the copulation time for antennating a female and about 67 % of the copulation time was used for stroking her during subsequent copulations, this was proportionately longer than during the first copulation when an average of about 27 % of copulation duration was used by a male for antennating a female and about 56 % for stroking a female (Figure 4.6). Generally, the stroking and antennation durations were shorter in absolute term in subsequent copulations when compared to the initial one (virgin male and virgin female (Figure 4.6).

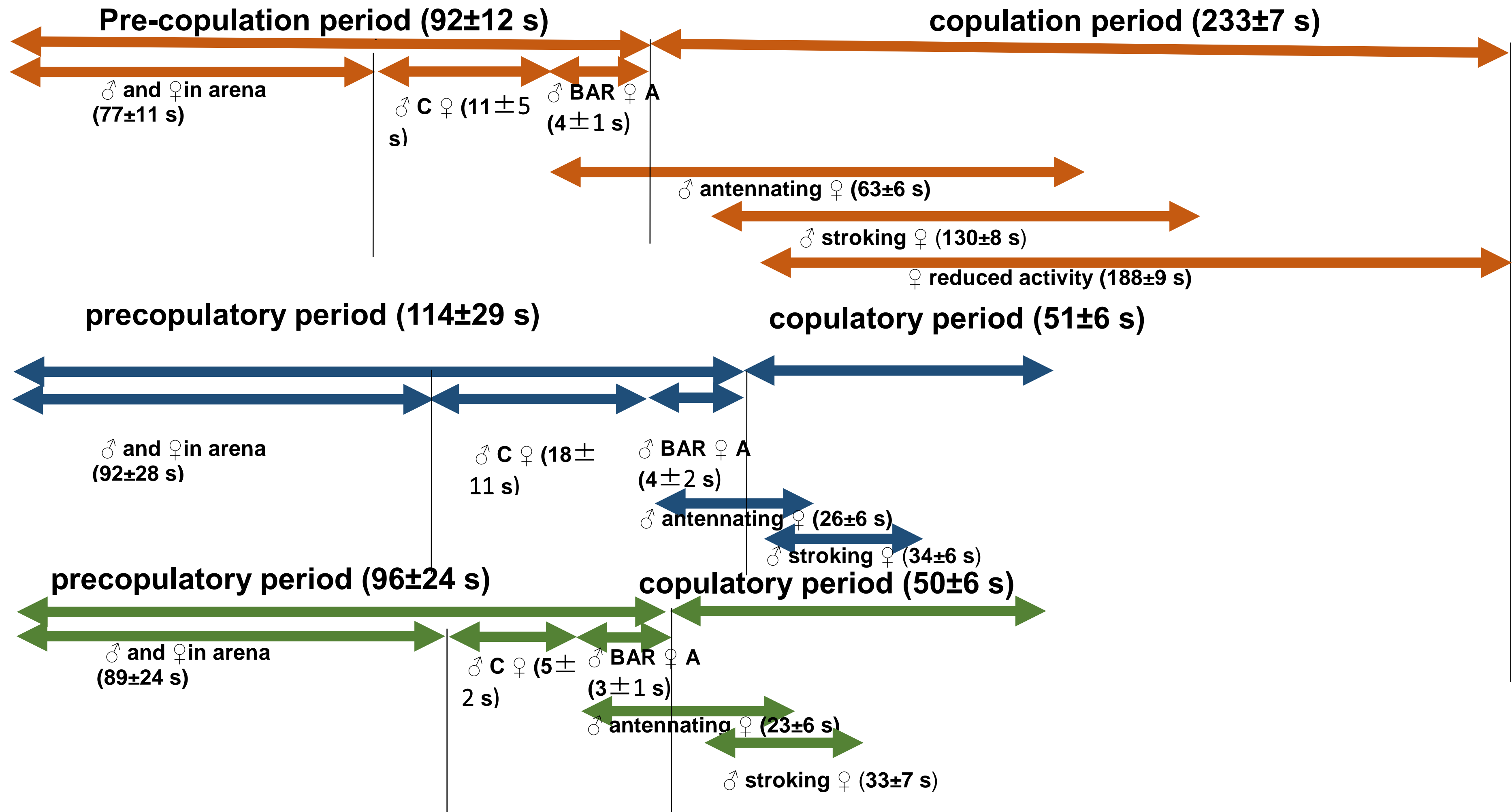


Figure 4.6 Sequence of copulation behaviour in virgin male and virgin female, virgin males and once copulated females and virgin males and twice copulated females *F. occidentalis*. c =climbed; A=Abdomen BAR = bend abdomen round female abdomen. Specific behaviours in a few of the samples could not be observed due to position during copulation in the arena or difficulty of viewing. The numbers are mean ± standard error.

4.4.3 Could the observed re-copulation and reduction in copulation duration after initial copulation be due to age difference or because of possible inbreeding in laboratory colonies?

The proportion of virgin females (3-5 days old) that copulated with virgin males (3-5 days old) was not significantly different from the proportion of virgin females (5-7 days old) that copulated with virgin males (3-5 days old) ($\chi^2_{(1)} = 0.0$, $P = 1.00$) (Table 4.4)**.** The mean copulation durations were 219 and 233 s respectively. These two copulation durations were not significantly different from one another (Mann-Whitney, $W = 2696.5$, $P = 0.43$).

There was no significant difference in the proportions of virgin females (3-5 days old) that copulated and re-copulated two days later with virgin males (3-5 days old), regardless of whether males and females used for rearing virgin males and females were laboratory-sourced or field-sourced ($\chi^2_{(1)} = 1.685$, $P = 0.29$ and $\chi^2_{(1)} = 0.021$, $P = 1.0$ respectively) (Table 4.5). There was also no significant difference between the copulation durations at first copulation and subsequent copulation regardless of whether males and females used for rearing virgin males and females were laboratory- sourced or field-sourced (Mann-Whitney, $W = 2698.5$ $P = 0.70$ and Mann-Whitney, $W = 501.0$, $P = 0.07$ respectively) (Figure 4.7)

4.4.4 Does multiple copulation stimulate egg production by females?

We did not find any evidence that single or multiple copulations stimulate egg production. The average rate of egg production was generally low (< 2 eggs / 2 days) (Figure 4.8) because all thrips used survived only on water (without pod and pollen) throughout the experiment. There was no significant difference in the number of eggs laid regardless of whether the females were virgin or had copulated once with males (Mann-Whitney, $W = 291$, $P = 0.25$). There was also no significant difference between the number of eggs laid when females were

Table 4.4 The number of females that copulated when virgin females (3-5 days old) were introduced to virgin males (3-5 days old) compared with the number of females that copulated when virgin females (5-7 days old) were introduced to virgin males (3-5 days old). $\chi^2_{(1)} = 0.0$, $P = 1.00$

Mating status	3-5 days old females	5-7 days old females
Copulated	65 (83%)	15 (83%)
Did not copulate	13 (17%)	3 (17%)

Table 4.5 The numbers and percentages of copulation of both field-sourced and lab-sourced reared thrips at both 1st copulation (virgin males and virgin females) and 2nd copulation (virgin male and copulated females). Only the females that copulated the first time were tested again for second copulations. Pearson Chi-Square between copulation status and the field and lab sourced thrips for 1st and 2nd copulations ($\chi^2_{(1)}$ = 1.69, P = 0.29) and ($\chi^2_{(1)}$ = 0.02, P = 1.00) respectively. 1st copulation trial (virgin male and female, 3-5 days old), 2nd copulation trial (Virgin male, 3-5 days old and copulated female 5-7 days old (these were females that copulated at first copulation trial).

	Laboratory	Field
1st Copulation trial		
Copulated	65 (83%)	16 (94%)
Did not copulate	13 (17%)	1 (6%)
2st Copulation trial		
Copulated	29 (64%)	8 (67%)
Did not copulate	16 (36%)	4 (33%)

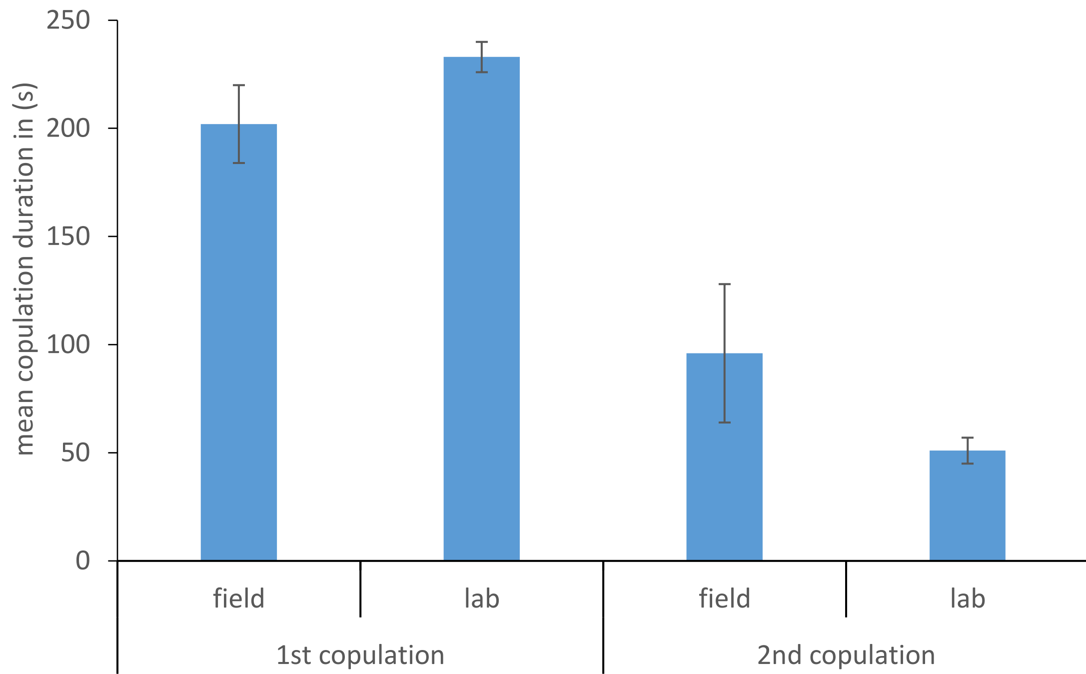


Figure 4.7 The mean copulation durations of both field-sourced and lab-sourced reared thrips at both 1st copulation (virgin males and virgin females) (Mann-Whitney, W = 2698.5 P= 0.70) and 2nd copulation (virgin male and copulated females) (Mann-Whitney, W = 501.0 *P*= 0.07).

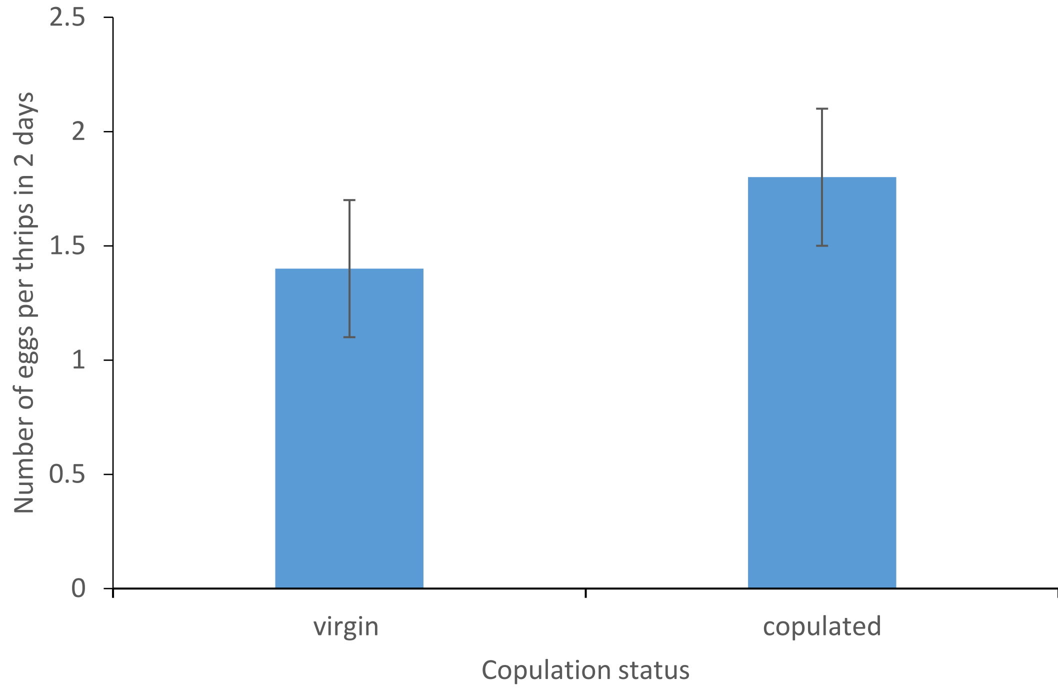


Figure 4.8 The number of eggs ± SE laid by virgin (n = 18) and copulated females (n = 17) after two days. Females were 4-6 days old. (Mann-Whitney, W = 291, *P* = 0.25)

copulated once or more than once (Mann-Whitney, W = 291, P = 0.25) (Figure 4.9). It is possible that the number of replicates used was too small to detect a difference.

4.4.5 At what time does sperm get transferred during copulation?

In order to find out the time at which male sperm was transferred into the spermatheca of females during copulation, copulating males and females were interrupted after different durations (virgin, 20-30 s, 45-55 s, 80-91 s, full copulation). They were then re-copulated again at six to seven days. The mean copulation durations for subsequent copulations for those whose initial copulations were interrupted at 20-30 s, 45-55 s, 80-91 s were not significantly different from females that were left as virgins before subsequent copulation (Figure 4:10). These means were however significantly different from the mean of subsequent copulation duration (63 s) for females that were allowed to complete copulation without interruption (Figure 4.10).

When the eggs laid by females were reared to adult, only females with full copulation (without interruption) produced females (Table 4.6). Though one female was found in just one replicate in both virgins and females interrupted at 20-30 s. This female was likely mistakenly introduced into the culture during counting or possibly at the larva stage.

When the duration of copulation between a male and a female was further interrupted at different durations (88-91 s, 148-151 s, 202-214 s), this similar experiment confirmed that sperm was not transferred until after 91 s. In this experiment, after copulations were interrupted at 88-91 s, 148-151 s, 202 - 214 s they were re-copulated again at 13 days later to check if the copulation duration was reduced as had been observed in females that had already copulated. The mean duration (249 s) of subsequent copulation for those interrupted at 88-91 s was significantly longer than those interrupted at 148-151 s and 202-214 s with mean subsequent copulation durations of 51 s and 61 s respectively

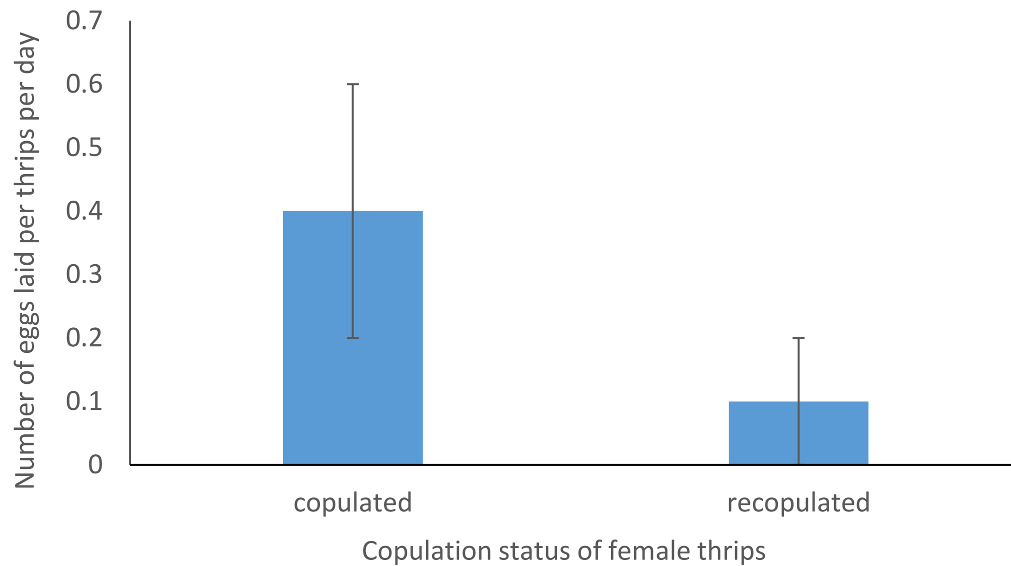


Figure 4.9 The number of eggs ± SE laid by copulated females (copulated once with a male) (n = 11) and re-copulated females (copulated again once or twice after two days) (n = 9).The copulated females were copulated with males picked from the culture. Females used were 4-6 days old. (Mann-Whitney, $W = 128$, $P = 0.23$).

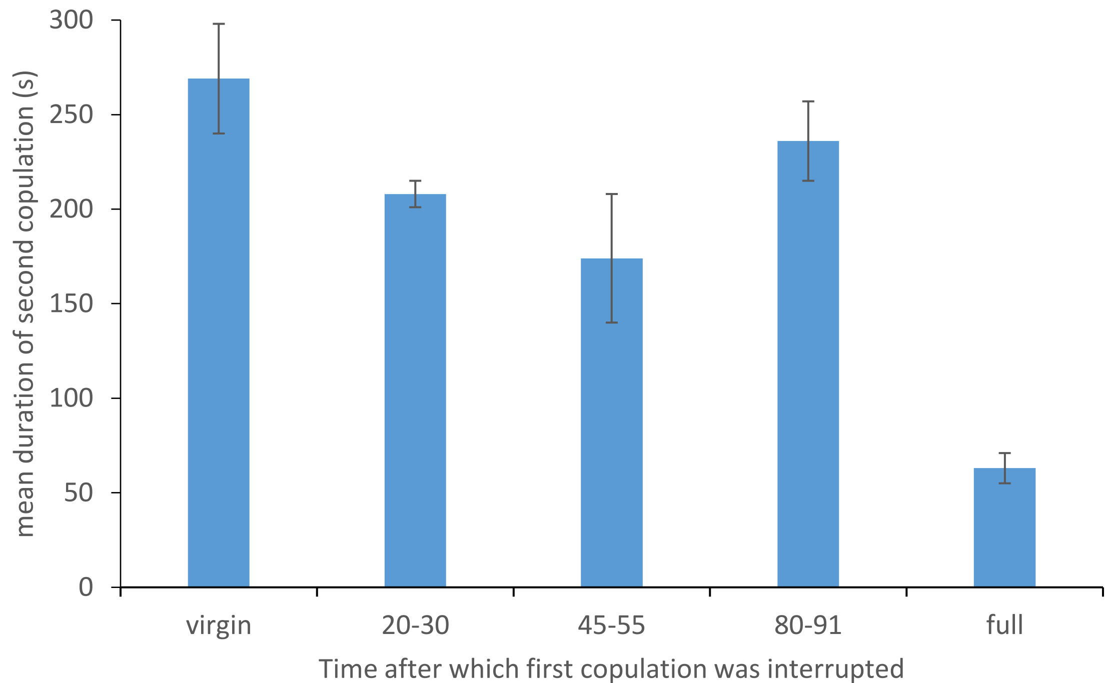


Figure 4.10 The mean duration of copulation ± SE. These mean durations were for repeated copulations at 6-7days after initial copulations of virgin females (4-5 days old) were interrupted at different durations. Subsequent copulation was full copulation without interruption and was done with virgin males (2-4 days old). There were two controls, virgin: females that did not copulate at all the first time, full: Females that were allowed complete copulation without interruption at the first time. Mean with the same letter are not significantly different from one another. n=5. (H = 15.91, df = 4, P = 0.003).

Table 4.6 Minimum number and sex observed from the offspring produced when copulations between a culture male and virgin female were interrupted after different durations. Interuptn: Interruption durations. S: Sex, Full cop: Full copulation * Females here may be contamination during rearing process.

	Replicates													
	1		2		3		4		5		6		7	
S.	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀
Interuptn														
20 s -30 s	20	**0**	13	1*	7	0	8	0	13	0	9	0	8	0
45 s-55 s	10	0	16	0	14	0	1	0	10	0	15	0	8	0
88 s-91 s	5	0	9	0	9	0	6	0	19	0	18	0	-	-
Virgin	9	0	17	0	14	0	12	1*	16	0	23	0	7	0
Full cop.	4	9	1	18	1	7	-	-	4	8	0	6	0	12

(one-way ANOVA, $F_{(2,6)} = 100$, $P < 0.0004$) (Figure 4.11). This suggests that at most after 148 s sperm has been transferred to the female. Though most of the F1 generations of the insects interrupted at different durations before re-copulation died (because the food source dried out), the few offspring that were rescued confirmed that females offspring were produced by females interrupted at 148-151 s and 202-214 s, but no female was found in samples with copulation interrupted at 88-91 s, confirming that the females interrupted at 148-151 s and 202-214 s had their eggs fertilised while the copulation interrupted at 88-91 s did not have their eggs fertilised.

The short subsequent copulation duration observed after 13 days in females with copulation interrupted at 148-151 s and 202-214 s suggests that short copulation in a copulated female (4.4.2) persists for most of their adult life.

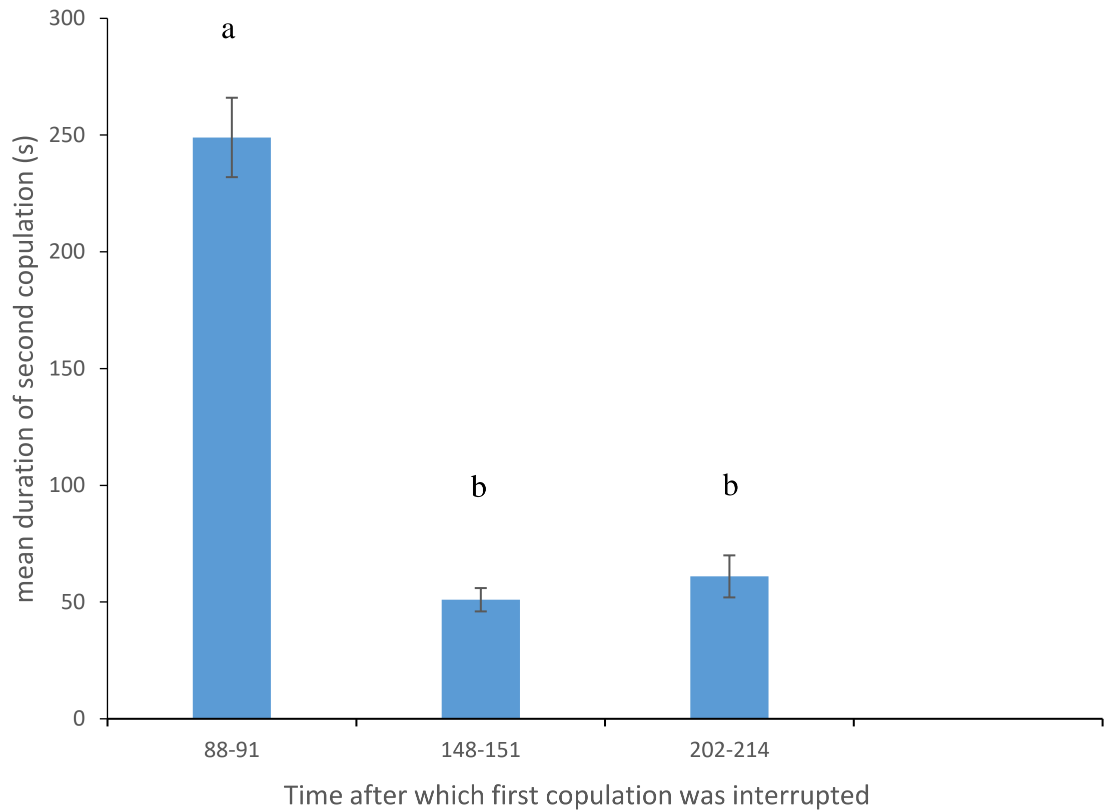


Figure 4.11 The mean duration of copulation ± SE 13 days after the initial copulations of males with virgin females (5-8 days old) were interrupted at different durations. Subsequent copulations were done with virgin males (3-4 days). (One-way ANOVA, $F_{(2,6)} = 100$, $P<0.0004$). Means with the same letter are not significantly different.

4.5 Discussion

4.5.1 Does virgin female *F. occidentalis* copulate multiple times?

It has been widely assumed that a female *F. occidentalis* refuses further copulations for at least five days after her first copulation (Terry & Schneider, 1993; Li *et al.*, 2015b; Terry & Schneider, 1993; Terry & Dyreson, 1996; Terry, 1997; Olaniran *et al.*, 2013; Li *et al.*, 2014). This claim originated from research carried out by Terry & Schneider (1993). However, it was observed in this chapter that virgin female *F. occidentalis* copulated multiple times with virgin males regardless of whether males were introduced to the female in rapid succession or spaced apart over a minimum of 2 days between copulations. In our research, all males used were virgins with up to 45 replicates tested while Terry & Schneider (1993) used both virgin males and males isolated for three days and the number of replicates tested was only eight. The difference in observation may have to do with copulation status of the males used as this may influence copulation response to an already copulated female (Chapter 5). It may also be due to the low number of replicates tested in their research. Re-copulation in female *F. occidentalis* has been previously observed to occur in the field especially with an extra male that remained on the female while the initial copulation was going on (Terry & Dyreson, 1996). This study therefore followed up from where Terry & Dyreson (1996) stopped and showed for the first time a clear demonstration of multiple copulation by female *F. occidentalis* with different males in a controlled environment both in immediate successions and over days.

Female re-copulation frequency with virgin males was as high as 64% in the laboratory. This high rate of re-copulation suggests that these females were receptive to virgin males. Most of the females left for two days after first copulation accepted copulation from virgin males almost immediately without consistent flipping of abdomen to dislodge the males. An

unreceptive female could consistently flip her abdomen to reject a virgin male regardless of how persistent the male may be (up to 40 flips in 10 minutes was observed in a female that did not copulate with a male). This therefore implies that re-copulation in most of the females was not to avoid male harassment which may be associated with aggressiveness in a virgin male attempting to copulate with a female. The field observation by Terry and Dyreson (1996), showed that a female entered a swarm and copulated with the first male she encountered and only 13.9 % of those females observed copulated more than once. The differences in percentage re-copulation may be because of the complexity of field situation and the difficulty of knowing the copulation status of both males and females in the field.

This study therefore showed very clearly that a female *F. occidentalis* that accepted copulations after the first copulation, consistently had its copulation duration significantly reduced to about a quarter of the length of the copulation duration of a virgin female and virgin male. Similar reduction in the duration of subsequent copulation was observed in the field (Terry & Dyreson, 1996), this has not been experimentally demonstrated in the laboratory. The reduction in subsequent copulation duration after initial copulation has not been reported in any other thrips species where multiple copulation has been observed (Crespi, 1988a-b; Gilbert & Simpson, 2013; Li *et al.*, 2015b).

There is no indication that time to first contact indicates readiness to copulate. The time to first contact between those that copulated again (2 days later) and those that did not was not significantly different and there was also no significant difference between the time to first contact for those that copulated as virgin and those that re-copulated later. This suggest that time to first contact was random and did not reflect willingness to copulate.

4.5.2 Is copulation with an already copulated female successful?

The short copulation durations of subsequent copulations may not always be successful i.e. resulting in transfer of sperm and fertilisation of the female. When copulation between a male and a virgin female was interrupted after different durations, females interrupted at 91 s after the start of copulation produced male offspring. In *F. occidentalis*, haplodiploidy (through arrhenotoky) is the only mode of reproduction, therefore production of female offspring is an indication of successful fertilisation. A virgin female produces only male offspring. Therefore, at 91 s of copulation, sperm was not transferred to the female. The mean copulation duration of subsequent copulations in this research both in rapid successions and over days was often shorter (39-51 s) than 91 s suggesting that these copulations did not lead to sperm transfer or fertilisation in females. Terry and Schneider (1993) also noted in a laboratory experiment that a virgin female that copulated with a virgin male with a duration of less than 70 s did not produce female offspring thus suggesting that such copulations were not successful. This therefore implies that during copulation, other activities such as transfer of accessory materials to the female took place for at least 91 s before sperm transfer. In *E. americanus*, a species of thrips in which copulation lasts for over 6 minutes, accessory gland materials were first transferred to the female and spermatozoa was only transferred at about 3 minutes after phallus insertion (Krueger *et al.*, 2017).

The fact that the sperm bundle in the spermatheca of copulated females are often single and intact and always placed close to the spermatheca duct has led to the proposition that successful copulation occurs only once in most Thripidae including *F. occidentalis* (Heming, 1995). Therefore, the observed short copulation in already copulated female may imply that a male stopped the copulation process after detection of a barrier within the female. There have been suggestions that the sperm bundle and the enveloping material in Thripidae is a special form of ‘spermatophore’ that coagulates within the spermatheca of the female

(Heming, 1995; Krueger *et al.*, 2017). If the coagulating materials were produced in a male but coagulate in the female, then it is difficult to conclude that the regular observation of a single sperm bundle within most copulated females implies that females copulate only once. This is because it is possible that sperm and secretions from further copulations by males may merge to form a single coagulant with the sperm bundle that is already in the copulated female. This may be the case in a very few long copulation durations observed in females that had already copulated in this study.

4.5.3 Why is the copulation duration in a copulated female short?

Possible reasons for the reduction in the duration of subsequent copulations after the initial one include:

1. the spermatheca of the female was full of sperm, therefore sperm could not be transferred.
2. detection of a mechanical barrier in copulated female.
3. Reduced motivation after detecting the female was already mated

It is very unlikely that the reason for short copulations observed in female *F. occidentalis* (already copulated) was due to a filled female spermatheca. This is because re-copulation duration after 13 days in a female that copulated just once and had laid fertilised eggs for up to about 13 days was also short. If the reason for short copulation duration was due to filled spermatheca, then it could be assumed that the duration should increase once the sperm in the spermatheca has been depleted for some days. More so, the phallus of the male does not reach the spermatheca of the female during copulation.

The second and likely reason for short copulation duration in an already copulated female may be because the male detected a barrier during copulation. The detected barrier may be from materials transferred from the first male that had previously copulated with the female.

In *F. fusca*, the capsulated sperm bundle presumably formed in the female was called a spermatophore by Heming (1970a), and it is always attached to the spermathecal duct (Heming, 1995). Heming (1970a) observed that this 'spermatophore' has an extension termed a 'spermatophore tube' which opens up into the spermathecal duct just before the spermathecal duct opens into the dorsal side of the vagina. This suggests that there may be remains of secretions produced by a male (possibly from the materials used in the formation of a capsule round the sperm bundle) extending from the spermathecal duct into the vagina. These remains may be what was detected by the males and stopped the copulation process. Alternatively, the remains may also be a mechanical barrier preventing male accessory secretions or sperm from passing along the spermatheca of an already copulated female. Male *F. occidentalis* have two accessory glands that open into the ejaculatory bulb and produce secretions that flow together with the sperm into the bulb (Dallai *et al.*, 1997; Kumm, 2002). These secretions may therefore be involved in the formation of the mechanical barrier. The female can however control the exit of the sperm from the spermatheca through the 'spermatophore tube' (if hollow) or through the spermathecal duct with the aid of the lateral dilator muscle insertion attached to the wall of the spermathecal duct (Heming, 1970a; Dallai *et al.*, 1996). A spermatophore and spermatophore tube have been found to function as a mating plug in several species of insects, and this has been widely reviewed (Parker, 1970; Simmons, 2001), these spermatophore were however transferred to the vagina of the female where it blocks access by males. The variation in copulation duration was associated with detection of a spermatophore in the female by the male during copulation in stalk-eyed fly *Cyrtodiopsis whitei* (Lorch *et al.*, 1993).

4.5.4 Is there an antiaphrodisiac pheromone?

In this research, when a virgin male was introduced to a copulated female (2 days after initial copulation), about 27 % of the virgin males did not copulate with these already copulated females even though they made contact with the females. Some of the males climbed on the females but did not bend their abdomen beneath that of the female to proceed with copulation. This suggests that at least some of these virgin males could detect if a female had already copulated. In Thysanoptera, it is only in *E. americanus* that males have been identified to detect female copulation status using an antiaphrodisiac pheromone (Krueger *et al.*, 2016). The evidence of an antiaphrodisiac pheromone in *F. occidentalis* is discussed later in Chapter 6. It is not clear why it was only a few of the virgin males that avoided copulation with a copulated female since it is expected that males should avoid copulating with a female if he can assess her copulation status since such copulation may not be successful. This will be discussed further in Chapter 5. However, at this point we may assume that a virgin male has nothing much to lose and therefore could attempt copulation in case some of his sperm can manage to get to the female's spermatheca despite a possible barrier. Alternatively, a virgin male's assessment of an already copulated female might be somewhat inaccurate. Therefore, the male may stop progressing with the copulation process when it detects a blockage thus preventing sperm wastage as suggested by Lorch *et al*. (1993) in *Crytodiopsis whitei*.

4.5.5 Does a female benefit from multiple copulation with virgin males?

This research gave clear evidence that females copulate again with virgin males. The question is why will she do that if such copulations may not be successful considering the cost associated with multiple copulation such as energy cost committed to periods of copulation and exposure to predation (Thornhill & Alcock, 1983; Franklin *et al.*, 2012). It is

expected that the average benefit of such subsequent copulations should outweigh its negative impact (Walker, 1980; Arnqvist & Nilsson, 2000) otherwise it will not be advantageous. One possible benefit a female may get from multiple copulation is that such copulations produce a substance which contributes to egg production, fecundity, oviposition and egg viability in females (Boggs & Gilbert, 1979; Hou & Sheng, 1999; Singh & Mishra, 2010). No increase in fecundity was observed when females were copulated multiple times and compared with females that copulated only once, though the number of replicates used may be too small to draw any conclusion yet and this will require further investigations. However, since it is likely that males transfer accessory secretions first to the female before sperm transfer in *F. occidentalis* as observed in *E. americanus* (Krueger *et al.*, 2017), then copulated female *F. occidentalis* may trick the male to release such material for her benefit. This research however did not investigate if males transfer any material to a female that has already copulated but this question may be better answered by using histological methods, genetic or molecular marker and other biotechnology approaches. The limited time scope of this research did not give room for such further study.

Another reason why a copulated female may allow further copulations as observed in this study is to prevent cost associated with male harassment or copulation attempts, a situation called 'convenience polyandry'. A high rate of harassment has been reported in females paired with virgin or mated males in *Thrips tabaci*, a species in which females copulate multiple times (Li *et al.*, 2015b). A female may therefore re-copulate if the cost of avoiding harassment is more than the cost of re-copulation (Rowe *et al.*,1994) Male harassment may however not be the only reason for multiple copulation in female *F. occidentalis* considering the fact that already copulated females were likely attracted to aggregation pheromone produced by male *F. occidentalis* (Hamilton & Kirk, 2003; Dublon, 2009; Olaniran, 2013).

Why will a female that has already copulated be attracted to an aggregation of males if she is trying to prevent harassment from such males?

4.5.6 Sequence of activities during copulation

Observation from this study showed that copulation can be interrupted by dropping a copulating couple on a cold petri dish placed permanently on a bowl of ice and carefully separating them using a fine brush. Fertilisation of virgin females did not take place when the females were allowed to copulate for 91s (they produced male offspring only) while fertilisation took place in those that were allowed to copulate for 147 s (they produced male and female offspring). As stated earlier, the first 91 s may be for the transfer of accessory material to the female but the ‘delay’ in sperm transfer may also have to do with a male preparing or making a female ready for sperm transfer or even the male preparing for the transfer of sperm. It is however interesting to note that the male stroked the female for an average of 130 s (stroking starts about 4 s after the male has secured attachment (Chapter 3). This end of stroking time was between the first 91–147 s of copulation, during which sperm was observed to be transferred to the female. This may therefore suggest that a male stopped stroking the female after sperm was transferred to the female, thus affirming the role that male stroking may be playing in ensuring that a female allowed a male to successfully transfer sperm. Reduction in antennation and stroking duration in copulated females when compared with a virgin female is also an indication that these two behaviours may be associated with successful sperm transfer. Although in a male where stroking did not occur because the female was not calm (she was walking around), sperm was still transferred to the female.

Chapter 5

Variation in copulation behaviour in males and females

5.1. Introduction

5.1.1. Factors affecting male copulation behaviour in insects

In most organisms, males invest less in parental care when compared to females. Males therefore compete for females while females choose among males for the male that is worth her investment (Thornhill, 1979). A male's reproductive success therefore depends on the number of successful inseminations or availability of receptive females while females tend to discriminate among males thus resulting in what was described as an 'undiscriminating eagerness in males and discriminating passivity in females' (Bateman, 1948). This is however not always the case, for example, in species where males contribute to paternal care, female-female competition and male mate choice may result in 'sex role reversal' (Gwynne, 1991). If a male invests more than a female in parental care or mating effort there may be selection for male choice (Edward & Chapman, 2011). In addition, a situation where a male contributes other materials apart from sperm during copulation results in a 'partial role reversal' and a male mate choice with female choice and/or male competition may result (Bonduriansky, 2001).

Male choice is however possible in some species where males do not contribute to parental care (Edward & Chapman, 2011). If, for example, the benefits of accessing a female are more than the cost and there is variability in the quality of available females, then male choice will occur (Edward & Chapman, 2011). In species where males do not contribute to parental care, the conditions of either the male or the female or even both sexes, such as their ages, quality of the female and available male sperm resources might necessitate the need for judicious male sperm allocation. In *Drosophila melanogaster*, a male tends to become choosy as resources become limiting due to the change in its condition (recently mated) (Byrne & Rice, 2006). This therefore suggests that a subsequent mating investment is expected to favour male choice in a situation where investment reduces with additional copulation (Bonduriansky, 2001). In the scorpion fly *Panorpa cognata*, males show variation and strategic allocation of sperm; choosiness for high- quality females was only observed in males with limited resources or in poor condition (Engqvist & Sauer, 2001). Similarly, in the parasitoid wasp *Trichogramma turkestanica,* males that were not fed were choosier than those that were fed (Martel *et al.*, 2008) and even in a fish (*Pseudomugil signifier*), males have been shown to respond adaptively to change in cost of choosing (Wong & Jennions, 2003).

Another important factor that may determine male choice is the level of male experience. That is, whether males are kept continuously with females (experienced) or isolated (not experienced). Males that were deprived of females tended to be less choosy than males exposed to females thus suggesting that level of experience of a male can result in male choice (Baxter *et al.*, 2015). Though it is believed that insects unlike birds and mammals do not learn in relation to sexual behaviour, since they have a short life as adults and may often copulate immediately after eclosion cycle (Alexander *et al.*, 1997), recent evidence has

shown the role of insect learning in sexual behaviour and choice in *Drosophila* (Dukas, 2005a-b, 2006).

One of the conditions for male choice is variability in the quality of available females (Edward & Chapman, 2011). Males generally allocate more sperm to females with higher fertilisation potential which is often dependent on their condition and age (Wedell *et al.*, 2002). Male *Panorpa cognata* transfer more sperm to females from high nutrient treatments than to females that are in poor condition (Engqvist & Sauer, 2003). The reason for such strategic sperm transfer may be because females in good condition and that are well fed are likely to lay more eggs than females in poor condition. Similarly, some male insects tend to have preference for younger females over older ones (Simmons *et al.*, 1994). In the bushcricket *Requena verticalis*, males prefer young to old females and males of these species have been suggested to use the age of the female as a strategy for rejecting already mated females (Simmons *et al.*, 1994). *Drosophila melanogaster* also releases more sperm to young females than to older ones (Lüpold *et al.*, 2011). Wedell *et al*. (2002) have suggested that a male may invest more sperm in a virgin female than a mated one if virgin females are readily available but mated females are scarce and unproductive. Similar results suggesting that males show preference for virgin females have been reported in other insect species (Carazo *et al.*, 2004; Martel *et al.*, 2008; Assis *et al.*, 2017).

In species of insects where females copulate once but males copulate multiple times, available females are expected to be scarce and there may be no reason for male choice (Bonduriansky, 2001), rather males may have to compete for available virgin females. In *Echinothrips americanus*, a thrips species of the family Thripidae, adult males stay close to and guard a female pupa and they eventually copulate with the female immediately after emergence (Krueger *et al.*, 2016). This behaviour clearly suggests strong competition for virgin females since most females do not copulate again with males for at least 30 days test

period (Li *et al.*, 2014; Krueger *et al.*, 2015a). In *Frankliniella occidentalis*, there may also be competition for females since fighting has been observed within male aggregation swarms (Terry & Gardner, 1990; Terry & Dyreson, 1996), though the reason is not yet clear. However, female *F. occidentalis* re-copulate multiple times with virgin males, but may be ineffective if sperm transfer does not occur (Chapter 4). Female *F. occidentalis* have been observed to copulate with the first male encountered within an aggregation in the field and then reject advances by other males (Terry & Gardner, 1990; Terry & Dyreson, 1996) suggesting that females do not choose males within an aggregation. However, no research has clearly demonstrated female or male choice in *F. occidentalis*.

5.1.2. Assessing female copulation status

A male's tendency to choose a female based on her copulation status will depend on his ability to recognise any evidence that the previous male had left, such as a mating plug or chemical cues, or through signals the copulated female emits by herself on her own cuticle after copulation (Bonduriansky, 2001). Alternatively, a male may assess a female's mating status using other cues such as age as suggested in *Requena verticalis* (Simmons *et al.*, 1994). A male is therefore able to assess a female's quality through visual, olfactory or tactile means and some species may be able to combine these mechanisms (Bonduriansky, 2001). Use of a chemical cue has great potential in mate assessment among different organisms (Johansson & Jones, 2007). Several pheromones have been identified in adult male thrips of different species (Hamilton *et al.*, 2005; Olaniran *et al.*, 2013; Akella *et al.*, 2014), and the latest being an antiaphrodisiac pheromone which a male *Echinothrips americanus* uses to assess female copulation status (Krueger *et al.*, 2016).

5.1.3. Resource allocation in male Thripidae

Multiple copulations are not uncommon in male thrips (Lewis, 1973; Terry & Schneider, 1993; Strauss & Karban, 1995; Terry, 1997). There is in fact no known report of single mating in male thrips. A male *F. occidentalis* can successfully copulate with several virgin females within a swarming period (Terry & Schneider, 1993) but the quantity of sperm or other accessory secretions transferred with consecutive copulations were not determined (Terry & Schneider, 1993). Spermiogenesis in several male Terebrantia is accomplished at adult eclosion (Heming, 1975; Krueger *et al.*, 2017) and it is unclear if prospermatogeny will result in sperm limitation that may influence male copulation strategy and behaviour (Krueger *et al.*, 2017). In *E. americanus*, though a male can inseminate up to 10 females with the same quantity of spermatozoa, the quantity of spermatozoa in the testis of the male reduces with increased copulations and increased age (Krueger *et al.*, 2017). If this is similar in *F. occidentalis*, it suggests that an old male that has copulated with several females may need to make a trade-off between current copulation and future investment; thus an experienced male with lower resources may allocate his resources more carefully when compared with a virgin male with plenty of resources left (Engqvist & Sauer, 2001).

There is a contradiction between the observation by Terry & Schneider (1993), that female *F. occidentalis* do not copulate for several days (over 5 days) with males after initial copulation, and the observation in Chapter 4 that female *F. occidentalis* do copulate multiple times with virgin males in immediate succession and over days. One possible reason for such difference in observation may be because the males used by Terry & Schneider (1993) were not all virgins. This suggests that there may be variation in copulation between males and females tested under different conditions or copulation status.

5.1.4. Aims of study

The general aim of this research was to understand the copulation strategy in male and female thrips. This includes the study of possible variation in copulation behaviour between male and female *F. occidentalis* of different copulation status or condition.

This chapter specifically tested

1. for possible male and female choice in *F. occidentalis* and
2. the stage at which a male or a female makes copulation decisions in the sequence of pre-copulation activities.

Hypothesis:

There is mate choice in *F. occidentalis*

Males make decision to copulate with a female at specific times during the pre-copulation stage.

5.2. Materials and Methods

Pilot experiments showed that culture males and culture females (picked from the laboratory culture) did not copulate, while virgin males and virgin females did (Chapter 4). Therefore, comparison was made between crosses involving virgin and culture males with virgin and culture females in all possible combinations. This revealed the variation in copulation and the sequence of behavioural activities leading to copulation between a male and a female. In a pair that did not copulate, it also showed at what point during pre-copulation activities a decision was made to either copulate or not.

5.2.1. Rearing of adult 3-5 days old virgin males and females

Virgin males and females needed for the comparison discussed above (5.2) were reared on bean pods as described in 3.2.1. The date of emergence of adults was written on the pot used for holding the tubes containing individual insects that emerged the same day. The sex at emergence was also distinguished to avoid later mistakes. In all the experiments, 3-5 days old male and female adults were used.

5.2.2. Picking adult males and females from the culture

As discussed above (5.1), crosses between virgin males and females were compared with crosses between culture male and culture females in all possible combinations. Culture males and females were picked from the culture maintained on pot chrysanthemum at Keele University. The colony was in three cages and the details of the processes involved in the maintenance of the culture have been described in Chapter 2. In order to pick individual thrips, chrysanthemum flowers from two of the cages were carefully tapped over a white bowl. The white bowl containing mixed sex and mixed age thrips was then covered to prevent thrips escape. When needed, the appropriate sex of thrips was picked from the bowl using a slightly moist fine brush and placed in the arena.

5.2.3. The bioassay and video recording

The variation in copulation or activity leading to copulation between a male and a female (virgin and culture) in crosses of every combination was tested in order to identify the possible source of variation (male or female or both) and attempt to predict the reasons for such variation.

The four crosses tested were:

1. Virgin males introduced to virgin females.

2. Virgin males introduced to culture females.

3. Culture males introduced to virgin females.

4. Culture males introduced to culture females.

The order of the treatments was randomised in such a way that each of the four treatments were done before starting another set and these were replicated 16 times. An individual adult female was first placed carefully within the 5 mm diameter arena and covered with a glass cover slip to prevent her from escaping (see Chapter 2). The glass cover slip was carefully opened and a male was introduced into the same arena usually within 5 minutes and the arena was covered immediately to prevent the pair from escaping. Whenever a culture male and a culture female were paired, they were picked from different bowls of thrips collected from a different cage. The arena with the thrips was placed on the stage of the camera-mounted dissecting microscope (see Chapter 2) and recorded for approximately 10 minutes. If any pair finished copulation before 10 minutes, recording was stopped, however if the pair were still copulating after 10 minutes, they were left until they finished copulation. All experiments were performed at a temperature of 25±1 °C.

Since there was a high rate of copulation in virgin males and females reared on bean pods while the culture males and females reared on chrysanthemum rarely copulated (Figure 5.1), it was necessary to test if the differences observed were due to host plant used in order to remove the confounding factor of host plant. Therefore, two other rearing cultures (mixed age and mixed sex) were set up with beans as host plant, in the oviposition pots described in Chapter 2. A male from one of the pots was introduced to a female from another pot within an arena as described in the last chapter. The percentage copulation in paired bean-culture-reared male and female thrips was compared with results from the chrysanthemum-culture-reared male and female thrips and virgin males and females reared on bean pods.

5.2.4. Observations

All the experiments to test the variability among crosses between virgin male and female and culture male and female were carried out between 08:00 and 13:00 h for consistency in behaviour over several days in order to avoid changes in behaviour that may be associated with time of the day. The video of pre-copulation and copulation process was recorded and saved on a SD memory card. The video was later played back to identify and measure the sequence of behaviours displayed in the pre-copulation and copulation phases, which include contact, climbing, bend abdomen and copulation.

The proportion that copulated among all possible crosses between a culture male and female and a virgin male and female may predict the possibility of a male or female choice based on female copulation status. Therefore, if males copulated with females of a particular status more than females of another status, it may predict male preference for the female they copulated with more. However, high rates of copulation in a paired male and female may not simply mean female acceptance or choice of that male, it may be because of male persistence despite several female rejection attempts. If a female copulated with a male just because the male was persistent, it was expected that there will be more rejection attempts in such a female than in a female that accepted copulation from a male not based on male persistence. Therefore, to test this, the number of rejection attempts in females was counted in pairs that copulated in order to compare like with like. The number of rejection attempts was measured as the number of abdominal flips by females (one flip is when a female raises her abdomen to about 45° or higher and brings it down again) between the time at first contact to the beginning of male stroking. A male often starts stroking a female a few seconds after the male aedeagus has been attached to the female during copulation (Chapter 3), so this was a convenient end point that can be seen easily.

In a few of the pairs, some behaviour could not be measured because of difficulty associated with viewing under the microscope. Such behaviours were not included in the analysis.

5.2.5. Statistical analysis

The non-parametric Kruskal-Wallis test was used (because most of the parameters were not normally distributed) in Minitab 17 (Minitab Inc., USA) to compare

1. The durations of specific behaviours at the pre-copulation and copulation stages.
2. The number of flips among the different crosses tested.

Significant differences were compared using multiple comparison Holm's adjusted *P*-values, a modification of the Bonferroni procedure, as suggested by Wright, (1992) after multiple Mann-Whitney tests.

Chi square tests used exact probability to allow for small sample size using IBM SPSS Statistics 21 (IBM Corp., New York). Chi square was used for the following tests

1. The proportion of individuals that expressed behavioural activities before and during copulation among the crosses and

2. The proportion of males and females that copulated in the chrysanthemum-cultured colony compared with those in the bean-cultured colony

5.3. Results

5.3.1. Is there variation in copulation behaviours among crosses between virgin and culture male and female thrips?

Possible variations in the proportion of copulation among thrips of different copulation status were tested by comparing all possible crosses among virgin or culture male with virgin or culture female thrips. There was an interaction between male status and female status ($\chi^2_{(1)}$

= 5.94, P = 0.03) (Table 5.1). There was no male effect ($\chi^2_{(1)}$ = 2.81, P = 0.13), but there was a female effect ($\chi^2_{(1)}$ = 8.40, P = 0.05) (Table 5.1). Copulation tended to occur if there was at least one virgin in a pair and copulation was rare when both were from the culture (Table 5.1). Regardless of the status of males (whether virgin or culture) introduced to virgin females, there was no difference in the proportion that copulated with the virgin female ($\chi^2_{(1)}$ = 1.03, P = 1.00) (Table 5.1). All the virgin males copulated with virgin females introduced to them and 94 % of culture males copulated with virgin females introduced to them (Table 5.1). However, male status influenced the proportion of males that copulated with culture females: 69 % of virgin males copulated with culture females while only 6% of culture males copulated with culture females ($\chi^2_{(1)}$ = 13.3, P = 0.001) (Table 5.1). Virgin males copulated proportionately more with virgin females than with culture females ($\chi^2_{(1)}$ = 5.93, P = 0.04) . Similarly, the proportion of culture males that copulated with culture females was significantly reduced when compared with the proportion of culture males that copulated with virgin females ($\chi^2_{(1)}$ = 24.5, P < 0.001).

Copulation durations were compared in all crosses where copulation occurred at least once. This includes: virgin male and virgin female vs virgin male and culture female vs culture male and virgin female. There was an overall significant difference among the crosses (H = 20.4, df = 2, P <0.001) (Table 5.2). The mean copulation duration between virgin male and virgin female (216 s) and culture male with virgin females (208 s) were significantly longer (Mann–Whitney test with Holm's adjusted P, P < 0.001 and P = 0.004 respectively) than the copulation duration between virgin males and culture females (55 s) (Table 5.2).

5.3.2. Evidence for pre-copulation male choice based on female copulation status

To test whether a male can distinguish between females based on their copulation status and to test for pre-copulation choice by males based on female status (virgin or culture), the

Table 5.1 The numbers and percentages of individuals that copulated out of 16 replicates for four different combinations of culture and virgin males and females observed for up to 10 minutes.

		Females	
		virgin	Culture
Males	Virgin	16 (100 %)	11 (69 %)
	Culture	15 (94 %)	1 (6 %)

Table 5.2 The mean time in seconds ± SE (number of replicates) for successful phases from one behaviour to the other. The Kruskal-Wallis test was used to test for differences in duration of activities. Differences in mean within rows were compared using multiple comparison with Holm's adjusted *P*-values after multiple Mann-Whitney tests. Means with the same letter within a row were not significantly. Number of replicates decreases because not all behaviours were completed or led to copulation.

	Male and female types introduced into the arena				
Behavioural phase	virgin ♂ with virgin ♀	virgin ♂ with culture ♀	culture ♂ with virgin ♀	culture ♂ with culture ♀	*P*-value
Start to first contact	29.6a ± 8 (15)	31.1a ± 12 (15)	91.5a ± 31 (14)	69.6a ±18 (15)	*P* = 0.08
First contact to start of climb	3.9a ±1 (15)	2.1a ± 1 (15)	10.2a ± 8 (14)	21.0a ±10 (10)	*P* = 0.29
Start to end of climb	0.7a ± 0.1 (16)	5.8ab ± 5 (16)	1.0ab ± 0.2 (15)	140.8b ± 67 (5)	*P* = 0.01
End of climb to bend abdomen	0.4a ± 0.2 (16)	0.9a ± 0.3 (15)	0.5a ± 0.2 (15)	0.0a ± 0 (3)	*P* = 0.26
Copulation duration	216.3a±14 (16)	54.8b ± 7 (11)	208.3a ± 16 (15)	202.0 ± 0 (1)	*P* <0.001

proportion of males that engaged in series of activities (contact, start climb, end climb, bend abdomen and copulation) leading to copulation were compared between virgin and culture females (Figure 5.1).

100 % of virgin males contacted females regardless of her status and 94 % of culture males contacted females regardless of her status (Table 5.3). However, after male contact with a female, there was a difference in the sequence of pre-copulation activities between culture males and females vs virgin males and females (Figure 5.1).

The behaviour of culture males was different after contact with either virgin or culture females. When culture males were introduced to a succession of females, all the 94 % of males that contacted virgin females also climbed, bent the abdomen beneath and copulated with virgin females, and these were significantly more than the proportion of males that climbed ($\chi2_{(1)}$ = 14.7, P < 0.001), bent the abdomen beneath ($\chi2_{(1)}$ =18.3, P < 0.001) and copulated ($\chi2_{(1)}$ = 24.5, P < 0.001) with culture females (Tables 5.1 and 5.2). Only a small percentage of culture males bent the abdomen beneath (19%) and copulated (6%) with culture females (Tables 5.1 and 5.3). A culture male tended to move away from a culture female after contact or climbing, even when culture females did not show signs of rejection (flipped the abdomen to dislodge the male). This suggests a pre-copulation choice in culture males based on female copulation status. However, when virgin males were introduced to females in succession, 100 % of the males climbed the female regardless of her status and there was no significant difference between the proportion of virgin males that bent the abdomen beneath that of the virgin females when compared with culture females ($\chi2_{(1)}$ = 1.0 P =1.00) (Table 5.3).

Among the crosses, the time between each of the pre-copulation activities did not differ between, start to first contact (H = 6.9, df = 3, P = 0.08), first contact to start of climbing

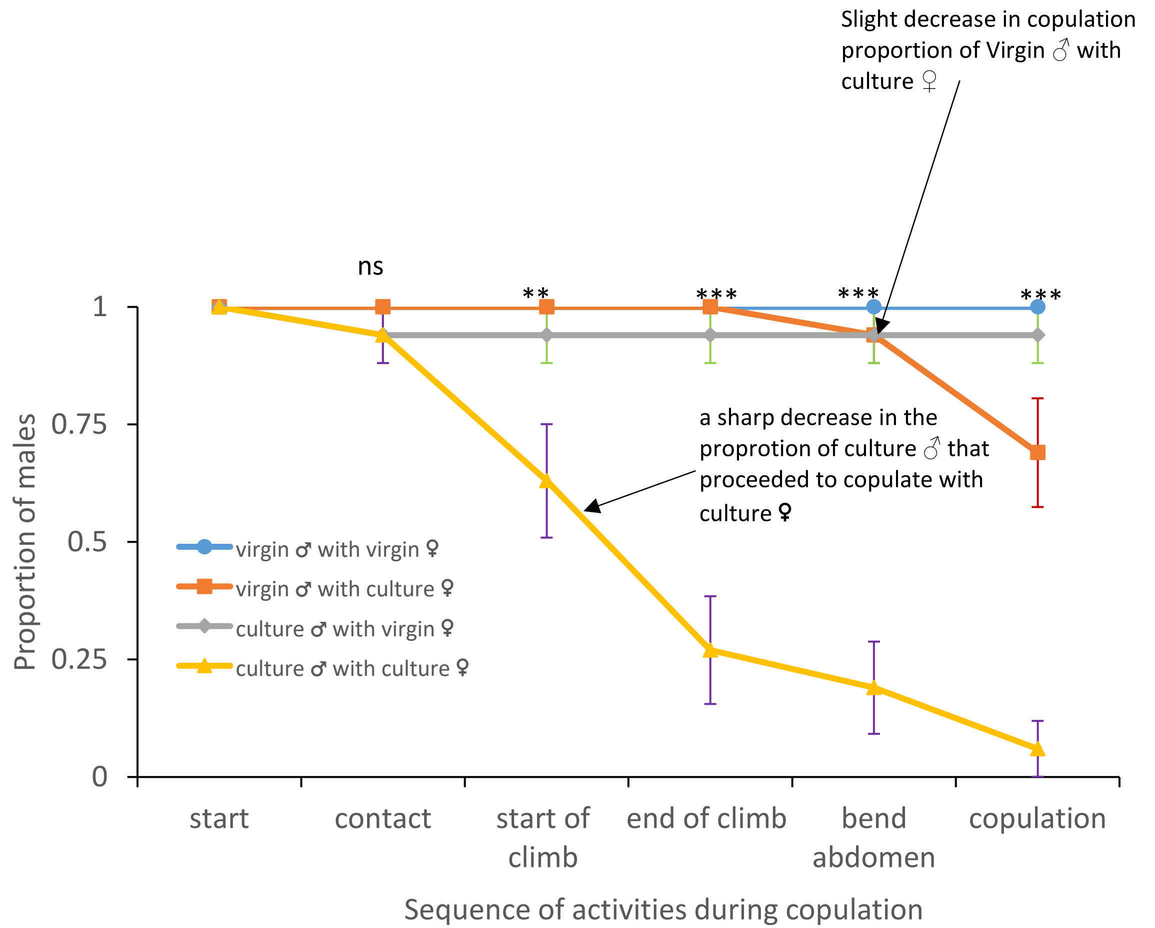


Figure 5.1 Variation in the sequence of behaviour leading to copulation among crosses between virgin and culture male and female thrips. The mean proportion of males ± SE. Contact (χ^2 = 2.07, df = 3, P = 1.000), climb (χ^2 =15.88, df = 3, P = 0.003), mount (χ^2 =37.90, df = 3, P < 0.001), bend abdomen (χ^2 =39.97, df = 3, P < 0.001), copulation (χ^2 =36.85, df = 3, P < 0.001), ns=not significant. **= P<0.01, *** = P<0.001

Table 5.3 The number of copulations/total replicates and percentages of individual that climbed, bent the abdomen, and copulated for four different combinations of culture and virgin males and females and observed for up to 10 minutes.

			Females	
		Activity	Virgin	culture
Males	Culture	Climbed	15/16 (94 %)	4/15 (27 %)
		Bent the abdomen	15/16 (94 %)	3/16 (19 %)
		Copulated	15/16 (94 %)	1/16 (6 %)
	Virgin	Climbed	16/16 (100 %)	16/16 (100 %)
		Bent the abdomen	16/16 (100 %)	15/16 (94 %)
		Copulated	16/16 (100 %)	11/16 (69 %)

(H = 3.8, df = 3, P = 0.29) and end of climbing to male bend abdomen (H = 4.0, df = 3 P = 0.26) (Table 5.2). However, the time from start of climbing to end of climbing was significantly different among the four crosses (H = 10.6, df = 3, P = 0.01), suggesting that at this point a decision was being made by males. It took a longer time for culture males to climb on culture females when compared to virgin males and virgin females (Mann–Whitney test with Holm's adjusted P, P = 0.03) (Table 5.2). The start time of any behaviour was when the behaviour first happened while the end time was when the behaviour ended, regardless of the number of times a male moved away before completing the activity.

5.3.3. Is there evidence of pre-copulation female choice based on male status?

The proportion of virgin or culture females that copulated with culture or virgin males were compared to determine if females made a choice based on the status of the males. There was no significant difference between the proportion of virgin females that copulated with virgin males when compared with culture males ($\chi^2_{(1)}$ = 1.03, P = 1.00) (Table 5.1). Virgin females did not show signs of rejection to males regardless of their status (virgin or culture) confirming that virgin females did not make a choice based on a male's status before copulating with the male.

Culture females copulated significantly more with virgin males than culture males ($\chi^2_{(1)}$ = 13.3, P = 0.001) (Table 5.1). Despite the high proportion of culture females that copulated with virgin males when compared to culture males, this did not necessarily suggest that culture females were showing preference for virgin males. Females may be copulating with virgin males after a series of rejection attempts (abdominal flips). I therefore measured the number of abdominal flips in all pairs that copulated. The number of female abdominal flips were compared among culture females that copulated with virgin males, virgin females that copulated with virgin males and virgin females that copulated with culture males. There was

an overall difference in the number of abdominal flips observed in all crosses tested (*H*=13.1, df = 2, *P* = 0.001). Significantly more abdominal flips were observed in culture females that copulated with virgin males (Mann–Whitney test with Holm's adjusted P, *P* = 0.01) than in virgin females that copulated with virgin or culture males (Figure 5.2). Culture male and culture female pairs were not included in the analysis since only one pair copulated out of the 16 pairs tested. Culture males did not proceed with copulation after contact or climbing despite the fact that the culture females did not show any physical sign of rejection. This therefore suggests that the increase in the proportion of females that copulated with virgin males when compared with culture males was not an indication of female pre-copulation choice.

5.3.4. Does food source influence the copulation proportion in culture males and females?

I tested if host plant played a role in higher rate of copulation (100%) observed in virgin male and virgin female reared on bean pods when compared with very low rate of copulation (6 %) observed in culture male and female reared on chrysanthemum. To do this, another culture of mixed age and mixed sex thrips were reared using bean pods. The rate of copulation in cultures reared on bean pods were compared to those reared on chrysanthemum and also with virgin male and virgin female reared on bean pods. The proportions of culture males and culture females that copulated in thrips reared on chrysanthemum were not significantly different from the proportion of culture male and culture females that copulated in thrips reared on bean pods ($\chi^2_{(1)} = 1.71$, $P = 0.53$) (Table 5.4). However, the proportion of culture males and culture females that copulated in thrips reared on bean pods was significantly lower than virgin males and virgin females reared on bean pods ($\chi^2_{(1)} = 16.00$, $P < 0.001$) (Table 5.4).

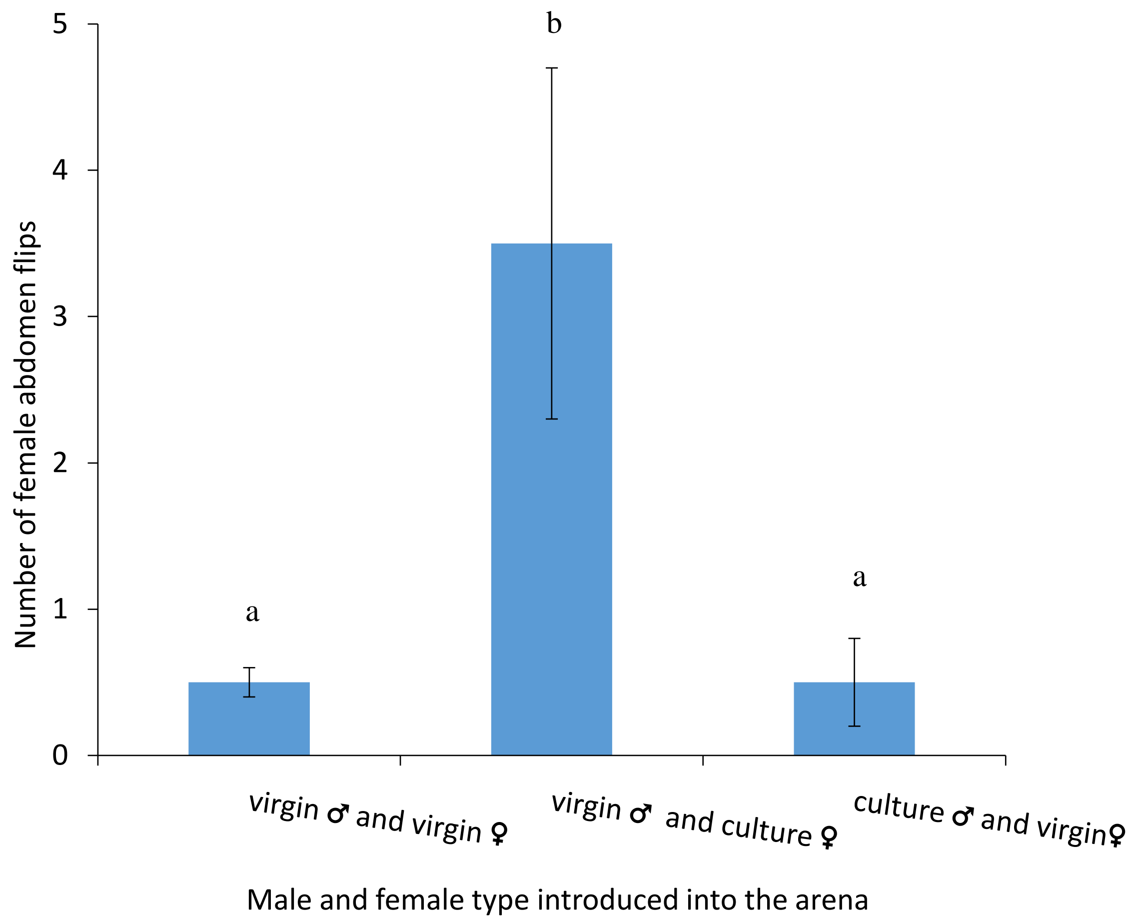


Figure 5.2 The mean ± SE of the number of female abdominal flips between first contact and start of male stroking female in crosses leading to copulation between virgin and culture male and female thrips. H=13.1, df=2, P = 0.001. Only pairs in which copulation occurred were included in the analysis so as to be able to compare like with like.

Table 5.4 The number of females that copulated when chrysanthemum culture males were introduced to chrysanthemum culture females compared with the number of females that copulated when bean pod culture males were introduced to bean pod culture females and bean pod virgin female. $\chi^2_{(2)} = 30.23$, $P < 0.001$

Mating status	Chrysanthemum culture ♂ with ♀	Bean pod culture ♂ with ♀	Bean pod virgin ♂ with virgin ♀
Copulated	1 (6%)	2 (25%)	16 (100 %)
Did not copulate	15 (94%)	6 (75%)	0 (0 %)

5.4. Discussion

5.4.1. Variation in copulation behaviour in virgin and culture male and female thrips

This research shows that there are variations in copulation behaviour between virgin males and females and males and females picked from the culture. Most of the virgin females (over 90%) copulated with males regardless of their status (virgin or culture) suggesting that virgin females showed indiscriminate copulation between males. However, there was variation in the proportion of culture males and virgin males that copulated with culture females; for example, the proportion of virgin males that copulated with culture females was higher than the proportion of culture males that copulated with culture female. In addition, the sequence of activity leading to copulation differs in virgin males or culture males that were paired with culture females. This suggests that there is a difference between the copulation strategy of virgin males and culture males, and it also reveals the complex nature of variation in copulation within the laboratory culture, an example of what may happen in a field situation.

There was also variation in copulation duration among the crosses tested. Short copulation duration was observed when culture females copulated with virgin males when compared with when virgin females copulated with virgin or culture males. The short copulation duration observed in culture females implies that these females had likely copulated at least once. This short copulation (55 s) was similar to the reduced copulation duration (51 s) that characterised a female that had already copulated (Chapter 4). It has been suggested in Chapter 4 that short copulations observed in females that had copulated at least once may not be successful.

5.4.2. Is there evidence of female choice based on male copulation status?

The observed increase in the proportion of culture females that copulated with virgin males when compared with culture males can only be because of two reasons or a combination of them.

1. Culture females were making a choice between virgin and culture males

2. Virgin males were more persistent than culture males (which may show no or limited attempts to copulate with culture males) and therefore copulated with the females regardless of rejection attempts by females.

This research did not suggest that virgin or culture females were making a choice among males based on their copulation status. Virgin females copulated with males regardless of their status. However, though culture females copulated more with virgin males than culture males, this could not be interpreted as a culture female choice of virgin male over culture male because most of the culture females that copulated with the virgin males first attempted to reject the males by flipping the abdomen before they eventually copulated with these virgin males. Culture females flipped the abdomen significantly more in crosses between virgin males and culture females than virgin or culture males with virgin females. This therefore suggests that virgin males were persistent even when culture females attempted to reject virgin males. It is possible that male harassment is costly to female *F. occidentalis* for example, in *Thrips tabaci* females experienced fitness cost (reduced fecundity and oviposition) as a result of continuous exposure to males and possibly male harassment (Li *et al.*, 2015b). Therefore, culture female *F. occidentalis* might copulate with persistent virgin males in order to avoid the cost of continuous rejection such as male harassment or sexual attempts (Thornhill & Alcock, 1983; Parker, 1984; Rowe, 1992). This situation has been termed ‘convenience polyandry’ (Thornhill & Alcock, 1983).

Virgin female *F. occidentalis* were not observed to choice between males at least until the male bent the abdomen beneath the female abdomen. Similar observation in *Thrips tabaci* indicated that the frequency of copulation by females was not influenced by the copulation status of the male (Li *et al.*, 2015b). Virgin female *F. occidentalis* may however show such discrimination when sperm or accessory materials become extremely limiting in males. Male *F. occidentalis* can inseminate up to 10 females in 3 hours (Terry, 1997) and male *E. americanus* can inseminate up to 10 females with the same amount of spermatozoa (Krueger *et al.*, 2017), but the accessory materials decrease with the number of previous copulations (Krueger *et al.*, 2017). Terry & Schneider (1993) observed that some virgin females rejected males that had copulated with several virgin females. This research has therefore not eliminated the possibility of female choice in *F. occidentalis,* but has suggested that such choice may not be between a virgin male and a male that has copulated just once or a few times.

5.4.3. Evidence of male choice based on female copulation status

The mating strategy used by a virgin male appears to be different from that of a culture male. While a virgin male was observed to attempt copulation (by bending his abdomen beneath that of the female) with a female regardless of her status, culture males attempted copulation with virgin females but not with culture females. This research showed that only one of the 16 pairs of the culture males and the culture females copulated. In most cases, a culture male contacted or climbed a culture female (at least the first two times) and moved away (without bending the abdomen beneath that of the female) even though the female did not flip the abdomen to reject the male. As suggested above, the reason why a culture female did not copulate with culture males introduced to her may be because a culture male did not proceed with the copulation process once he could identify that the female was already copulated.

There is therefore clear evidence that a culture male showed preference for a virgin female over a culture female while a virgin male did not show such preference.

Culture males might therefore be able to assess female status after contact or climbing before making a decision to either bend his abdomen beneath that of the female or not (depending on her copulation status). This strongly suggests that a culture male could differentiate between a copulated female and a virgin female after contact and will therefore proceed to copulate with a virgin female but avoid a culture or copulated female (this will be discussed further in Chapter 6). However, it is important to note that such discrimination will benefit the male because attempting to copulate with an already copulated female may lead to waste of time and resources since such copulation may not be successful (as suggested in Chapter 4).

The average copulation duration of virgin males and culture females was about 55 s, an average similar to those observed in copulated females (Chapter 4) and the only culture female that copulated with a culture male copulated for 202 s, a duration similar to what was observed when males (virgin or culture) copulated with virgin females. Culture males and females were from a mixed culture and there will be some virgin females present in the culture. This strongly suggests that the only culture female that copulated with a culture male was a virgin female.

5.4.4. Why were culture males more choosy?

As discussed above, a culture male was observed to show low preference for culture females but they copulated readily with virgin females. Why then did a culture male appear more choosy than a virgin male? Before attempting to answer this question, I eliminated the possibility that the difference observed in crosses between virgin male and female (reared on bean pods) and culture males and females (reared on chrysanthemum) were due to

different host plant (food source) by raising another culture from beans pod. There was no significant difference in the proportion that copulated in these two food sources, while the proportion of virgin males and virgin females reared on bean pods were significantly higher than the proportion of culture males and culture females reared on bean pods. Therefore, the only answer to this question may be the possible differences between a virgin male and a culture male.

Two important conditions that may differentiate culture males from virgin males include: 1. The culture males were likely older on average than the virgin males (3-5 days old).

2. The culture males might have copulated multiple times and might be depleted of costly resources while virgin males have plenty of resources available.

According to Bonduriansky (2001), a male becomes choosy as the resources become limiting due to additional copulation, therefore a male judiciously allocates the limited resources as observed in *Drosophila melanogaster*. Culture males were likely older and would have copulated with several females, since male and female *F. occidentalis* can live for up to 20 and 40 days respectively (Bryan & Smith, 1956). They may therefore be limiting in vital resources. Though I did not compare available resources between virgin males and culture males, in *E. americanus*, a thrips species in the same family as *F. occidentalis*, the quantity of spermatozoa and accessory materials in the testis of the male reduced with increasing copulations and age (Krueger *et al.*, 2017). This suggests that production of spermatozoa or accessory secretions may be costly to male *F. occidentalis* and these materials may become limiting as they copulate with more females. Culture males may therefore be limiting in costly resources such as sperm and accessory materials and may therefore strategically avoid available culture females in order to trade-off current copulation opportunity (which may not lead to transfer of sperm to females and so not increase his paternity advantage) for future

investment, that is, the possibility of copulating with a virgin female in the future. Virgin females are expected to be of higher quality than culture females since they are likely to utilise the sperm of any male that successfully copulates with them while culture females are expected to have copulated and may not be willing to accept further copulations.

However, for virgin males, the strategy was different, they attempted copulation with females regardless of their copulation status. Most virgin males (69%) copulated with culture females in the 10 minutes test period. In all these cases, copulation duration was short suggesting that these culture females had already copulated. Virgin males have plenty of resources available to them, and they therefore have nothing much to lose but rather will increase their paternity chances by attempting copulation with any female they encountered. This is especially true if there is a chance of their sperm being transferred to a copulated female. A culture male with limited resources therefore allocates his resources with caution (strategically to females of higher quality) when compared to virgin males (Engqvist & Sauer, 2001; Martel *et al.*, 2008).

Several other male insects have been suggested to show preference for virgin females (Carazo *et al.*, 2004; Martel *et al.*, 2008; Assis *et al.*, 2017) or females of high quality or fertilisation potential (Simmons *et al.*, 1994; Wedell *et al.*, 2002; Engqvist & Sauer, 2003). The quality of females may be associated with age for example some male insects prefer younger females to older ones (Simmons *et al.*, 1994; Lüpold *et al.*, 2011). In this research, however the reason why a culture male preferred a virgin female may not only be because virgin females used were young, since culture males copulated readily with older virgin females (6-7 days old) (Chapter 4).

Culture males may likely have copulated with females of different copulation status while in the culture. They were therefore likely to be more experienced than virgin males. Baxter

et al. (2015) have suggested that *Drosophila* males exposed to females tend to be choosier than isolated males. Further investigation will however be needed to confirm if culture males can learn female copulation status since they possibly produce an antiaphrodisiac pheromone (Chapter 6).

5.4.5. How does a male assess the female copulation status?

It is likely that a male *F. occidentalis* assesses a female status using an antiaphrodisiac pheromone. This will be discussed in more detail in Chapter 6. Culture males seem to detect this pheromone before copulation. This may therefore help in culture male choice because any pheromone that can indicate that a female has already copulated will likely be of more benefit to the male than the female (Johansson & Jones, 2007).

Chapter 6

Evidence for an antiaphrodisiac pheromone

6.1. Introduction

A previous experiment (Chapter 5) showed that a higher proportion of males from the culture copulated with virgin females than with females picked from the culture. These culture males contacted and sometimes climbed on culture females but did not proceed to copulate with such females even when the females showed no sign of rejection. However, after climbing on virgin females, culture males proceeded to bend the abdomen beneath and copulated with virgin females. Therefore, it is likely that culture males can differentiate between a virgin female and a female picked from the culture. It is very likely that most females from the culture have copulated at least once and that may be the reason why culture males did not proceed with copulation after climbing on culture females. A culture male may therefore have developed a mechanism for assessing the mating status of a female. The mechanism may be visual, olfactory, tactile or a combination of these mechanisms (Bonduriansky, 2001). The experiment suggested that a male uses an olfactory mechanism in the identification of the copulation status of the females.

6.1.1. The use of chemical cues in determining the copulation status of female insects

Females of several species of insects do not copulate again after initial copulation due in part to the fact that they become less attractive to males after initial copulation (see review by Gillott, 2003). The reason for the loss of attraction in copulated females could be that male insects alter the odour of females by marking the females with an antiaphrodisiac pheromone during or after copulation (Thomas, 2011). A male may therefore use this antiaphrodisiac compound to assess the copulation status of a female before deciding on whether or not to copulate with her. Usually, an antiaphrodisiac pheromone should be of benefit to the discriminating sex because this pheromone indicates the mating status of the female and a male after using the pheromone for assessment is able to preferentially copulate with virgin females (Johansson & Jones, 2007). Since an antiaphrodisiac pheromone transferred from a male to a female reduces her attractiveness and prevents other males from courting or copulating with the female especially in species where females mate more than once (Brent & Byers, 2011; Thomas, 2011; Yamane, 2013; Laturney & Billeter, 2016), sperm competition from other future males will be reduced and this is especially beneficial to a signalling male that has a potential sperm competition disadvantage (Engqvist & Taborsky, 2017). Alternatively, when a male uses the assessment of a female's mating status to determine the possibility of sperm competition, he may regulate the volume of sperm to be transferred to the female in order to maximise his fitness (Larsdotter-Mellström *et al.*, 2016; Malouines, 2017). A male therefore generally increases his chance of fertilising more eggs by applying an antiaphrodisiac pheromone that will reduce sperm competition from other males (Malouines, 2017). A male may also benefit from marking a female as mated in species where females become refractory after copulation because identifying a female's status reduces the time cost associated with courting an unreceptive female.

Applying an antiaphrodisiac pheromone by a male may also benefit females (especially those that copulate only once), by preventing male harassment after copulation (Thomas, 2011). A polyandrous female may however be disadvantaged when a male marks her with an antiaphrodisiac pheromone because it prevents her from benefiting from multiple copulations (Arnqvist & Nilsson, 2000; Malouines, 2017). This may therefore lead to a potential sexual conflict between males and polyandrous females (Chapman *et al.*, 2003).

In *E. americanus*, the only thrips species with an identified male-produced antiaphrodisiac pheromone (Krueger *et al.*, 2016), application of an antiaphrodisiac pheromone on copulated females appears to benefit both sexes. Females of this species of thrips copulate only once for at least a 30 days test period (Li *et al.*, 2014; Krueger *et al.*, 2015a) and may therefore benefit from being marked by male-produced antiaphrodisiac pheromones since this will prevent unnecessary harassment from future males. Two compounds in *E. americanus* (dimethyl adipate and dimethyl glutarate) were found on the head and thorax regions of males but were not found on virgin females (Krueger *et al.*, 2016). Dimethyl adipate was also found on females reared with males, while dimethyl glutarate was not found on such females, however males did not copulate with a virgin female on which either of the compounds was applied (Krueger *et al.*, 2016). It is not clear from this study if all the females analysed as mated were confirmed to have copulated and if the transfer of the compound was actually during copulation or during other male-female interactions (Krueger *et al.*, 2016). Males of this species have been observed to stay close to pupae of female thrips and therefore copulate with females after emergence (Krueger *et al.*, 2016), a situation that suggests strong competition among males for virgin females. Males will therefore benefit from marking females with an antiaphrodisiac pheromone after copulating with her since this will reduce the time and energy cost associated with attempting to copulate with an unreceptive female that has already copulated, and specifically, a male that applied the

antiaphrodisiac pheromone on the female also benefits by not wasting time attempting to copulate with the same female.

The manner in which the cuticle of females may change after copulation is not restricted to application of a male-produced antiaphrodisiac pheromones, though this appears to play an important role in many species (Thomas, 2011). In some species, females produce compounds that make them less attractive to males after copulation (Schiestl & Ayasse, 2000), some reduce the production of aphrodisiac pheromone or change their cuticular hydrocarbon profile or cuticular volatiles and become less attractive to males (Ayasse *et al.*, 1999; Polidori *et al.*, 2017). Change in hydrocarbon profile is not expected to be immediate, though a change in as little as 30 minutes after copulation has been associated with loss of attraction in *Leptothorax gredleri* (Oppelt & Heinze, 2009), but the changes observed in 30 minutes were so small that it is difficult to conclude that a male could detect it.

In some lepidopterans, sex pheromone production in females ceases after copulation due to a signal received from the male reproductive system (e.g. Ramaswamy *et al.*, 1996; Wedell, 2005), therefore making females less attractive to males.

6.1.2. Is 7-methyltricosane an antiaphrodisiac pheromone?

Three male-produced compounds have been identified in adult male *F. occidentalis*: neryl (*S*)-2-methylbutanoate, (*R*)-lavandulyl acetate and 7-methyltricosane (Hamilton *et al.*, 2005; Olaniran *et al.*, 2013). Neryl (*S*)-2-methylbutanoate serves as an aggregation pheromone that attracts both sexes of *F. occidentalis* in flight (Hamilton *et al.*, 2005) while 7-methyltricosane is a contact pheromone (Olaniran *et al.*, 2013). The role of (*R*)-lavandulyl acetate is still not clear but it reduced trap catch in the field (Sampson, 2014) and arrested females but made males more active in a laboratory study (Olaniran, 2013). This

identification of multiple pheromones in *F. occidentalis* suggests that pheromones play an important role in reproductive activities in this species.

The contact pheromone, 7-methyltricosane is less volatile when compared with other known male-produced compounds (Olaniran, 2013; Olaniran *et al.*, 2013). Males and females spent more time in the vicinity after contact with a glass bead coated with cuticular hydrocarbon 7-methyltricosane, but there was no long-distance attraction as was observed with the aggregation pheromone (Olaniran *et al.*, 2013). The specific role of 7-methyltricosane has not been clearly demonstrated, but it has been suggested to play a role in mate choice through species or mate recognition or even mate assessment (Olaniran, 2013). Adult females from a mixed-age culture (presumably mated) placed in contact with the compound were observed to lift the abdomen, a rejection behaviour (Olaniran *et al.*, 2013), which led Olaniran (2013) to suggest that adult males may be transferring the compound to the adult females during copulation in order to make her less willing to copulate with other future males. During copulation, a male *F. occidentalis* antennates a female's head or antennae with his own antennae and also strokes the dorsal body part of her thorax and abdomen during copulation (Terry & Schneider, 1993) (Chapter 3). These behaviours suggest that a male may be rubbing 7-methyltricosane on the female by antennation or by rubbing 7-methyltricosane on the female with his legs during stroking. These two behaviours were also observed in *E. americanus* (Krueger *et al.*, 2017), a thrips species in which antiaphrodisiac pheromone was extracted from the head-thorax region of virgin males but none was found in the abdomen region (Krueger *et al.*, 2016). It is therefore possible that a male produces an antiaphrodisiac pheromone from the leg arolium and applies it while stroking.

6.1.3. Aims of study

The aims of this chapter were to:

1. test whether a male *F. occidentalis* can differentiate between a virgin female and a culture or copulated female and to identify the stage at which a male discovers the copulation status of a female.
2. attempt to identify chemical compound(s) a male uses in the detection of the copulation status of the female by extracting the cuticular hydrocarbons of virgin males and virgin females and comparing them with those of copulated females.
3. confirm the male copulation strategy observed in chapter 5

Hypothesis: Virgin males uses chemical cues applied on the female to detect her copulation status

6.2. Materials and methods

6.2.1. Rearing of adult virgin males and females

To test if a virgin male can detect the copulation status of a female, it was necessary to rear virgin males and females. Virgin males and females were reared using the method described in Chapter 2. They were maintained individually on a small portion of bean pod in a modified microcentrifuge tube until needed for experiment. Some of the virgin males were also used for the GC/MS analysis of the cuticular hydrocarbons.

6.2.2. Copulated females

This research compared virgin females with copulated females and also measured the duration of copulation in dead copulated females. It was therefore necessary to be sure of the copulation status of any female used for the experiment. To confirm that females

designated as copulated females have indeed copulated, virgin males were used to copulate virgin females. To copulate the female, a virgin male (3-5 days old) was introduced to a virgin female (3-5 days old) placed in the arena described in Chapter 2. The pairs were allowed to copulate, and after copulation, the female was removed and placed separately into a modified microcentrifuge tube (Chapter 2) and covered with the snap-on lid attached to the tube (Chapter 2).

6.2.3. Freezing of adult female thrips

When a culture male was introduced to a culture female, they did not copulate despite the fact that males contacted and sometimes climbed the female. The reason for no copulation in this pair may be due to culture male choice after assessing the female's status or due to mechanical resistance from the female. To remove the possibility of female mechanical resistance, the females used for this research were frozen and defrosted before use. Live males of *Scirtothrips aurantii* (a thrips species of similar size to *F. occidentalis*) have been shown to copulate with frozen dead females. A similar approach was therefore used in this experiment. Preliminary experiments showed that none of the females picked from the laboratory culture recovered after they were frozen for 40 minutes at a temperature of between -15 to -23 ^{o}C. In this research, virgin and culture females or copulated females were kept individually in a microcentrifuge tube and were placed inside a domestic freezer for at least 40 minutes at between -15 to -25 ^{o}C (the freezer temperature varied from time to time). They were then taken out of the freezer and allowed to defrost for a minimum of 10 minutes before they were used for the experiment. Females used for the experiments were dead and none of the females were observed to recover by moving around throughout the period of the experiments. However, some females observed during the video playback to move parts of the body possibly as a result of the process of dying were not included in the analysis.

6.2.4. The bioassay and video recording

A male that can detect an antiaphrodisiac pheromone on a copulated female, is expected to move away after making contact or climbing on the female since such a pheromone will repel him, while such a male should copulate with a virgin female on which there is no antiaphrodisiac pheromone except if the virgin female resists the male. This was similar to the behaviour observed in live culture males introduced to live females in chapter 5. However, because the females were alive, it was possible that lack of copulation observed in some females may be due to female resistance and not detection of an antiaphrodisiac pheromone by males. To test if males can detect an antiaphrodisiac pheromone on thrips, live female behavioural or mechanical cues or resistance was removed by using dead females. There were two experiments to achieve this purpose:

1. Crosses between live culture males introduced to dead virgin females were compared with crosses between live culture males introduced to dead culture females and this was replicated 30 times.
2. In the second experiment, in order to confirm if the result obtained in 1 above was due to differences in copulation status of females (since culture females were expected to have copulated), copulated females (females that were deliberately copulated once with males) were compared with virgin females of the same age. Also, the responses of virgin males were compared with males picked from the culture in order to confirm the copulation strategies observed in chapter 5. The test therefore compared four different crosses and was replicated 10 times. The crosses included:

a. Virgin males introduced to dead virgin females.
b. Virgin males introduced to dead copulated females.
c. Culture males introduced to dead virgin females.

d. Culture males introduced to dead copulated females.

In both experiments, treatments were randomised in such a way that each of the treatments was observed before starting another set so that treatments were not confounded with time. The stage during the series of activities leading to copulation in which a male stopped proceeding with the copulation process in pairs that did not copulate was also observed in order to identify at what stage a male detected an antiaphrodisiac pheromone in copulated females.

An individual dead adult female was first placed carefully (on her legs or side when it was difficult to make her stand) close to the edge of the 5 mm diameter arena (described in Chapter 2) and a male was introduced into the same arena usually from the other edge opposite where the dead female was, using a slightly moist brush, and the arena was covered with a glass cover slip to prevent the male from escaping (see Chapter 2). Whenever a culture male was used, it was picked from the bowl of thrips collected from the laboratory culture as described in Chapter 5. The arena with the thrips was placed on the stage of the camera-mounted dissecting microscope (see Chapter 2) and recorded for approximately 10 minutes as discussed in Chapter 5. All experiments were performed at a temperature of 25±1 °C.

6.2.5. Observations

The research was carried out between 08:00 and 13:00 h for consistency in behaviour over several days to avoid changes in behaviour that may be due to differences in time of day. The video of activities before and during copulation was recorded and saved on a SD memory card. The video was later played back to identify and measure the sequence of behaviours displayed in the pre-copulation and copulation phases as described in Chapter 5. The durations of all activities leading to copulation including copulation duration were measured. For any pair where some behaviour could not be measured because of difficulty

associated with viewing under the microscope that behaviour for that pair was not included in the analysis.

6.2.6. Collection and analysis of cuticular hydrocarbons

Individual adult virgin males and adult virgin females were reared on bean pods and isolated in individual microcentrifuge tubes as larvae or pupae before emergence as adults. From these, 50 adult males and 100 adult females were collected together into separate microcentrifuge tubes of virgin males and virgin females. 100 females reared on bean pods were also copulated once with a virgin male and were collected into centrifuge tube as copulated females. These insect samples were transferred into a separate glass vials (2 ml). In order to obtain extracts, 1 ml of HPLC-grade hexane was added to each vial containing thrips using a 500 µl hexane-washed Hamilton syringe and the vial was covered with the vial lid. To allow the cuticular hydrocarbon, but not the internal contents, to dissolve into the hexane, the sample was left for about 5 minutes after which the extracts in hexane was drawn out using the syringe and transferred into another empty similar glass vial. This was then left opened until all the hexane had completely evaporated, so that it can be reconstituted to the appropriate concentration and volume during the GC-MS analysis. The extract was labelled before keeping the vial in the freezer until needed. The vial containing the cuticular hydrocarbon was then sent to Dr Falko Drijfhout in the School of Chemical and Physical Sciences for the analysis of the cuticular hydrocarbons using GC-MS. Analysis of the reconstituted extract (5µl) was done on an HP7890 GC system that was coupled to an HP5975 Network Mass Selective Detector (Agilent). The capillary column was coated with ZB-5HT (30 m×0.25 mm i.d., 0.25 µm film; Phenomenex, Macclesfield, Cheshire, UK). The manual injection (2 µl) was splitless (250 °C), and helium (1 ml min −1) was used as the carrier gas. The temperature of the oven was held at 50 °C for 2 min, before increasing to 200 °C at 25 °C min −1 , then to 260 °C at 3 °C min −1 , and then to 320 °C at 20 °C min

−1 , and held for 2 min. The mass spectrometer was operated in the EI mode at 70 eV, scanning from 40 to 500 amu at 1.5 scans sec −1 . Hydrocarbon compounds were subsequently identified using a mass spectral library (NIST 08), comparing diagnostic ion fragments, and calculating the RIs from retention times of n-alkanes.

6.3. Statistical analysis

The durations of behaviours were compared with Mann-Whitney or Kruskal-Wallis tests using Minitab 17 (Minitab Inc., USA). Where there were significant differences after Kruskal-Wallis tests, significant differences were compared using multiple comparison with Holm's adjusted *P*-values, a modification of the Bonferroni procedure, as suggested by (Wright (1992). The chi-square test was used for calculating differences in the proportion of individuals. Chi-square was used with exact probability in order to allow for small sample size, using IBM SPSS Statistics 21 (IBM Corp., New York).

6.4. Results

6.4.1. Can culture male *F. occidentalis* differentiate between dead virgin females and dead culture females?

When culture males were separately introduced to both dead virgin females and dead culture females, significantly more males proceeded to bend the abdomen beneath that of the dead virgin females when compared to dead culture females ($\chi^2_{(1)}$=13.46, $P < 0.001$) (Figure 6.1). Only about 14% of culture males bent their abdomen beneath that of the dead culture females when compared to about 60 % of the culture males that bent the abdomen beneath that of the dead virgin females. There was no significant difference between the proportion of culture males that contacted ($\chi^2_{(1)}$= 0.09, P = 1.00), started climbing ($\chi^2_{(1)\ (1)}$ =1.83, P = 0.279), or ended climbing ($\chi 2_{(1)}$ =4.34 P =0.065) virgin females when compared to culture females (Figure 6.1). However, the proportion of males that carried out each sequential

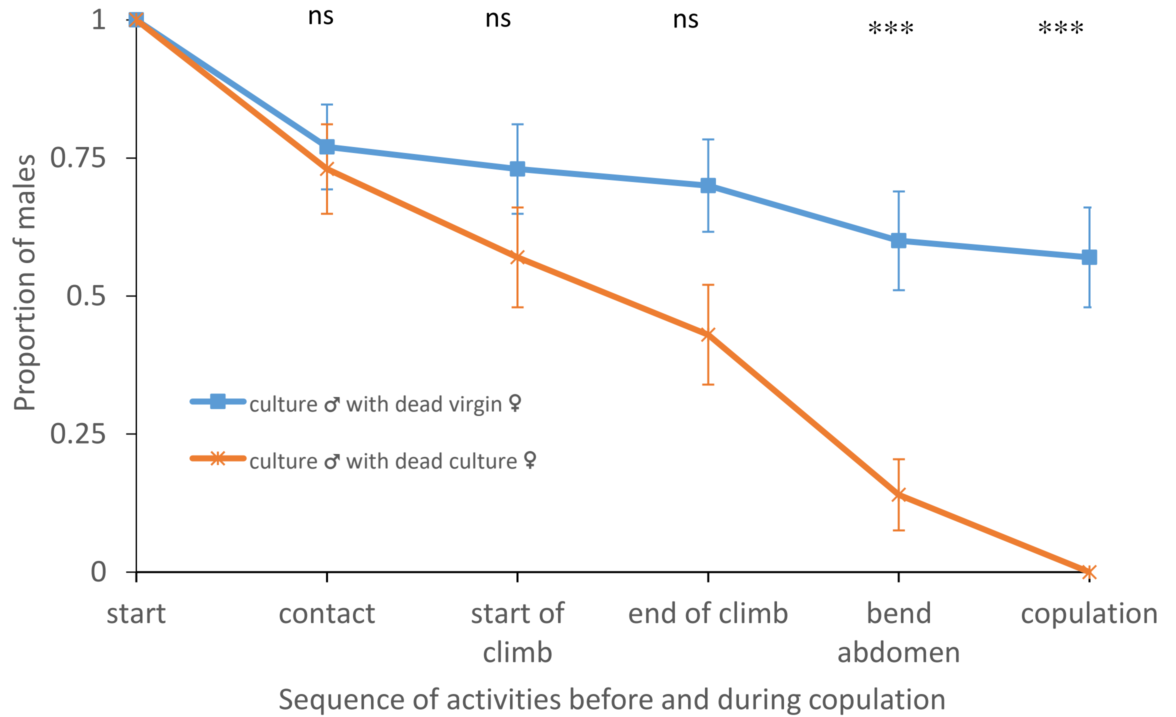


Figure 6.1 Variation in the sequence of activities leading to copulation between culture males and dead virgin or dead culture females. Proportion of males ± SE. Summary of analysis: contact ($\chi^2_{(1)} = 0.09$, $P = 1.00$), start climb ($\chi^2_{(1)} = 1.83$, $P = 0.279$), end climb ($\chi^2_{(1)} = 4.34$, $P = 0.065$), bend abdomen ($\chi^2_{(1)} = 13.46$, $P < 0.001$), copulation ($\chi^2_{(1)} = 23.72$, $P < 0.001$). Key: ns = not significant , ***=significant at $P < 0.001$

activity decreased as they progressed from contact to bending of the abdomen in culture females when compared with virgin females. Males therefore likely gradually discovered the difference between culture females and virgin females as they progressed from contact to bending of the abdomen (Figure 6.1).

In a similar manner, there were no significant differences in time to first contact (W = 597, P = 0.13), or first contact to start of climbing (W = 395, P = 0.32) between culture males crossed with dead culture female and dead virgin female (Table 6.1). The likelihood that a culture male made a decision not to proceed with copulation with culture females towards the end of climbing is further indicated by the fact that the time from start of climbing to end of climbing was longer when culture males were introduced to dead culture females than when culture males were introduced to dead virgin females (W = 335, P = 0.04) (Table 6.1). The start time of any behaviour was when the behaviour first happened while the end time was when the behaviour ended, regardless of the number of times a male moved away before completing the activity. None of the culture males copulated with dead culture females while about 57% of culture males copulated with frozen virgin females for a mean duration of 245 s (Figure 6.1).

6.4.2. Does male copulation status (virgin or culture) influence his pre-copulation strategy and copulation response to female copulation status?

I tested whether female copulation status influenced the activities leading to copulation by culture males or virgin males. There were differences in the response of males (culture or virgin) to pre-copulation activities in females based on her status. In virgin males for example, there were no significant differences between the proportion of males that contacted ($\chi^2_{(1)}$= 2.2, P = 0.47), started to climb ($\chi^2_{(1)}$ = 0.4, P = 1.00), ended climbing ($\chi^2_{(1)}$ = 0.4, P = 1.00), bent the abdomen ($\chi^2_{(1)}$ = 0.3, P = 1.00) and copulated ($\chi^2_{(1)}$ = 1.8, P = 0.37)

Table 6.1 The mean durations ± SE (n) for successful phases in the sequence of behaviours. The start time of any behaviour was when the behaviour first happened while the end time was when the behaviour ended, regardless of the number of times a male moved away before completing the activity and this was the reason for the long durations observed for some of these activities

Summary of analysis: Start to first contact (Mann-Whitney, $W = 597$, $P = 0.13$) and first contact to start of climb (Mann-Whitney, $W = 395$, $P = 0.32$), Start of climb to end of climb (Mann-Whitney, $W = 335$, $P = 0.04$) and start of climb to end of climb (Mann-Whitney, $W = 189$, $P = 0.13$). Key: ns = not significant, * = significant at $P < 0.05$

	Culture male		
Behaviour duration (s)	with dead virgin female	with dead culture female	Significance level
Start to first contact	114.20 ± 30 (23)	57.4 ± 21 (22)	ns
First contact to start of climb	24.40 ± 13 (22)	53.20 ± 23 (16)	ns
Start of climb to end of climb	7.73 ± 4 (22)	77.8 ± 38 (13)	*
Start of climb to bend abdomen	20.2 ± 14 (18)	220 ± 133 (4)	ns
copulation	245.1±20 (16)	-	-

with either dead copulated females or dead virgin females (Figure 6.2). However, in culture males, even though similar proportions of males contacted and climbed females regardless of their copulation status, none of the culture males proceeded to bend the abdomen beneath or copulate with dead copulated females compared to 80 % that bent the abdomen beneath and 50 % that copulated with dead virgin females ($\chi^2_{(1)} = 13.3$, $P = 0.001$ and $\chi^2_{(1)} = 6.7$, $P = 0.03$ respectively) (Figure 6.2). There was therefore a difference in strategy between a virgin male and a culture male. A culture male stopped the copulation process after climbing on a copulated female, while the virgin male proceeded to copulate with her regardless of her copulation status. There was an overall general difference in the time taken for a male to climb a female in all crosses tested ($H = 20.4$, df = 3, $P < 0.001$) (Figure 6.2). It took significantly (Mann–Whitney test with Holm's adjusted P, < 0.05) longer time for culture males to climb a dead virgin or dead copulated female than when virgin males were introduced to dead virgin or dead copulated females (Table 6.2)

6.4.3. Does copulation status influence copulation duration in dead females?

There was an overall difference in the copulation duration in all crosses that copulated (H=7.9, df=2, P=0.02) (Table 6.2). When virgin males were introduced to copulated dead females, the copulation duration was significantly reduced (Mann–Whitney test with Holm's adjusted P, $P = 0.03$) to about 43 s (Table 6.2) when compared to 209 s when virgin males copulated with dead virgin females.

6.4.4. Amount of 7-methyltricosane found on males, virgin and copulated females

The peak area of 7-methyltricosane extracted using an ion chromatogram (EIC) m/z = 112 based on the peak areas showed that males had a peak area 27,335,327. The peak area of 7-methyltricosane found on copulated females (2,754,762) was over four times higher than

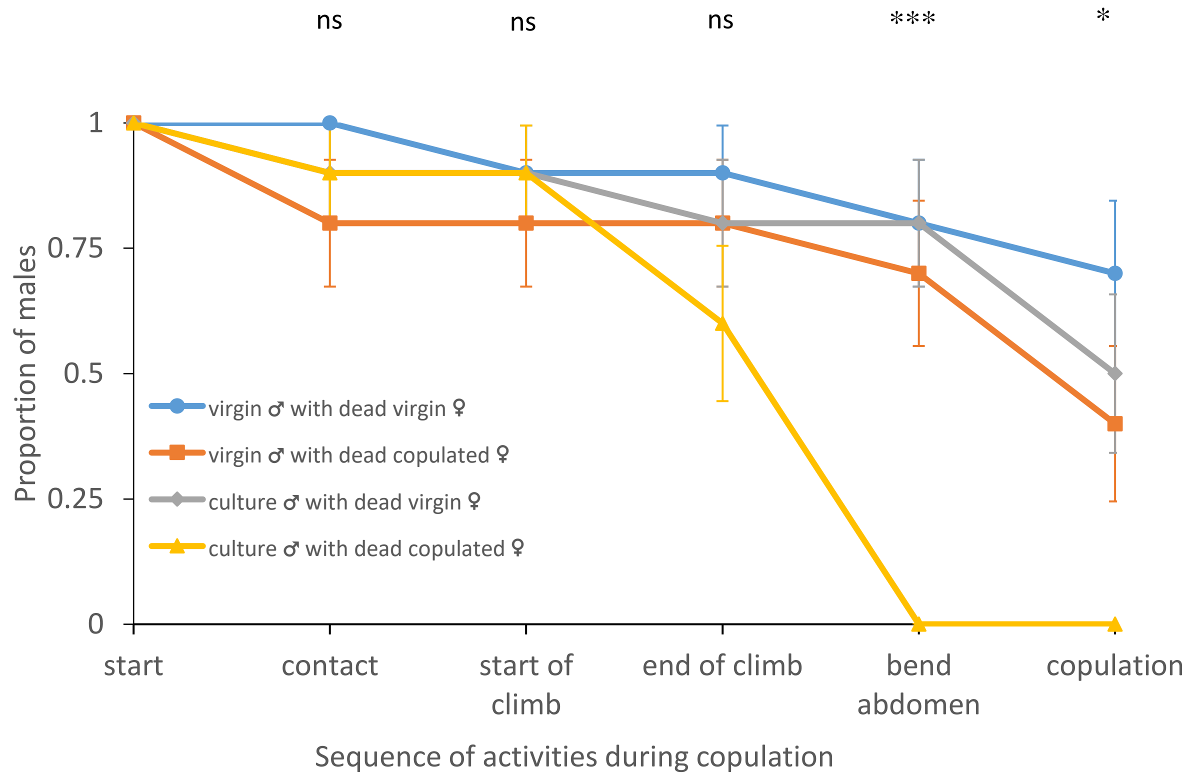


Figure 6.2 Variation in the sequence of activities leading to copulation between live virgin males or culture males with dead virgin females or dead single-copulated females. The mean proportion of males ± SE. Contact ($\chi^2_{(3)} = 2.22$, $P = 0.89$), start of climb ($\chi^2_{(3)} = 0.7$, $P = 1.00$), end of climb ($\chi^2_{(3)} = 2.7$, $P = 0.56$), bend abdomen ($\chi^2_{(3)} = 18.31$, $P < 0.001$), copulation ($\chi^2_{(3)} = 10.83$, $P = 0.017$). ns= not significant; * = $P<0.05$; *** = $P<0.001$

Table 6.2 The mean ± SE (n) duration of successful phases in the sequence of behaviour. Kruskal-Wallis tests was used for the analysis. Holm's adjusted *P*-values were used for multiple comparisons after a series of Mann-Whitney tests. Means followed by the same letter along the row are not significantly different. Number of replicates decreases because not all behaviours were completed. The duration of activities from contact to bend abdomen was sometimes interrupted and this was the reason for long duration observed in some of these activities.

	Male and female types introduced into the arena				
Behaviour duration (s)	virgin ♂ with virgin ♀	virgin ♂ with copulated ♀	culture♂ with virgin ♀	culture ♂ with copulated ♀	P-value
Start till first contact	53.5a ± 30 (10)	125.9a ± 65 (8)	137.4a ± 64 (9)	124.4a ± 47 (9)	$H = 3.4$, df = 3, $P = 0.34$
First contact to climbing	17.7a ±7 (9)	5.1a ± 1 (8)	10.0a ± 5 (9)	27.4a ± 15 (9)	$H = 1.1$, df = 3, $P = 0.77$
Start of climb to end of climb	2.6ab ± 2 (9)	0.4a ± .2 (8)	38.2b ± 25 (9)	169.7b ±74 (6)	$H = 20.4$, df = 3, $P < 0.001$
End of climb to bend abdomen	0.8a ± 0.4 (8)	46.7a ± 45 (7)	1.3a ± 0.4 (8)	-	$H = 3.0$, df = 2, $P = 0.23$
Copulation duration	209.0a ±10 (7)	42.8b ± 3 (4)	165.4ab± 38 (5)	-	$H = 7.9$, df = 2, $P = 0.02$

that on virgin females (627,972) (Table 6.3). No standard error is given because there was only one replicate of each extract.

Table 6.3 The peak area of 7-methyltricosane extracted from the cuticle of virgin males, virgin females and copulated females using an ion chromatogram (EIC) at m/z = 112.

F. occidentalis	**Area of ion chromatogram (EIC) m/z = 112**
Virgin males	27,335,327
Virgin females	627,972
Copulated females	2,754,762

6.5. Discussion

6.5.1. Can a culture male detect female copulation status?

In this study, it was confirmed that a male from the culture can detect the copulation status of a female. After making contact and climbing on a dead copulated female, none of the live culture males tested proceeded to bend the abdomen beneath that of the dead copulated female in order to copulate with her. However, when a live culture male was introduced to dead virgin females about 80% of the culture males proceeded to bend their abdomen beneath that of the females and about 50% of the culture males copulated with dead virgin females.

A similar lack of copulation was observed when a culture male was introduced to a live culture female (Chapter 5) or a dead culture female (this chapter). Though the copulation status of the females from the culture was unknown, this research clearly showed a similar trend between culture females and females that had copulated once. This therefore, confirms the assumption (Chapter 5) that most females from the culture were already copulated. A culture male may benefit from the ability to differentiate between a virgin and an already copulated female since virgin females will likely be of higher quality (fertilisation potential) than females that have already copulated in which subsequent copulation may not be successful (Chapter 4). This is especially true since some of the copulated females may not accept further copulations from males (Chapter 4) even if subsequent copulations could occasionally be successful. Other male insects have been observed to allocate more sperm to females with higher fertilisation potential, which is often dependent on their condition and age (Wedell *et al.*, 2002). This research therefore further confirms that a culture male can

distinguish a virgin female from a copulated one, and thus strategically copulate with virgins but not copulated females.

6.5.2. Can a virgin male detect female copulation status?

A virgin male was not observed to show any discrimination between a virgin and an already copulated female (at least until they bent the abdomen beneath that of the female). Even though a lower percentage of virgin males copulated with dead copulated females when compared with dead virgin females (40% vs 70%), this difference was not significant. It is possible that a few of the virgin males also discriminated against a copulated female, which may explain the lower percentage copulation observed in virgin males that copulated with copulated females when compared to virgin females. In Chapter 4, some of the virgin males that did not copulate with one-time copulated females were observed to climb but not bend the abdomen beneath that of the copulated female. However, as suggested in the previous chapter, it is likely that most virgin males strategically disregarded the copulation status of the female and proceeded with copulation because they still have plenty of accessory or sperm resources available to them (see Chapter 5).

6.5.3. Which cue was used to assess female copulation status?

A male insect can assess a female's quality through visual, olfactory or tactile means and some species may be able to combine these mechanisms (Bonduriansky, 2001). If a culture male *F. occidentalis* uses a visual cue in accessing a female's copulation status, it may be expected that there should be differences in time to first contact between a male paired with a dead virgin female and a male paired with a dead copulated female except if it is a short-range visual cue. Though a male appeared to see a female at close range before contact (Chapter 3), he did not appear to be able to distinguish the copulation status at this short range since the proportion of males that contacted and even climbed on dead females was

also not different in these two cases. Moreover, there are no obvious physical differences between a virgin and copulated female when live females were used (Chapter 5) suggesting that females did not alter their appearance after copulation. This therefore suggests that the cue used was neither visual nor tactile.

Behavioural changes (associated with change in copulation status) in the copulated females (Thomas, 2011) or mechanical resistance by copulated females could also be ruled out as a reason for the observed lack of copulation or bending of the abdomen by culture males since the females used were dead. Size differences between a culture male and a copulated female could also not be the reason for no copulation since the culture males copulated with dead virgin females that were of similar age and picked from the same batch of reared females. If it had been due to size differences, males usually would still have attempted copulation by at least bending the abdomen beneath that of the female even if they could not attach the abdomen tip to the ventral tip of the female's abdomen (Chapter 3). It was also not due to detection of a mechanical barrier (Chapter 4) since none of the culture males bent the abdomen beneath that of the dead copulated female, therefore there was no way they could have had a contact with any mechanical barrier within the female's body. Therefore, the likely means of assessing the copulation status of a female by a male was the use of a chemical cue.

The use of chemical cues by males in differentiating a copulated female from a virgin one is common among insects (Thomas, 2011; Malouines, 2017) and it is already known in one species of thrips (Krueger *et al.*, 2016). Several pheromones have been identified in male *F. occidentalis* but none in females (Kirk & Hamilton, 2004; Hamilton *et al.*, 2005; Olaniran *et al.*, 2013; Kirk, 2017). In this research, no behaviour suggested that a male avoided a copulated female before contact since there is no difference in time to first contact between a male and a dead virgin female vs a male and a dead copulated female, therefore there is no

evidence of production of a long-range repellent pheromone after copulation in females. The fact that a culture male only detected the copulation status of a female after contact suggests that the mechanism involves the use of a contact cuticular hydrocarbon which is usually less volatile and can only be detected at close range or after contact (Matthews & Matthews, 2010). The second possible reason as stated above involves change in female cuticular hydrocarbon to become less attractive. This is very unlikely since copulated females used for this research were often transferred into the freezer less than 2 hours after copulation. Although, the possibility of an immediate (30 minutes) change in cuticular hydrocarbon has been associated with a female ant *Leptothorax gredleri* becoming less attractive to males (Oppelt & Heinze, 2009), the changes observed in 30 minutes appeared so small that it casts doubt on whether it was actually detectable to a male.

The third and most likely mechanism by which a male *F. occidentalis* identifies the mating status of a female is by detecting a male-produced antiaphrodisiac pheromone applied to mark the female during copulation. The culture males likely detected the antiaphrodisiac pheromone on a copulated female after climbing on her because most of the culture males climbed copulated females after contact but did not proceed to bend the abdomen beneath her. During copulation, a male *F. occidentalis* usually stroked the dorsal part of the female thorax and abdomen with his mid leg, this behaviour that suggests a male may be applying a pheromone on the female or applying substances found on the arolium as an antiaphrodisiac pheromone on the females as he strokes her with the arolium.

Extracts of cuticular hydrocarbon of copulated females, virgin female and males showed more 7-methyltricosane in virgin males and copulated females than in virgin females. This compound has been previously identified as a male-produced contact pheromone in *F. occidentalis* (Olaniran *et al.*, 2013) and may therefore play a role in the detection of the copulation status of the female by a male *F. occidentalis*. A male *F. occidentalis* produces

7-methyltricosane and applies to the substrate an amount almost equal to that found on his cuticle, thus suggesting this pheromone plays an important role in male behaviour (Olaniran *et al.*, 2013). The application of this compound on the substrate led Olaniran *et al.* (2013) to suggest that a male may be using this compound in substrate marking and mediating fighting in male thrips as well as the pheromone acting as a male recognition pheromone. A male might also apply it on the female during copulation thus adapting the function of the compound into detection of female copulation status.

Pheromone production is expected to be costly to the male in order to ensure its reliability in female assessment (Johansson & Jones, 2007). Therefore, production of 7-methyltricosane will likely be costly if is used as an antiaphrodisiac pheromone except if the compound serves other functions, considering the quantity they produced (Olaniran *et al.*, 2013). Therefore, the benefit of assessment which may include prevention of future copulations or sperm competition from other males (Simmons, 2001) should be more than the cost to male *F. occidentalis.*

However, since the synthetic form of this compound has not been tested, it cannot be confirmed that 7-methyltricosane functions as an antiaphrodisiac pheromone in *F. occidentalis*. Other minor compounds or even blends of compounds which may include 7-methyltricosane are other options of possible antiaphrodisiac pheromones.

Traces of 7-methyltricosane found on virgin female *F. occidentalis* (though at lower quantity when compared to what was observed on copulated females or males) confirmed previous detection of traces of this compound on females (Olaniran *et al.*, 2013). Though females might have mechanically picked up the compound as suggested by Olaniran *et al*. (2013), it is clear from our observation that females also produced it in small amounts since the compound was detected on isolated virgin females. However, 7-methyltricosane is more of

a male-specific pheromone since the quantity found in males is much more when compared to a trace amount found in virgin females. It is therefore possible that this compound is below threshold or dispersed rather than localised in virgin females.

The two antiaphrodisiac compounds (dimethyl adipate and dimethyl glutarate) detected in *E. americanus* (the only known thrips species with an antiaphrodisiac pheromone) (Krueger *et al.*, 2016) or any similar compound were not found in the extract from male *F. occidentalis*, which may suggest variation in the compound used as an antiaphrodisiac pheromone among Thripidae.

6.5.4. Further evidence of a mechanical barrier preventing copulation in an already copulated female

As previously observed (Chapter 4), when a live virgin male copulated with a live copulated female, there was a consistently shorter copulation duration whether the copulation was in quick succession or separated by days. In this chapter also, when a virgin male copulated with a dead copulated female the copulation duration was significantly shorter when compared with copulation between a virgin or culture male and a dead virgin female. The use of dead females in this chapter therefore confirms that the observed reduction in copulation duration was not due to female behaviour but may likely be due to male detection of a physical obstruction.

There is an important question that should be asked. Why would a virgin male proceed with copulation with a copulated female if he can detect an antiaphrodisiac pheromone and when such copulation is likely to be shorter and thus ineffective? It is possible that a virgin male may be able to put sperm close to the spermathecal duct where it may still be useable by the female despite the possible barrier since the barrier in copulated females is likely located at the far end of the spermathecal duct (Chapter 4). Alternatively, it is also possible that there

is a small chance of the male's sperm getting into the female's spermatheca and dissolving into the spermatozoa bundle of an already copulated female. This may especially be possible if this already copulated female had the initial copulation with an old male that had copulated multiple times. Such males may transfer small sperm ball since the amount of sperm transferred by males to the female likely decreases with increase in the number of previous copulations, as observed in *E. americanus* (Krueger *et al.*, 2017). Less choosy virgin males may therefore still copulate with copulated females if there is a small chance of paternity.

Chapter 7

Species recognition

7.1. Introduction

In animals, an individual should be able to recognise its own kind before proceeding to mate. Mate recognition is therefore a subcategory of species recognition involving the ability of an individual to recognise other individuals in the same species as potential mates (Padian & Horner, 2013). Species recognition, however, involves the ability to recognise conspecifics for any appropriate social purposes (Padian & Horner, 2013). Mechanisms involved in species or mate recognition in insects include the use of chemical cues (Blows & Allan, 1998; Singer, 1998; Ginzel & Hanks, 2003; Ryan & Sakaluk, 2009), acoustic signals (Claridge, 1985; Percy *et al.*, 2006; Pennetier *et al.*, 2010; Derlink *et al.*, 2014), and visual or tactile cues (Gorb, 1998; Miller & Fincke, 1999; Tibbetts, 2002; Ryan & Sakaluk, 2009; Winfrey & Fincke, 2017).

The use of insect cuticular hydrocarbons for species, sex and colony recognition has been well documented (Singer, 1998; Martin & Drijfhout, 2009). Males of some species depend on their antennae for female sex recognition (Murakami & Itoh, 2003; Ryan & Sakaluk, 2009) before or after contact with the cuticle of the female. In some insects, cuticular

hydrocarbons are species and sex specific, therefore conspecific mates recognise one another upon contact with the antennae (Singer, 1998).

A male *F. occidentalis* produces aggregation pheromone which attracts both sexes of the same species (Hamilton *et al.*, 2005) and there is indication that this pheromone is species specific (Broughton & Harrison, 2012). This aggregation pheromone may therefore play a role in species recognition just before thrips landing, by attracting both sexes of the same species. Male *F. intonsa* produces the same headspace volatiles (neryl (*S*)-2-methylbutanoate and (*R*)-lavandulyl acetate) as *F. occidentalis* (Zhang *et al.*, 2011; Zhu *et al.*, 2012). Differences in ratio between the two compounds were claimed to play a role in interspecies recognition between *F. occidentalis* and *F. intonsa* (Zhu *et al.*, 2012), but this was not clearly demonstrated since synthetic forms of these compounds have not been tested.

At close range, a male thrips benefits from being able to identify conspecific females in a situation where more than one species is found on a crop. Male *Thrips major* and *Thrips fuscipennis* aggregating together in a flower of *Calystegia sepium* (L.) have been shown to copulate only with conspecific females despite the close morphological similarities and differences in population ratio observed during aggregation between males and females of these two species (Kirk, 1985a). Chemical cues extracted from the body of *Scirtothrips aurantii* have been shown to be used by males to recognise a female of the same species especially after antennal contact with the female, thus confirming that there is a chemical cue involved in mate recognition in *Scirtothrips aurantii* (Rafter & Walter, 2013). A male *F. occidentalis* has been observed to sometimes pause at a close range before contact or just after contacting a female using his antenna (Chapter 3) and a female without antennae has been observed to reject a male placed with her in a laboratory experiment (Terry & Schneider, 1993). This therefore suggests that a close-range cue may be involved in species or mate recognition in *F. occidentalis*.

Previous analysis of the cuticular lipids of adult *F. occidentalis* showed that the substances on the cuticle are made up of hydrocarbons and free fatty acids (Golebiowski *et al.*, 2007). A male-produced contact pheromone, 7-methyltricosane, identified in cuticular extracts of *F. occidentalis* has been suggested to play a role in mate recognition since it was produced abundantly in males when compared to females (Olaniran *et al.*, 2013). Therefore, thrips, like several other insects, likely depend on hydrocarbon compounds present in the lipid layer of its cuticle for a male to recognise females of the same species.

7.1.1. Aims of study

The aims of this chapter are to

1. test if a male *F. occidentalis* can differentiate between a virgin female of his own species and that of thelytokous *Thrips tabaci* and to identify the stage in copulation behaviour at which a male recognises if a female is of his own species or not.
2. analyse and compare the cuticular hydrocarbons of female *F. occidentalis* and female *T. tabaci* in order to predict the possible compounds that may be involved in species recognition by males. Only thelytokous *Thrips tabaci* is known to occur in UK.

Hypothesis: A male *F. occidentalis* can differentiate between a virgin female of his own species and that of thelytokous *Thrips tabaci* using female cuticular hydrocarbon.

7.2. Materials and methods

7.2.1. Rearing adult virgin female *F. occidentalis* and female *Thrips tabaci*

To test if a male picked from the culture can detect a female of its own species, it was necessary to rear adult virgin female *F. occidentalis* and female *Thrips tabaci*. Virgin female *F. occidentalis* were reared using the method described in Chapter 2. They were maintained individually on a small portion of bean pod in a modified microcentrifuge tube until needed

for experiment. *Thrips tabaci* were collected from glasshouses in Essex, UK and maintained on bean pods and pollen grains (*Pinus sylvestris*) in small pots each layered with four filter papers. The pots were placed in a humidity cage using a similar method to that described for oviposition and rearing pots in Chapter 2. Since *Thrips tabaci* are not known to produce males and were always virgin females, there was no need to isolate them as individuals before they emerged as adults. They were therefore picked from the pot when needed for an experiment.

7.2.2. Culture males

Male *F. occidentalis* that were to be used to test for possible different responses to virgin female *F. occidentalis* and *Thrips tabaci* were picked from the culture maintained in the laboratory (see Chapter 2).

7.2.3. Freezing of adult female thrips

In order to eliminate live female behaviour that could influence mating behaviour or even mating decisions by males and females, all the females used for this research were frozen at the same time for 90 minutes with the temperature of the freezer fluctuating between 6 and -24 ^{o}C using the same method described in Chapter 6. They were then defrosted for a minimum of 10 minutes before use. None of the frozen and defrosted females used were observed to move any part of the body or show any sign of life and were considered to be dead.

7.2.4. Bioassay and video recording

To test if a culture male *F. occidentalis* can detect a female of his own species and differentiate it from that of another species, two bioassays were set up. In the first bioassay, crosses between live culture male *F. occidentalis* introduced to virgin female *F. occidentalis* in a 5 mm diameter arena (described in Chapter 2) were compared with crosses between live

culture male *F. occidentalis* introduced to female *T. tabaci* and this was replicated 9 times. In the second experiment, dead females were used to remove possible behavioural or mechanical cues that a live female may produce that might alter a male's behaviour. Live culture male *F. occidentalis* were introduced to dead virgin female *F. occidentalis* and compared with crosses between live culture male *F. occidentalis* introduced to dead female *T. tabaci* and this was replicated 9 times. An individual dead adult female was first placed carefully (on her legs or side when it was difficult to make it stand) near the middle of the 5 mm diameter arena (described in Chapter 2) and a male was introduced into the same arena usually from the edge of the arena using a slightly moist brush. The arena was covered with a glass cover slip to prevent the male from escaping (see Chapter 2). The culture male used was individually picked from the bowl of thrips collected from the laboratory culture as described in Chapter 5. The arena with the thrips was placed on the stage of the camera-mounted dissecting microscope (see Chapter 2) and recorded for approximately 10 minutes. The arena containing the thrips was left for over 10 minutes if the pair was still copulating at 10 minutes. All experiments were performed at a temperature of 25±2 °C.

7.2.5. Observations

The research was carried out to test if a male *F. occidentalis* can recognise a female of his own species, therefore the bioassay set up was observed under the microscope to be able to view the behaviour of the male before and after contact with the female. The video of activities before and during copulation was recorded and saved on a SD memory card. The video was later played back to identify and measure the sequence of behaviours displayed in the pre-copulation and copulation phases as described in Chapter 5. When live culture male *F. occidentalis* were introduced to live females *F. occidentalis*, the activities were only observed as they happened under the microscope and not recorded and therefore the times between activities after contact was not noted. Therefore, the duration of activities after

contact could not be compared with when live culture males were introduced to live *T. tabaci*. In a few of the pairs, some behaviour could not be measured because of difficulty associated with viewing under the microscope. Such individual behaviours were not included in the analysis.

7.2.6. Collection and analysis of cuticular hydrocarbons

Individual adult virgin female *F. occidentalis* (n=100) reared on bean pods and isolated as larvae in individual microcentrifuge tubes, before emergence as adults, were collected together 3-5 days after emergence into a 1.5 ml separate microcentrifuge tube (the same females used in Chapter 6). Adult female *T. tabaci* (n=100) mass reared on bean pods were also collected into a microcentrifuge tube. They were then transferred into a separate glass vial (2 ml). 1 ml of HPLC-grade hexane was added to the vial containing the thrips using a 500 µl hexane-washed Hamilton syringe and the vial was covered with the vial lid for 5 minutes in order to obtain extracts. After about 5 minutes, it was expected that the cuticular hydrocarbon would have dissolved into the hexane but not the internal contents, therefore the extracts and hexane were drawn out using the syringe and transferred into another empty similar glass vial. This was then left open until all the hexane had completely evaporated. It was necessary to evaporate completely in order to be able to reconstitute to standard concentration and volume before GC-MS analysis. Evaporating the hexane did not lead to loss of compounds since cuticular hydrocarbons are less volatile. The extract was labelled before keeping the vial in the freezer until needed. The extract was then reconstituted and the vial containing the cuticular hydrocarbon was sent to Dr Falko Drijfhout in the School of Chemical and Physical Sciences for the analysis of the cuticular hydrocarbons using GC-MS, following the procedure described in Chapter 6 (6.2.6).

7.3. Statistical analysis

The difference in duration of behaviours was tested with a Mann-Whitney test using Minitab 17 (Minitab Inc., USA). The chi square test was used to test for differences in the proportion of individuals. Chi square was used with exact probability in order to allow for small sample size using IBM SPSS Statistics 21 (IBM Corp., New York).

7.4. Results

7.4.1. Can culture male *F. occidentalis* differentiate between virgin females of the same species and female *T. tabaci*?

Regardless of whether females were or alive or dead, the numbers of culture males that initiated climbing on a virgin female *F. occidentalis* were significantly more than those that initiated climbing on *T. tabaci* ($\chi^2_{(1)}$ =7.14, P = 0.02; $\chi^2_{(1)}$ =6.92, P = 0.03 respectively) (Figure 7.1 and 7.2), suggesting that they could differentiate between species after contact. After contact, 100% of culture males climbed and bent the abdomen beneath that of female *F. occidentalis* and 100 % of the males copulated with live females while 67 % copulated with dead females, however none of the culture males got to the stage of bending the abdomen beneath that of the female *T. tabaci* regardless of whether the female was alive or dead (Figures 7.1 and 7.2).

7.4.2. At what stage does a culture male identify females of his species and differentiate them from female *T. tabaci*?

All culture males contacted females regardless of the species. However, after contact, a very low percentage (only 25 %) of the males started climbing a female *T. tabaci* and only 13 % completed the climbing, when compared with the 89% of live virgin female *F. occidentalis* that were climbed by live culture males (Figure 7.1). When females were frozen,

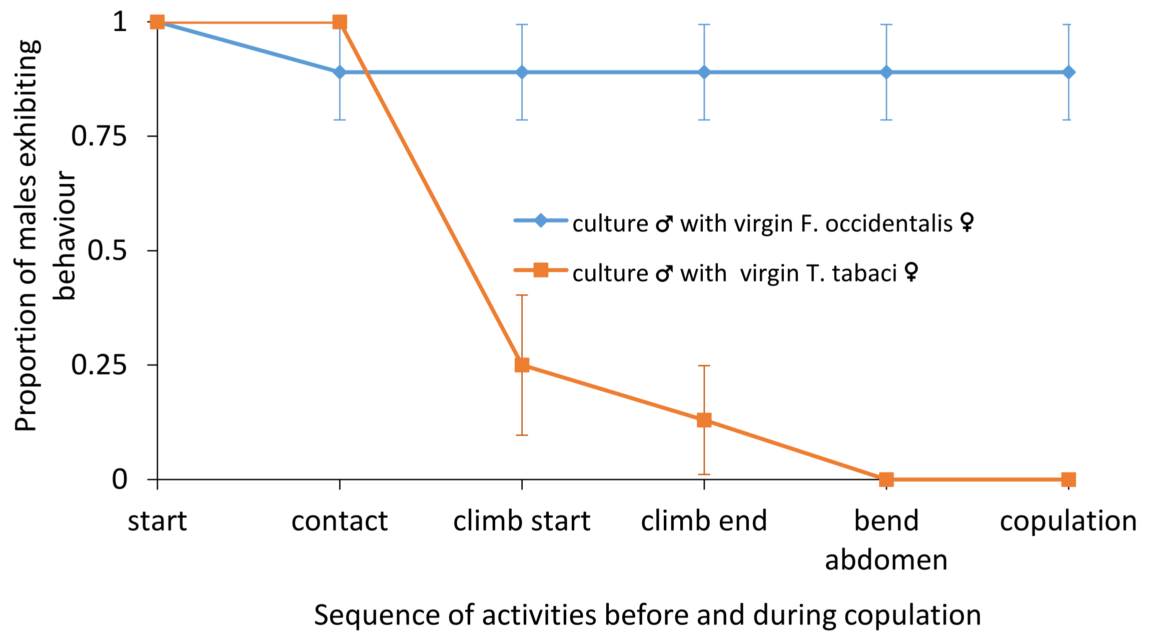


Figure 0.1 Variation in the sequence of activities leading to copulation between live male *F. occidentalis* with virgin female *F. occidentalis* or female *T. tabaci*. The proportion of males exhibiting the behaviour ± SE. Contact ($\chi^2_{(1)} = 1.06$, $P = 1.00$), start of climb ($\chi^2_{(1)} = 7.14$, $P = 0.02$), end of climb ($\chi^2_{(1)} = 9.92$, $P = 0.003$), bend abdomen ($\chi^2_{(1)} = 14.4$, $P < 0.001$), copulation ($\chi^2_{(1)} = 14.4$, $P < 0.001$) ns = not significant

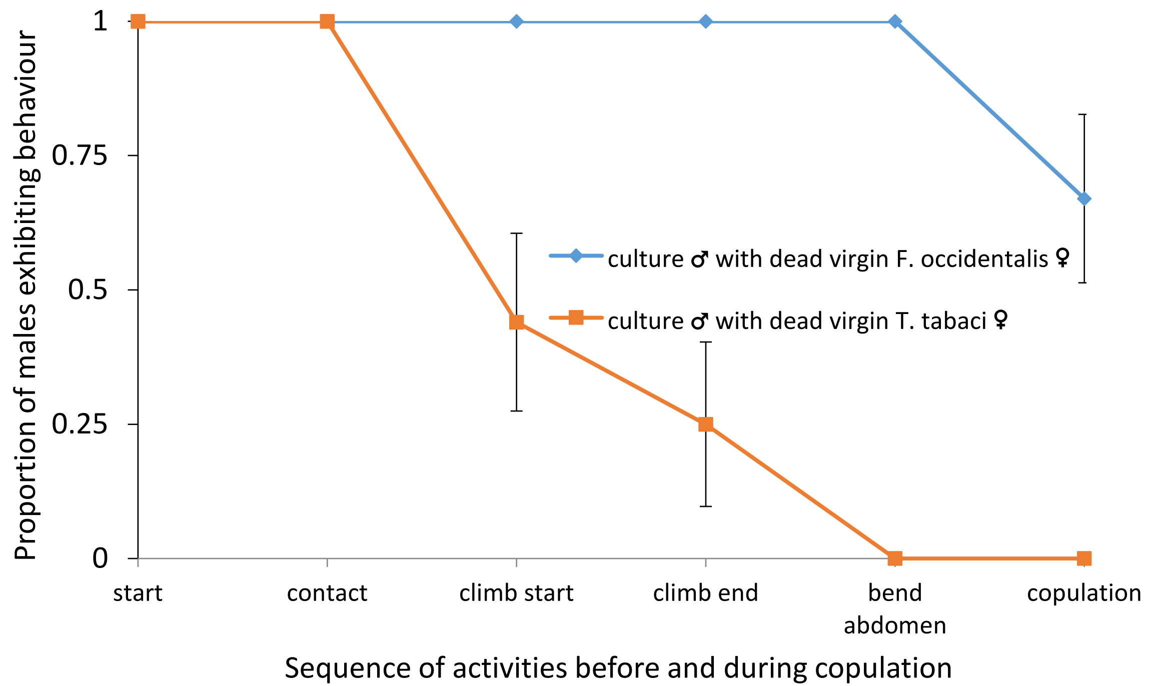


Figure 0.2 Variation in the sequence of activities leading to copulation between live male *F. occidentalis* with dead virgin female *F. occidentalis* or dead female *T. tabaci*. The proportion of males exhibiting the behaviour ± SE. Contact (-), start of climb ($\chi^2_{(1)}$ =6.92, P = 0.03), end of climb ($\chi^2_{(1)}$ =10.43, P = 0.002), bend abdomen ($\chi^2_{(1)}$ =18.0, P < 0.001), copulation ($\chi^2_{(1)}$ =9.0, P < 0.009)

only 25 % of culture male *F. occidentalis* completed climbing on dead *T. tabaci* compared to 100 % on virgin female *F. occidentalis* (Figure 7.2). However, the time to first contact between culture males and dead *T. tabaci* (63.8±33) was not significantly different (Mann-Whitney, W = 63.5, $P = 0.67$, n=8.) from the time to first contact between culture males and virgin female *F. occidentalis* (38.3 ± 10). It took a significantly longer time after contact before culture male *F. occidentalis* started climbing dead *T. tabaci* when compared to the time it took to climb dead virgin *F. occidentalis* (Mann-Whitney, W = 33, $P = 0.02$) (Table 7.1). It took a longer time ($P< 0.05$) for culture males to contact dead *T. tabaci* when compared to the time it took him to contact dead female *F. occidentalis* (Table7.1).

7.4.3. Identification of cuticular hydrocarbons of virgin female *F. occidentalis* and thelytokous *T. tabaci*

GC-MS analysis of cuticular hydrocarbons in the *n*-hexane extract of adult female *F. occidentalis* and *T. tabaci* is shown in figure 7.3. The hydrocarbons identified, retention times and the peak areas are given in table 7.2. In both adult female *F. occidentalis* and *T. tabaci*, the hydrocarbon profile consisted of a mixture of *n*-alkanes (C25-C29), and branched monomethyl and dimethyl alkanes. 7, 9, 11 MeC25 and 5MeC25 were more abundant in *F. occidentalis* while only trace amounts were found in *T. tabaci* (Table 7.2). Also, 9,XdiMeC25, 5,XdiMeC27, 3MeC25 and 4MeC26 were found in *F. occidentalis* but were absent in *T. tabaci*. Of all the major compounds present in *T. tabaci,* only pentacosanone and heptacosanone were found abundantly in *T. tabaci* but absent in *F. occidentalis* (Table 7.2).

Table 0.1 The mean ± SE (n) duration of successful phases between behaviours involving male *F. occidentalis* introduced separately to female *F. occidentalis* and *T. tabaci*. Mann-Whitney tests was used to compare durations. * = $P < 0.05$, ns=not significant. Number of replicates decreases because not all behaviours were completed.

Behavioural score	Culture male *F. occidentalis*		Significance level
	with frozen virgin female *F. occidentalis*	with frozen female *T. tabaci*	
Start to first contact	21.1 ± 12 (9)	110.8 ± 37 (9)	*
First contact to start of climb	3.2 ± 1 (9)	260.0 ± 86 (3)	*
Start of climb to end of climb	1.4 ± 1 (9)	2.5 ± 1 (2)	Ns
Start of climb to bend abdomen	24.8 ± 13 (9)	-	-
Copulation	239.5±15 (6)	-	-

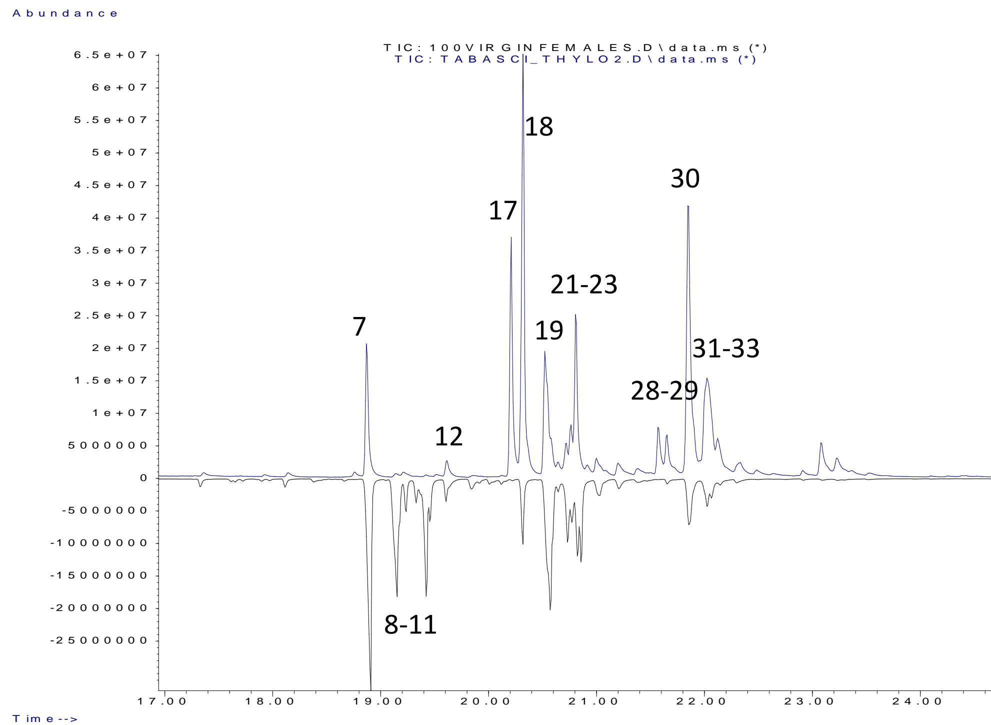


Figure 0.3 Gas chromatograms, from C23 to C29, of hexane extracts of adult female thelytokous *T. tabaci* (top) and adult virgin female *F. occidentalis* (bottom). The peak numbers correspond to peak numbers in Table 7.2 (Source: F. Drijfhout).

Table 0.2 Cuticular hydrocarbons of adult female *F. occidentalis* and female *T. tabaci* (Source: F. Drijfhout). X-unknown position of the methyl groups, *=Location of ketone group unknown (tentative identification). ND=Not detected.

Peak number	Retention time	Hydrocarbon Name	Peak Area	
			Female *F. occidentalis*	Female thelytokous *T. tabaci*
1	17.333	C_{23}	26109673	ND
2	17.62	9,11MeC23	6376193	ND
3	17.656	7MeC23	7405371	ND
4	17.903	3MeC23	ND	ND
5	18.118	C24	25706350	ND
6	18.384	?	21289353	ND
7	18.909	C25	7.59E+08	1.08E+08
8	19.153	7,9,11MeC25	5.42E+08	Trace
9	19.237	5MeC25	91107637	Trace
10	19.333	9,XdiMeC25	68282619	ND
11	19.426	3MeC25	5.13E+08	ND
12	19.609	C26	93060610	18097267
13	19.847	XMeC26	47988180	ND
14	19.917	?	16006207	ND
15	20.012	4-MeC26	27522295	ND
16	20.122	?	25044371	ND
17	20.212	Pentacosanone*	ND	1.71E+08
18	20.32	C27	1.78E+08	3.28E+08
19	20.576	7;9;11;13MeC27	7.31E+08	1.6E+08
20	20.647	5MeC27	40358324	13781598
21	20.736	9,XdiMeC27	2.16E+08	28301409
22	20.775	7,XdiMeC27	1.25E+08	37949671
23	20.828	3MeC27	2.26E+08	1.38E+08
24	20.861	5,XdiMeC27	2.34E+08	ND
25	21.028	?	1E+08	29441992
26	21.211	?	53017044	19715047
27	21.388	?	21497657	14551314
28	21.577	Heptacosanone*	ND	43824515
29	21.659	C29	20286230	48450680
30	21.862	7;9;11;13MeC29	2.26E+08	2.98E+08
31	22.029	diMeC29	1.13E+08	1.92E+08
32	22.068	7,XdiMeC29	68513951	55155881
33	22.304	?	22131893	21082470
34	23.085	?	ND	39692465
35	23.231	?	ND	21203526

7.5. Discussion

This research chapter confirms that a male *F. occidentalis* can identify a female of his own species and differentiate it from a female *T. tabaci.* A male *F. occidentalis* benefits from his ability to identify conspecific females since this will prevent him from wasting time courting or copulating with a female of another species, therefore the ability of an insect to recognise its own species is important for successful copulation and reproduction (Singer, 1998).

The time before contact was longer when a male *F. occidentalis* was introduced to a dead female *T. tabaci* compared to the time before contact when a male *F. occidentalis* was introduced to a dead female *F. occidentalis*. This may suggest that chemical or visual cues are involved in species recognition in male *F. occidentalis*; however, this duration difference may be an experimental artefact because the evidence of differences in durations conflicted with time from start till first contact when live females of these two species were used. Female *T. tabaci* are generally smaller than female *F. occidentalis,* therefore a live male could easily see, approach and contact a larger dead female (*F. occidentalis*) placed in the middle of the arena than a smaller one (*T. tabaci*) placed in a similar position

The fact that similar percentages (89-100 %) of males made antennal contact with females of both species (either live or dead females) before differences in behaviours were observed in males' interactions with the two species suggested that antennal contact with a female helps a male in confirming and recognising a conspecific female. Lewis (1973), stated that there are sense cones on the antennae of thrips that help sexes find each other. Previous experiments (Chapter 3) suggested that initial antennal contact was important for copulation. Males of other insects have been shown to depend on their antennae for female sex recognition (Singer, 1998; Murakami & Itoh, 2003; Ryan & Sakaluk, 2009). Few live male *F. occidentalis* completed climbing on female *T. tabaci* when compared to female *F.*

occidentalis even when they were paired with dead females. Since the use of dead females helps to eliminate female behaviours, it therefore implies that a male *F. occidentalis* after contact with a female *T. tabaci* recognised her as a different species and did not initiate copulation with her and moved away after climbing even in a no choice situation.

The cuticular extract of the hydrocarbons of female *F. occidentalis,* in the current study, was made up a mixture of *n*-alkanes (C25-C29), branched monomethyl and dimethyl alkanes which is similar to what has been previously identified in *F. occidentalis* (Golebiowski *et al.*, 2007; Olaniran *et al.*, 2013). Cuticular hydrocarbons have not been previously identified in *T. tabaci* and this appears to be the first report of the cuticular hydrocarbons in *T. tabaci*. When the hydrocarbon profile of *T. tabaci* was compared with *F. occidentalis*, both species have the same linear alkanes C25-C29, branched monomethyl and dimethyl alkanes. A few of the methyl branched alkanes (9,XdiMeC25, 5,XdiMeC27, 3MeC25 and 4MeC26) were present in virgin female *F. occidentalis* but not in female *T. tabaci.* It has been shown that in the social wasp *Polistes dominulus*, linear alkanes are not involved in nestmate recognition while methyl branched alkanes played a role as a recognition cue (Dani *et al.*, 2001). This may therefore suggest that any of the methyl branched alkanes present in *F. occidentalis* and absent in female *T. tabaci* may be employed by a male in identifying conspecific females, although this does not exclude the possibility that species recognition may also be a function of blends of chemicals in different concentration or ratio especially in cryptic or close species.

The only two compounds identified in female *T. tabaci* but not in female *F. occidentalis* were the ketones pentacosanone and heptacosanone. It is not clear if a male *F. occidentalis* recognises his conspecific female by identifying a particular compound or mixture of compounds or by the absence of such compounds or by detecting foreign compounds. If the latter is true then the presence of pentacosanone and heptacosanone in female *T. tabaci* may

play a role in species recognition of female *F. occidentalis.* 7- and 8-pentacosanone and 8- and 9-heptacosanone have previously been identified in male antennae of *Helicoverpa zea* but were not detectable in *Helicoverpa virescens* (Böröczky *et al.*, 2008).

Chapter 8

General discussion

The general aim of this study was to investigate the activities involved in the pre-copulation, copulation and post-copulation of adult male and female *F. occidentalis* and to investigate the possible role of pheromones in these copulation processes. This study therefore explored the sequence of events that leads to copulation between an adult male and female *F. occidentalis* and brought to light the complex nature and variation in copulation that could suggest what happens in a field situation. During the study, species recognition by males and the possible role of an antiaphrodisiac pheromone in adult male choice were also investigated.

The specific objectives were to:

6. determine the sequences of activities and behaviour during the pre-copulation, copulation and post-copulation phases in virgin male and female *F. occidentalis* (achieved in Chapter 3 and summarised in 7.1).
7. identify behaviours that may predict pheromone involvement during copulation in *F. occidentalis* (achieved in Chapter 3 and summarised in 7.1).
8. identify the possibility of multiple copulation in female *F. occidentalis* (achieved in Chapter 4 and summarised in 7.1).

9. study possible male and female choice based on copulation status (achieved in Chapter 4 and summarised in 7.2).
10. attempt to identify the pheromones that may be involved in male assessment of female copulation status and male species recognition by males (achieved in Chapters 5 and 6 and summarised in 7.3 and 7.4).

8.1. Multiple copulation in adult *F. occidentalis*

The description of the sequence of copulation in adult *F. occidentalis* observed in this research is similar to what has been previously described (Terry & Schneider, 1993). A male made antennal contact with the female, climbed her back, bent the abdomen tip beneath that of the female's abdomen and inserted the aedeagus. During copulation, two important behaviours were consistently observed. A male antennated a female's head and stroked the dorsal surface of the female thorax down to the dorsal surface of the abdomen with one of the mid-legs (the one on top of the female). This study suggested that this behaviour may be to calm the female or for the application of a substance on the female's body to calm her or application of an antiaphrodisiac pheromone on the female to mark her as already copulated.

This thesis revealed that copulation behaviour in *F. occidentalis* is much more complex than a virgin female copulating once and rejecting further copulations from males as has been suggested previously (Terry & Schneider, 1993). The interpretation by Terry and Schneider (1993) was too general and was based on a sample of only eight females and the males used were not all virgin males. In this thesis, though a male *F. occidentalis* picked from the culture rarely copulated with a female *F. occidentalis* picked from the culture (Chapter 5), female *F. occidentalis* copulated multiple times with virgin males when males were presented in immediate succession and over days (Chapter 4). Subsequent copulations by females after the first one (between a male and a virgin female *F. occidentalis*) were significantly shorter

(50 s vs 230 s) (Chapter 4). This has opened up a whole new research area in the study of mating behaviour in thrips. It will be important to investigate further if females benefit from such multiple copulations. Some female insects have been reported to benefit from multiple copulations through increased fecundity, other material benefits (Boggs & Gilbert, 1979; Hou & Sheng, 1999; Lamunyon, 2000; Singh & Mishra, 2010) and even genetic benefits (Walker, 1980; Simmons, 2001; Snook, 2014) while some copulate multiple times to avoid male harassment (Thornhill & Alcock, 1983; Parker, 1984; Rowe, 1992). Though this research showed that a female likely gets harassed with subsequent copulations with virgin males (Chapters 5), the reasons why a female *F. occidentalis* copulated several times with virgin males will require further study may. We speculated that it may be to prevent male harassment or for material benefits from accessory secretions transferred by the males during copulation (Chapters 4 and 5).

The copulation duration of a male and a previously copulated female was short and this study provided evidence that these subsequent copulations may not always be successful (result in the fertilization of eggs that are laid). Bioassays showed that when copulation between a male and a virgin female *F. occidentalis* was interrupted at different stages, sperm was not transferred to the female for at least 90 s. As observed with *E. americanus* (Krueger *et al.*, 2017), a male *F. occidentalis* likely transferred accessory gland materials first before the spermatozoa were transferred. When an already copulated dead female was copulated with a live male, the copulation duration was also reduced; suggesting that reduction in copulation duration in a copulated female was not due to female behaviour. Therefore, in Chapter 4, it was suggested that the observed reduction in copulation duration may be due to a physical barrier detected by the male during copulation. The spermatheca of a copulated female always contains a sperm bundle (Heming, 1995; Kumm, 2002). The sperm bundle found in copulated females was suggested to be the type that is formed within the body of the female

using the accessory secretion produced by the male as has been suggested in *E. americanus* and *F. occidentalis* (Heming, 1995; Krueger *et al.*, 2017). The physical barrier detected by male thrips may therefore be the spermatophore-like extension observed in *F. fusca* and *F. occidentalis* and termed the spermatophore tube (Heming, 1970a, 1995). It may be formed by spermatozoa and secretion which was likely placed close to the spermathecal duct as observed in *E. americanus* (Krueger *et al.*, 2017). There is no known report of reduced copulation duration in other species of thrips that copulate multiple times.

8.2. Evidence of male choice in *F. occidentalis*

A male picked from the culture (likely to be older than 5 days old and to have copulated multiple times), did not continue with copulation with an already copulated female after climbing her but proceeded to copulate with a virgin female even when dead females were used for the experiment (Chapters 5 and 6). This study therefore showed clear evidence of a male choice. Males are likely to be more choosy when they invest more in mating effort (Edward & Chapman, 2011). This study therefore suggests that male choice could have been adopted due to the cost a culture male incurred during copulation. However, a virgin male attempted to copulate (by bending his abdomen beneath that of the female) and even copulated successfully with females regardless of their copulation status (even when dead females were used). This study therefore suggested that an older male that has copulated multiple times shows a preference for virgin females over copulated ones while a young virgin male (3-5 days) is less choosy and will attempt copulation with most females regardless of their copulation status. Some of the virgin males were also observed to avoid copulation with already copulated females but these were very few (Chapter 4).

Male mating choice will occur because investment in current or additional mating reduces the opportunity to invest in future mating, and selection is expected to favour the proper

allocation of limited resources (Bonduriansky, 2001). Therefore, male resource allocation theory has shown that males with limited resources and high mating cost will allocate resources more judiciously and show more bias to females with high reproductive potential when compared with males with plenty of resources (Engqvist & Sauer, 2001; Byrne & Rice, 2006). In *E. americanus*, males showed a decrease in the area of spermatozoa within the testis with increase in the number of copulations and age of males, the quantity of accessory material transferred to a female also reduced with increase in the age of males; with empty accessory glands observed in males that had copulated with 10 females (Krueger *et al.*, 2017), thus suggesting that culture males may be limited in resources and therefore more choosy. A culture male appears to assess a female's mating status and strategically invest his mating effort based on the reproductive potential (possibility of using his sperm to fertilise her eggs) and the available limited resources (sperm and accessory gland product). This is especially true if a male requires more mating effort as resources become limiting due to age and copulation frequency, and thus the need for cautious allocation of resources (Engqvist & Sauer, 2001). A young virgin male on the other hand with abundant resources disregarded the copulation status of the female and attempted copulating with an already copulated female. Though subsequent copulations with a copulated female may not be successful, there may be a slight possibility of a male forcing his sperm into the female and that may explain why a virgin male proceeded to copulate with a female that had already copulated, otherwise it would be biologically non-adaptive for the male. When such attempts fail or when a male detected a barrier, the virgin male may therefore withdraw the aedeagus thus resulting in short copulations observed in most copulation between a virgin male and an already copulated female (Chapter 4).

Since culture males were picked from a mixed age culture, another reason that may be suggested for choice is the possibility that culture males have learnt to avoid copulating with

an already copulated female. Therefore, since such copulations were usually unsuccessful, culture males will avoid proceeding with copulating with copulated females while an inexperienced virgin male would attempt copulation. It is however not clear if a male needs to learn to recognise a pheromone.

This research did not find any evidence of female choice based on the male's copulation status. This, however, does not rule out the possibility of female choice, since females are believed to make choices (Bateman, 1948), it showed however that such choice may not be based on male mating status but on other variables such as size difference, pheromone production, age etc. These were not investigated in this research.

8.3. How does a male assess female copulation status?

The culture male pre-copulatory preference for a virgin female rather than an already copulated female implies that a male must have a way of assessing the copulation status of the female. This study therefore confirmed this hypothesis by introducing males to dead females (both copulated and virgin). None of the males picked from the culture bent the abdomen beneath or re-copulated with females that had copulated once. The fact that most of these males do not proceed after climbing the female suggests that assessment of the female took place after contact with the female. Male antennal contact with the female suggests that the detection is at close range and may involve the use of a contact pheromone. Out of the three mechanisms a male insect often uses for female assessment (Bonduriansky, 2001), this evidence suggests that males use chemical cues for the assessment of females' copulation status. The use of chemical cues for detecting a female's mating status is common in insects (Thomas, 2011; Malouines, 2017).

The extracts of cuticular hydrocarbons in this research showed that 7-methyltricosane, a male-produced contact pheromone in *F. occidentalis* (Olaniran *et al.*, 2013), was found to

be five times more abundant in copulated females than in virgin females (Chapter 6). This compound, which is produced abundantly by males and applied to the substrate (Olaniran *et al.*, 2013), may be applied on females during copulation by the male or picked up from the arena by the female and used by males as an antiaphrodisiac female assessment pheromone. This research however did not prove that this compound was the antiaphrodisiac pheromone, this will need further investigation. Another thrips species *E. americanus* has been observed to apply antiaphrodisiac pheromone to mark a female during copulation (Krueger *et al.*, 2016). A male may therefore benefit from marking a female if the cost of marking is less than the cost of wasting time attempting to copulate with an already copulated female since such copulation may not be successful. The discovery of an antiaphrodisiac pheromone in thrips has potential applied use in pest management. If a male avoids a female due to this compound then it may be sprayed on the field and used for mating disruption. This may however be limited and expensive to use since the pheromone will have to contact virgin female insects to be effective and this is difficult to achieve considering the cryptic nature of thrips.

8.4. Species recognition in *F. occidentalis*

In another experiment, male *F. occidentalis* needed to make contact with a female in order to recognise the species. When culture males were introduced separately to virgin female *F. occidentalis* or thelytokous *Thrips tabaci*, most of the culture males contacted females with their antennae regardless of the species before proceeding to copulate with virgin females of their species and avoiding thelytokous *Thrips tabaci,* although some of the female thelytokous *Thrips tabaci* also moved away after making contact with male *F. occidentalis* or flipped the abdomen to reject a male (Chapter 7). However, even when dead females were used, after contact, out of about only 25 % of males that climbed the dead thelytokous *Thrips tabaci*, none of them bent the abdomen beneath that of the female when compared with the

100% of virgin males that bent the abdomen beneath that of the dead virgin female *F. occidentalis*. This study therefore highlighted the vital role cuticular hydrocarbons play in species recognition. Analysis of the cuticular hydrocarbons of female *F. occidentalis* and female *T. tabaci* suggests that any of the methyl branched alkanes present in *F. occidentalis* and absent in female thelytokous *T. tabaci* or blends of chemical in different concentration or a ratio of them may be employed by a male in identifying conspecific females.

It is possible that visual observation is also used in species recognition, since the time before contact was longer when a male *F. occidentalis* was introduced to a dead female thelytokous *T. tabaci* compared to the time before contact when a male *F. occidentalis* was introduced to a dead female *F. occidentalis.* The possibility of using visual cues especially in differentiating a conspecific species from another species that are completely different or dangerous such as a parasitoid is likely. Therefore, it may be difficult to rule out the use of vision in species recognition by a male thrips. Further research is necessary to compare male recognition of a female thrips with that of a parasitoid.

8.5. Future direction

This study has opened up a lot of questions regarding the variation that occurs in copulation in male and female *F. occidentalis*. It has clearly demonstrated that females copulate multiple times with virgin males and those subsequent copulation durations with copulated females are short and may not be successful. It will however be important to confirm if virgin males transfer accessory materials or even sperm to a female that has already copulated, using histological and biotechnological methods. Such findings will clarify why subsequent copulations after the first ones were short and it will confirm if accessory materials form a physical barrier and prevent further successful copulation in an already copulated female *F. occidentalis*.

Another important area to investigate further is the actual status of a male picked from the culture. Though it is likely that a male picked from the culture is older and has copulated multiple times, it will be necessary to investigate if this status was what resulted in culture males being more choosy than young virgin males. The possibility of learning by male thrips can also be investigated further.

This study showed that females do not appear to make choice based on the male's copulation status, but has not ruled out the possibility of choice in females. Therefore, further research is necessary to test if females make choices based on other parameters such as the age or size of the male.

This research showed evidence that an antiaphrodisiac pheromone is involved during copulation in *F. occidentalis* and has suggested 7-methyltricosane as the potential antiaphrodisiac pheromone. This compound needs to be tested on virgin females to confirm if it plays this role. It will also be interesting to investigate the site of production of this pheromone and compare it with the site of production of the already known antiaphrodisiac pheromone in thrips species (*E. americanus*) (Krueger *et al.*, 2016). In a similar experiment, it will be necessary to confirm the compound(s) involved with species recognition in male *F. occidentalis* by testing if the compounds found on virgin female *F. occidentalis* but not on thelytokous *Thrips tabaci* or vice versa could be involved in species recognition.

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