

# **Chemical ecology of moths**

**Role of semiochemicals in host location by *Ectomyelois ceratoniae* and mate guarding by *Heliothis virescens***

By

**Seyed Ali Hosseini**

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# Chemical ecology of moths

## Role of semiochemicals in host location by *Ectomyelois ceratoniae* and mate guarding by *Heliothis virescens*

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# 1

## General introduction

It is no secret that the current mode of agricultural production, largely based on the intensive application of chemical pesticides, has far-reaching harmful effects on the environment and on human health (Geiger et al. 2010, Goulson 2013, Mostafalou and Abdollahi 2013). Pest control strategies thus need to shift from using such xenobiotics to more environmentally friendly methods, as advocated in integrated pest management (IPM) programs. In line with this, under recent European Union regulation (EU 2009; see <http://eur-lex.europa.eu>), reduction in pesticide use is enforced and the use of non-chemical methods in IPM is highly promoted and encouraged. Integrated pest management is an ecosystem-based, long-term strategy to control agricultural pests and to reduce their damage by the combinatorial use of techniques such as the introduction of natural enemies, habitat manipulation through intercropping and other modified cultural practices, and the use of resistant plant varieties. Behavioral manipulation of the pest species is another environmentally friendly approach in IPM, which is defined as “the use of stimuli that either stimulate or inhibit a behavior and thereby change its expression” (Foster and Harris 1997). Even though there are quite a few examples of successful application of behavioral manipulation in IPM (e.g. Foster and Harris 1997, Rodriguez-Saona and Stelinski 2009, Eigenbrode et al. 2016), for many pest species efficient methods have yet to be developed.

### **1. Principal elements of behavioral manipulation in pest management**

For each deliberate manipulation of a pest behavior in IPM, it is essential to identify (1) a behavior that is related to insect pest damage, (2) a stimulus that can manipulate the behavior of the pest insect, and (3) a method that exploits the stimulus to help manage the pest insect (Foster and Harris 1997). As females play the main role in insect population dynamics (Caswell 2001), behavioral manipulation methods that include females or affect both sexes will be more efficient than those affecting only males; female sex pheromones would then be the technique of choice.

#### ***1.1. Behaviors that are related to pest damage***

The first step towards creating a behavioral manipulation method for IPM is to identify a behavior that is related to the pest damage. Some pest behaviors are directly related to pest damage (e.g. feeding and host searching behavior), and successful manipulation of these behaviors will ensure protection of the plant. Other behaviors, however, are unrelated to the plant resource but affect pest damage by affecting its population dynamics (e.g. mating behavior). Successful manipulation of an unrelated behavior may affect a local population but not completely protect the resource because of, e.g., immigration from outside populations into the area being protected, as has been observed in mating disruption of several moth species (Witzgall et al. 2010).

#### ***1.2. Stimuli that can manipulate pest behavior***

In principal, visual, tactile, acoustic, and chemical stimuli can be exploited for their use in behavioral manipulation (Foster and Harris 1997). However, chemical stimuli such as semiochemicals (compounds that transfer information between individuals of the same or different species) are the most efficient tools in many behavioral manipulation strategies, as olfaction is a major sensory modality of most insect species (Dethier 1947, Bernays and Chapman 1994, Hansson and Stensmyr 2011). In addition, there are a number of practical advantages of using semiochemicals over other types of stimuli: (1) semiochemicals can be reproducibly identified and produced as a result of recent advances in chemical analysis and synthesis techniques, (2) semiochemicals are easy to handle in the field and various parameters, such as release rate and stability over time, can be controlled, (3) semiochemicals have already been used widely and successfully in IPM programs, so that managers and



farmers are familiar with its concept, and will thus be inclined to earlier implement newly identified semiochemicals, and (4) semiochemicals are generally very specific and thus are expected to have only limited non-target effects.

The main semiochemicals that are used in insect pest management are long-range sex pheromones. Pheromones are semiochemicals involved in intraspecific communication that are produced by one or both sexes of a species. Sustainable pest management through manipulation of sexual communication of insect pests has been a major driving force for moth sex pheromone research, as many moth species are agricultural pests, and sex pheromones of > 1600 species have been identified by now (see Pherobase.com). Sex pheromones are used for a) monitoring, b) mating disruption, and c) mass trapping of pest populations (Rodríguez-Saona and Stelinski 2009, Witzgall et al. 2010). For instance, in the United States, mating disruption using female sex pheromones is used on >200,000 hectares per year as the dominant control tactic in the Slow the Spread (STS) program against the gypsy moth, *Lymantria dispar* (L.), the most severe and economically important forest pests in the area (Tobin and Blackburn 2007, Onufrieva et al. 2014). However, other kinds of semiochemicals have also been successfully exploited in pest management, such as host plant volatiles (Bruce et al. 2003, Knight et al. 2005), aggregation pheromones (Bakke and Lie 1989, Borden 1988, Blomquist et al. 2010), host marking pheromones (Katsoyannos and Boller 1980), and alarm pheromones (Vandermoten et al. 2012).

### **1.3. Methods that exploit semiochemicals to help manage the pest insect**

Different methods have been developed to use semiochemicals in insect pest management. In some of these, insect pest behavior is not directly targeted, but indirectly by enhancing the performance of natural enemies in biological control programs or to induce resistance in host plants. A good example where both mechanisms have been exploited is the wheat culture, in which spraying the plant activator *cis*-jasmones changes the volatile blend of the crop such that the wheat becomes more attractive to natural enemies that significantly suppress the aphids pests (Bruce et al. 2003). Semiochemicals are mainly used in methods that target pest behavior, the most important of which are summarized below.

#### **1.3.1. Monitoring**

Monitoring is important in the case of exotic pests, such as fruit flies in the “fruit fly free zone” in the citrus production area of Florida (Simpson 1993), and for determining the efficiency of pest management techniques such as mating disruption. To monitor insect populations, different insect attractants are exploited in association with a wide variety of trap types (Cardé and Elkinton 1984, Taylor 1991, Jones 1998). Usually, attractants in monitoring programs are synthetic copies of the sex pheromones, although food/fruit baits (Sussenbach and Fiedler 1999, Reddy et al. 2007) and host plant volatile-based attractants (Knight et al. 2005, Crook et al. 2008) have also been used in monitoring insects.

Monitoring of insect pest populations has been developed to effectively determine the time of emergence of specific life stages of insects and consequently the need for insecticide application as an alternative to prophylactic calendar-based sprays (Knight and Croft 1991, Witzgall et al. 2008, Jones et al. 2013), by using traps baited with different attractants in combination with temperature-dependent development models. In this approach, the timing of sprays may be more efficiently done if sprays are based on female population dynamics, which is not necessarily in accordance with that of males (reviewed by Allen et al. 2011). An example of where this method has been effectively implemented is the use of sticky traps baited with a kairomone to monitor the apple maggot fly, *Rhagoletis pomonella* (Walsh), an

important pest fly of introduced apples in North America (Stanley et al. 1987, Agnello et al. 1990). An action threshold of 8 flies per trap was developed for this pest and this reduced annual sprays by 70% while maintaining acceptable levels of control.

### **1.3.2. Mass trapping and attract-and-kill**

Semiochemicals can be exploited in mass trapping, defined as “capturing a sufficient proportion of a pest population prior to mating, oviposition or feeding so as to prevent crop damage” (Rodriguez-Saona and Stelinski 2009). Mass trapping and attract-and-kill are, in principal, variations of the same method. In attract-and-kill, a semiochemical-based lure is combined with a toxic substrate rather than, e.g., a sticky surface or liquid receptacle as used in mass trapping. One of the success stories with this approach is trapping the conifer bark beetle, *Ips typographus* (L.), with a synthetic aggregation pheromone, which has proven highly effective in reducing pest populations and preventing damage (Dimitri et al. 1992). However, efficiency of mass trapping/attract-and-kill methods in IPM programs has generally been low, and the method appears to be only effective at low densities of the pest (Knipling 1979). If males are targeted, they must be removed from the population prior to mating to have an impact. In theory, as most male insects mate more than once, it has been suggested that nearly 99% male trapping is required for effective crop protection (Roelofs et al. 1970, Rodriguez-Saona and Stelinski 2009), a situation that is difficult if not impossible to achieve (see El-Sayed et al. 2006 for a review).

More efficient mass trapping is likely achieved when females are targeted before oviposition. Long-range male sexual pheromones in species where females are the mate-searching sex, such as tephritid fruit flies, seem to be the attractant of choice in this approach (Jang et al. 1994, Hardie and Minks 1999). For example, methyl eugenol, a male pheromone precursor of *Bactrocera* flies, is a highly effective attractant that has been used for the eradication of these flies in the Pacific region, including Hawaii and California (Hee and Tan 2004, Vargas et al. 2008, El-Sayed et al. 2009). Mass trapping of females may be achieved by food baits, as for example has been done to eradicate the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), by using protein-hydrolysate with attract-and-kill methods in the United States (Jackson and Lee 1985).

### **1.3.3. Mating disruption**

The IPM strategy to disrupt mating in insects aims to prevent one sex from reaching the other. Mating disruption has been one of the most successful applications of long-range sex pheromones for direct pest control. Synthetic mimics of insect pheromones are used to saturate the environment and as a result blur the normal pheromone plumes of individual females, so that mating behavior is disrupted because the mate-searching males cannot find the individual point pheromone source anymore. This method has been used most effectively against the codling moth in fruit orchards worldwide (Agnello et al. 1990, Knight et al. 1998, Knight and Turner 1999, Witzgall et al. 2010).

### **1.3.4. Host finding disruption**

Disruption of the chemical communication between the host (resource) and the insect pest has also been a major goal in pest management. Host finding can be disrupted by repelling and/or deterring a host-searching insect from a host that is normally attractive. A number of botanical repellents/deterrents have been incorporated in pest management programs. For example, DEET (N,N-diethyl-*m*-toluamide) is widely used for personal protection against mosquitoes and biting flies, and the feeding-deterrent neem, which is extracted from the seeds of the neem tree (*Azadirachta indica* A. Juss.), is applied against a large number of crop pest species

(reviewed by Isman 2006). Host finding disruption may also be achieved by spraying attractant host-plant volatiles on the host plant. An example of this approach is the use of attractant crude oils extracted from almond seed to disrupt the host-finding behavior of the navel orange worm, *Amyelois transitella* (Walker), a pest of almonds in California (Van Steenwyk and Barnett 1987). Spraying a formulation of 5% crude almond oil on the trees significantly suppressed egg deposition in egg traps and reduced the infestation of nuts.

### 1.3.5. *Push-pull*

The most effective behavioral manipulation in IPM may be achieved by the push-pull strategy, which involves the behavioral manipulation of insect pests via the integration of attractive and unattractive stimuli in the following way. An unattractive stimulus is used to push the pest insect away from the crop, while luring them toward an attractive source (pull) from where the pest is subsequently removed (Cook et al. 2007). Neem seed extracts have been applied to cotton (*Gossypium hirsutum* L.) to protect (push) it from *Helicoverpa armigera* (Hübner) while using an attractive trap crop, either pigeon pea [*Cajanus cajan* (L.) Millsp.] or maize (*Zea mays* L.) to pull the pest population (Pyke et al. 1987). Another success story are cereal crops of sub-Saharan Africa where the push-pull strategy has been used against lepidopterous stem borers (Cook et al. 2007, Amudavi et al. 2009, Khan et al. 2010, Pickett et al. 2014). In these agricultural systems, homoterpenes such as (*E*)-4,8-dimethyl-1,3,7-nonatriene are used to repel (push) the pest away from the main food crop while leaf alcohols such as (*Z*)-3-hexen-1-ol function as attractants from the “pull” plants (Pickett et al. 2014).

## 2. Searching for new semiochemicals to use in behavioral manipulation methods

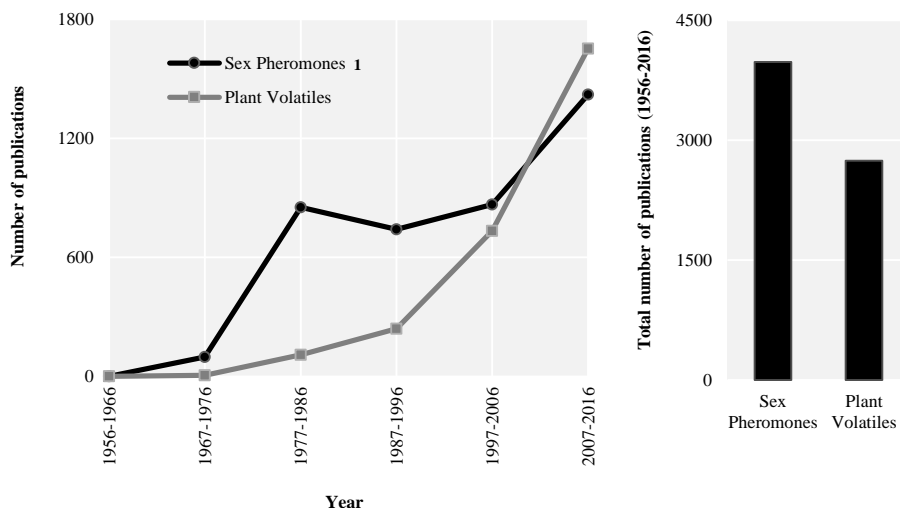
As mentioned above, behavioral manipulation methods will be more efficient if they target not only males but also females. For instance, mating disruption would be more efficient if both mate-searching and calling behaviors can be manipulated at the same time. In most pest species, especially moths, long-range female sex pheromones only affect behaviors of adult males. Semiochemicals that would affect females or both sexes may serve as alternatives or supplements to the long-range sex pheromones, especially when the sex pheromones are hard to produce, unstable, and/or inefficient. Therefore, it is important to focus research efforts on the identification of such semiochemicals and the establishment of methods that exploit them in pest management. In this thesis, I focus on host plant volatiles and pheromones that affect behaviors of both males and females.

### 2.1. *Host plant volatiles*

Host plant volatiles act as kairomones to herbivorous insects. Kairomones are infochemicals produced by an organism (the emitter) that benefit an individual of another species (the receiver), but harm the emitter. Plant volatiles play extremely important roles in an insect's life (Bernays and Chapman 1994). They are involved in host seeking behavior of females in search of oviposition substrates (Gothilf et al. 1975, Cossé et al. 1994, Yan et al. 1999, Landolt and Guédot 2008), in mate-finding behavior of males (Coracini et al. 2004, Landolt and Guédot 2008), and in detecting adult feeding sites in both sexes (Lin and Phelan 1991, Tingle and Mitchell 1992, Landolt and Guédot 2008). In some moth species, larvae also have been found to orient to host plant volatiles (Knight and Light 2001, Becher and Guerin 2009, Piesik et al. 2013).

Although sex pheromones have been the central element of insect behavioral manipulations, the extent to which plant volatiles influence host-plant localization in insects and thus their potential use in IPM has become increasingly apparent (Rodriguez-Saona and Stelinski 2009). This is most evident from the exponentially increasing numbers of studies on

the chemistry, the physiological and behavioral impacts, and application of host-plant volatiles in pest management over the last 20 years (Figure 1).



**Figure 1.** Number of publications that investigate sex pheromones or plant volatiles as tools for insect control, based on records from Web of Science (searched in All Databases, based on Topics). <sup>1</sup>Search terms: sex pheromones AND insect AND pest AND management. <sup>2</sup>Search terms: plant volatiles AND insect AND pest AND management.

### 2.1.1. Advantages and difficulties of using host plant volatiles in behavioral manipulation

Compared to long-range sex pheromones, the main advantage of host plant volatiles is that they can affect behaviors of both sexes (Gothilf et al. 1975, Cossé et al. 1994, Yan et al. 1999, Landolt and Guédot 2008). Moreover, they may also affect behaviors of immature stages, which usually are the life stages that inflict the plant damage (Knight and Light 2001, Becher and Guerin 2009, Piesik et al. 2013). There are also many examples of plant volatiles that synergize responses of male moths to female sex pheromone and thus enhance the efficiency of IPM methods that combine sex pheromones with plant volatiles (Ochieng et al. 2002, Yang et al. 2004, Schmidt-Buesser et al. 2009, von Arx et al. 2012). And not unimportantly, compared to pheromones, plant volatiles are often simple, commercially available, and cheap chemicals.

However, host plant volatiles may be generally less attractive than sex pheromones, as they have to compete with volatiles of the host plants in the field, especially in agriculture in which plants are usually grown as monocultures. This drawback may be overcome by using the stimuli at specific times, i.e. when or where the food source is absent, e.g., before fruiting time in fruit pest, or by using the stimuli to attract the pest to outside the agricultural area. Another drawback is that individual plant volatiles are generally not specific, and many are shared among a large group of plant species. As behavioral responses of pest insects to host plants are likely encoded by a specific volatile blend, it is essential to determine which host plants or plant parts are the most attractive sources for the pest, and through which volatile blends the insect is attracted to its hosts.

### 2.1.2. *Factors affecting insect behaviors in relation to host plant volatiles*

As choosing a poor host plant has immediate fitness consequences to the insect pest, any mechanism that increases its capacities to discriminate against inferior food sources is likely favored by natural selection. Fitness of a pest insect may not only be influenced by different plant species, but also by different varieties of a single host species (Bernays and Chapman 1994, Ventura et al. 1999, Tasin et al. 2005, Elanchezhyan et al. 2008, Sharon et al. 2009, Fotukiaii et al. 2013). The most susceptible hosts harboring the largest populations of an insect likely provide the most attractive cues to the insect (Ventura et al. 1999, Rull and Prokopy 2004, Elanchezhyan et al. 2008, Afzal et al. 2009, Gogi et al. 2010, Sobhani et al. 2015).

Insects synchronize their life cycle with that of their host to enhance fitness, optimize food intake and minimize the impact of environmental factors such as natural enemies and winter (Kooi et al. 1991, Zvereva 2002, Schoonhoven et al. 2005, Visser and Both 2005, da Silva et al. 2016). Synchronization can be achieved when the insects respond to the signals that are specific to the phenology of their host plants (Tasin et al. 2005, Proffit et al. 2007). It is well known that different phenological stages of the host differently affect insect attraction and oviposition behavior (Van Rensburg et al. 1988, Ramaswamy 1988, Sosa 1988, Spangler and Calvin 2000, Smyth et al. 2003, Tasin et al. 2005), which is likely due to the fact that different phenological stages but also different plant parts of the same phenological stage may emit distinctly different volatile blends (Bengtsson et al. 2001, Tasin et al. 2005, Vallat and Dorn 2005).

At high concentrations, an attractive host plant volatile may be unattractive or even repellent to the herbivorous insect (Finch 1978, Hern and Dorn 1999, Mewis et al. 2002). For example, it has been shown that doubling the amount of the attractant  $\beta$ -caryophyllene in a blend of host plant components, while keeping the concentration of the others constant, significantly reduced the attraction of female grape berry moth, *Paralobesia viteana* (Clemens) (Cha et al. 2011). Moreover, males, virgin females, and gravid females often respond differently to host-plant volatiles, because the integration of external stimuli and internal physiological state determines the threshold and ultimate outcome of the response of an insect to plant volatiles (Miller and Strickler 1984, Hern and Dorn 1999, Yan et al. 1999, Mechaber et al. 2002, Masante-Roca et al. 2007).

### 2.1.3. *Examples of behavioral manipulation with host plant volatiles*

Although host plant volatiles have recently been much studied in the context of pest management (Figure 1), there are only a few pest insects against which host plant volatiles are being used effectively, e.g. the codling moth, *Cydia pomonella* (L.) (Light et al. 2001, Knight et al. 2005, Knight and Light 2005a, Knight and Light 2005b, El-Sayed et al. 2013), the European grapevine moth, *Lobesia botrana* (Denis & Schifferrmüller) (Tasin et al. 2005, Tasin et al. 2006, Masante-Roca et al. 2007), and the Colorado potato beetle, *Leptinotarsa decemlineata* (Say) (Dickens 2000, Dickens 2002, Martel et al. 2005, Dickens 2006). The example par excellence is the codling moth, *Cydia pomonella*, a major pest in pome fruits and walnuts worldwide. Adults are attracted to the odor of apples (Wearing et al. 1973, Yan et al. 1999). Larvae and adults are also attracted to the plant volatile *E,E*- $\alpha$ -farnesene (Sutherland 1972, Hern and Dorn 1999). However, this terpene repelled female moths at high doses (Hern and Dorn 1999), and furthermore it has low environmental stability. A recent breakthrough in the development of an efficient plant volatile-based attractant was the identification of the pear ester ethyl (*E,Z*)-2,4-decadienoate, a volatile that is emitted from ripe Bartlett pears (Light et al. 2001). Field tests showed that traps baited with pear ester capture more codling

moths, both males and females, than pheromone-baited traps in orchards in which mating disruption is applied. The pear ester is stable, easy to synthesize, and readily released from dispensers, such as rubber septa (Knight and Light, 2005). Furthermore, the combination of the pear ester with codling moth sex pheromone considerably enhanced trap efficiency (Joshi et al. 2011). Most likely, codling moth females also use other volatiles from non-pear hosts to recognize suitable oviposition sites (Witzgall et al. 2005). For example, butyl hexanoate is an ester in apple odor that attracts mated female codling moths under laboratory conditions (Hern and Dorn 2004). The apple volatiles alone are not attractive in the field but they enhance the attractiveness of the pear ester when used in traps baited with the pheromone (El-Sayed et al. 2013).

### **2.2. Pheromones that do not only affect male behavior**

Pheromones are highly specific and often stable components that usually have no non-target effects. Apart from long-range male sexual pheromones in species in which females are the mate-searching sex, alarm and aggregation pheromones have also been successfully exploited in pest management strategies. These pheromones affect behaviors of both sexes, whereas host-marking pheromones (also called oviposition-detering pheromones) affect behaviors of gravid females only. For instance, aggregation pheromones have been applied successfully in attract-and-kill strategies against coleopteran pests (Lanier 1990), in particular against bark beetles (Borden 1988, Bakke and Lie 1989, Blomquist et al. 2010). Furthermore, host-marking pheromones are successfully exploited to control the cherry fruit fly, *Rhagoletis cerasi* (L.) (Katsoyannos and Boller 1980). Females of this insect smear some chemicals on cherry fruits directly after oviposition and these compounds inhibit oviposition by conspecific females on those fruits. Spraying an extract of this pheromone on cherry trees reduced fruit infestation by *R. cerasi* ten times (Katsoyannos and Boller 1980).

Chemical and behavioral analyses have also demonstrated that males of several moth species release odors during courtship that show aphrodisiac effects on female conspecifics, but inhibit mate searching behavior of conspecific males (Birch 1974, Birch and Hefetz 1987, Birch et al. 1990, Hillier and Vickers 2004, Hillier et al. 2007). Whether these close-range pheromones can be used in IPM has not been investigated so far. Because they simultaneously reduce female mate-finding activities such as movement and calling (Hillier and Vickers 2004) and repel conspecific males, male pheromones have great potential and should be more exploited in integrated pest management.

### **3. The study species of this thesis**

In this thesis, I focus on two moth species that are important agricultural pests, with the aim to develop behavioral manipulation methods to sustain the management of these two pests. The first pest species that I focus on is the carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae), which is a destructive fruit pest worldwide. It is known as the main limiting factor of pomegranate (*Punica granatum* L.) production in the Middle East. An efficient sex pheromone attractant is still missing in the control of this pest. The major female sex pheromone component [(*Z,E*)-9,11,13-tetradecatrienal] is unstable (Baker et al. 1991), so that an alternative stable mimic of this component, viz., (*Z,E*)-7,9,11-dodecatrienyl formate, is currently used in commercial sex pheromone lures. However, these lures are not very attractive in pomegranate orchards (Avand-Faghih et al. 2012, Dhoubi et al. 2016). In this thesis, I focus on pomegranate and pistachio to find a host plant volatile-based attractant to be used in carob moth pest management.

The second pest species that I focus on, the tobacco budworm, *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae), is an economically important pest of crops like tobacco, cotton, and chickpea in North and South America (Waldvogel and Gould 1990, Sheck and Gould 1993, Blanco et al. 2007). In this species, the male pheromone is identified as an aphrodisiac for females (Hillier and Vickers 2004). Using this species, I test the hypothesis that the male-specific hairpencil pheromone inhibits conspecific males from mating.

### 3.1. The carob moth

The carob moth (Figure 2) is a destructive worldwide polyphagous pest, attacking different fruits before and after harvest. It is recognized as the most important pest in the date fruit industry in the United States (Nay et al. 2006, Vetter et al. 2006). It is also a key pest of almonds [*Prunus dulcis* (Mill.) D. A. Webb] in Australia (Madge 2014), and in Europe it is frequently a problem in stored fruits and nuts, particularly almonds (Carter 1984). In the Middle East, *E. ceratoniae* is the most damaging pest of pomegranate in almost all pomegranate production areas, causing 30 - 80 % yield losses (Kashkuli and Eghtedar 1975, Shakeri 2004, Sobhani et al. 2015). Interestingly, date fruits have not been reported to be attacked by the carob moths in the Middle East, even though they are widely grown in the area. Pistachio is another host of this insect (Dhouibi 1982, Gothilf 1984, Mehrnejad 1992), especially in the Middle East (Mehrnejad 1992), although the carob moth has not been reported yet as an economic pest of pistachio before harvest. There are also a few records of carob moth on other host plants such as common fig, *Ficus carica* L. (Shakeri 1993), walnut, *Juglans nigra* L. (Balachowsky), and dried fruits, as well as on non-economic plants from a wide range of families (Doumandji-Mitiche and Doumandji 1982, Carter 1984).

The damage caused by larvae of carob moth on pomegranate is due to their feeding on the internal parts of the fruits, resulting in contamination with saprophytic fungi, which makes the fruits unmarketable and unfit for human consumption or the food processing industries (Shakeri 2004) (Figure 3). As oviposition and larval feeding occur inside the fruits and are thus hidden, commercial insecticides are not efficient against this pest (Shakeri 1993, Hoseini et al. 2014).

The carob moth has 3-5 generations per year on pomegranate in the Middle East, and fourth-generation larvae enter diapause at the end of the growing season, i.e. around October (Al-Izzi et al. 1985). The larvae resume feeding activity in spring. Fifth instars pupate in the fruit near the surface (near the calyx in uncracked fruit, also near cracks in cracked fruit) after they have made a hole in the peel (sometimes the top of the pupal case sticks out of the fruit). The insect pupates in the same fruit in which it fed as a larva (Al-Izzi et al. 1985, Shakeri 2004); females can mate in the night of emergence. There is no detailed information on the mating sites of carob moth, but we have observed mating couples on leaves of pomegranate in the field. Carob moth is highly polyphagous and it seems that the females that emerge in spring oviposit on plants other than pomegranate before they return to pomegranate. In pomegranate, female carob moth normally lays eggs inside the pomegranate crown (calyx) and in cracks of the fruits (Shakeri 2004, Talaie et al. 2010). Newly laid eggs are white and fertilized eggs become pinkish red within 48 h (Alrubeai 1987) (Figure 2a). Carob moths complete egg-to-adult development in a single pomegranate fruit (Shakeri 2004, Norouzi et al. 2008) and several larvae (occasionally more than 10) in different instars are generally found in a pomegranate fruit (Kashkuli and Eghtedar 1975, Al-Izzi et al. 1985, Shakeri 2004, Sobhani et al. 2015).



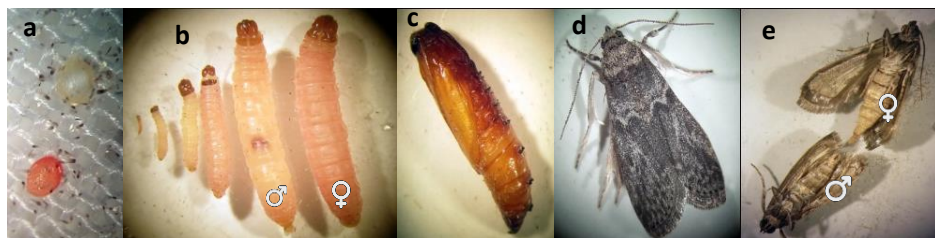


Figure 2. Carob moth, *Ectomyelois ceratoniae*, (a) eggs (a freshly laid, white egg and a pinkish red egg, ready to hatch), (b) the 5 larval instars, (c) pupa, (d,e) adults.

Pomegranate is native to Iran (Morton 1987) and there is a rich genotypic diversity of this plant species in the area (Sarkhosh et al. 2006, Sarkhosh et al. 2011). For instance, there are ~ 760 cultivars in the Yazd province of Iran alone (Behzadi 1998). Pomegranate orchards in most areas of the Middle East are typically composed of a mix of different cultivars with recently a tendency to increase the genotype diversity, especially in Iran which is one of the largest producers of this fruit in the world (Shakeri 2004, Sobhani et al. 2015). Peel cracking is a common phenomenon in pomegranate fruits and is considered as a major disorder (Shakeri 2004, Khalil and Aly 2013, Saei et al. 2014, Hoseini et al. 2014, Galindo et al. 2014). Recent studies have shown that different pomegranate cultivars have different susceptibilities to fruit cracking (Yuan et al. 2010, Saei et al. 2014), in addition to different susceptibilities to infestation by the carob moth (Moawad et al. 2011, Sobhani et al. 2015). Whether and how susceptibility of the cultivars to carob moth infestation is correlated with fruit cracking has been poorly studied.

Pomegranate continuously flowers during the growing season, so that different phenological stages are present to different extents at the same time. Signs of carob moth infestation become visible from the mid-growing season of the pomegranate, i.e. when most fruits are at a mature size (Hoseini et al. 2014, Sobhani et al. 2015). In this period, peels of many fruits are cracking (Figure 3).

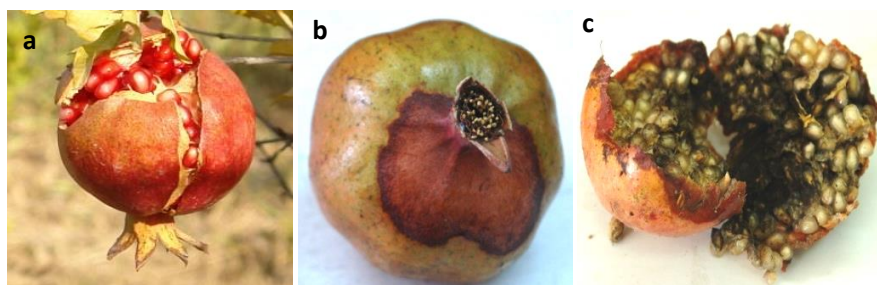


Figure 3. Fruit of pomegranate, *Punica granatum*, (a) cracked, (b) infested by carob moth, and (c) infested by carob moth and saprophytic fungi.

## 3.2. The tobacco budworm

*Heliothis virescens* is an economically important, polyphagous pest which occurs throughout the American continent (Fitt 1989). The insect has been reported to feed on more than 37 plant



species in 14 families (Waldvogel and Gould 1990, Sheck and Gould 1993, Blanco et al. 2007), and crops like tobacco, cotton, and chickpea belong to its economically important host plants (Blanco et al. 2007).

Female *H. virescens* normally produces from 300 to 500 eggs (Fye and McAda 1972). The insect has 5-7 larval stages, but mostly 5. Pupation occurs in the soil. Duration of the pupal stage is reported to be about 13 days at 25°C. Diapause is initiated by either low temperatures or short day length (Henneberry et al. 1993, Henneberry 1994). Longevity of moths is reported to range from 25 days at 20 °C to 15 days at 30 °C. There is no last-male sperm precedence in *H. virescens* (Lamunyon 2001). Females oviposit ~ 50% of their eggs after the first mating but significantly fewer eggs in each subsequent night (Proshold et al. 1982). Females re-mate every night or every other night (Raulston et al. 1975, Blanco et al. 2009). Males also mate only once per night and transfer a spermatophore that amounts to ~ 5-10 % of their body mass (Blanco et al., 2009). During courtship, male *tobacco budworm* display abdominal hairpencils and the hairpencils envelop the terminal end of the female's abdomen during mating (Figure 4).

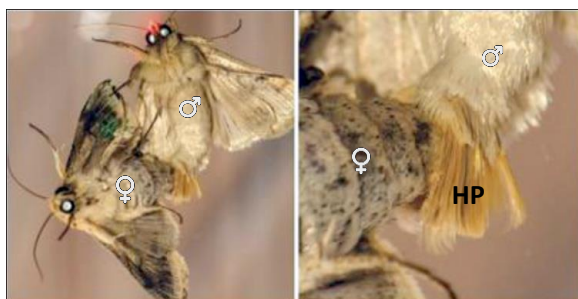


Figure 4. Tobacco budworm, *Heliothis virescens*, mating adults. HP = Hairpencils. © Jan van Arkel.

### Thesis outline

To identify new semiochemicals that can potentially be used to manipulate behaviors of the carob moth and the tobacco budworm, I investigated the host finding behavior of the carob moth and male-male competition in the tobacco budworm.

In **Chapter 2**, I investigated the association between fruit phenology and fruit susceptibility to the carob moth in pomegranate cultivars, with the aim to find the most susceptible host, assuming that this host provides the most attractive chemical cue to the insect. In a pomegranate orchard with 10 cultivars as a natural source of variation in host phenology, patterns of infestation by carob moth and fruit cracking were monitored during two consecutive years, 2013 and 2014.

In **Chapter 3**, I determined whether carob moths are differently attracted to different host plant materials, and whether the combination of pomegranate with virgin females enhances the attraction of males. I compared the attractiveness of pomegranate flowers, immature fruits, and cracked/uncracked mature pomegranates as well as mature pistachio using sticky delta

## Chapter 1

traps in pomegranate orchards of Iran. I also tested the attraction of the moths towards traps baited with headspace extracts of the two host plants.

As a first step towards the identification of host plant volatiles that mediate adult carob moths host finding and acceptance behavior, in **Chapter 4** I investigated the attraction of male, mated female, and virgin female carob moths to volatiles of different fruit stages of pomegranate and mature pistachio in a wind tunnel. I used coupled Gas chromatography-electroantennography (GC-EAG) to screen headspace extracts from pomegranate flowers, unripe fruits, healthy fruits, cracked fruits, and mature pistachio for bioactive compounds.

In **Chapter 5**, I tested the hypothesis that the close-range male pheromone of the noctuid moth *H. virescens* acts as an inhibitor in male-male competition. We also compared the reproductive output of females that mated once to those that mated two nights in a row to determine whether these anti-aphrodisiacs may affect female fitness.

In **Chapter 6**, I discuss the findings that I have presented in the previous four chapters in the context of their suitability in future IPM programs for the control of the carob moth and the tobacco budworm, two pest species of great economic importance.

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Seasonal pattern of infestation by the  
carob moth (*Ectomyelois ceratoniae*) in  
pomegranate cultivars

Seyed Ali Hosseini, Seyed Hossein Goldansaz, Seyedeh Masoumeh  
Fotoukchiaii, Steph B.J. Menken, and Astrid T. Groot

*Submitted to Crop Protection*

### Abstract

Pomegranate orchards in the Middle East are typically composed of a mix of different cultivars in which variation in fruit infestation by carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae), has been observed. However, seasonal variation in infestation and adaptation of the carob moth to this cropping system have not been explored. We monitored the progress of fruit infestation in 10 pomegranate cultivars during the growing season of two consecutive years in a pomegranate orchard in Iran. Overall, levels of infestation in fruits were strongly correlated with susceptibility to fruit cracking in pomegranate, so that cracked fruits and cracking-susceptible cultivars were infested the most. However, this pattern changed during the season. Infestation was first observed on cracking-susceptible cultivars. At this point almost all cracked fruits were infested. Towards the end of the season, infestation in uncracked fruits and cracking-resistant cultivars increased. Uncracked fruits seem better overwintering sites for carob moth as under simulated winter conditions, survival of insect larvae in uncracked fruits was > 3 times higher than in cracked fruits. Taken together, our data reveal that cracked fruits of pomegranate are the better host during the growing season, while uncracked fruits better sustain carob moth population in winter. It seems therefore advisable not to grow cracking-susceptible and cracking-resistant cultivars together in the same area.

### Introduction

Phytophagous insects synchronize their life cycle with growth stages and phenology of the host plants to enhance fitness, optimize food intake, and minimize the impact of adverse environmental factors such as natural enemies and winter (da Silva et al. 2016; Kooi et al. 1991; Schoonhoven et al. 2005; Visser and Both 2005; Zvereva 2002). Synchronization can be achieved when both insects and plants respond to the same environmental conditions or when insects respond to signals that are specific to the phenology of their host plants (Proffitt et al. 2007; Tasin et al. 2005).

Variation in suitability and availability causes phytophagous insects to display distinct preferences for particular plant species, cultivars, and even plant growth stages during the season (Jallow et al. 2004; van Asch and Visser 2007). Heteroecious, host-alternating aphids, for example, switch host plants during the season; they spend winter on tree or bush, but in summer they migrate to herbaceous plants, and at the end of season they return to the trees (Vilcinskis 2016). Also, population sizes of the trips *Frankliniella occidentalis* change over a season among chrysanthemum cultivars, because resistance against this insect develops differently among the cultivars (de Kogel et al. 1997).

The carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae), is a polyphagous destructive pest worldwide, attacking different fruits before and after harvest. This insect is recognized as the economically most important pest of pomegranate, *Punica granatum* L. (Lythraceae), in almost all pomegranate production areas of the Middle East, causing 30 - 80% yield loss (Kashkuli and Eghtedar 1975; Shakeri 2004; Sobhani et al. 2015). The pest larvae feed on internal parts of the fruit, resulting in contamination with saprophytic fungi, which makes the fruit unfit for human consumption and food processing industries, and thus unmarketable (Shakeri 2004). Since egg-laying and larval feeding activities occur within the fruits and are thus hidden from the outside world, commercial insecticides are not efficient



and thus not used against this pest. Carob moths have 3-5 generations per year on pomegranate. The last generation larvae enter diapause inside the fruit at the end of the season (Al-Izzi et al. 1985).

Pomegranate is native to Iran (Morton 1987), and there is a rich genotypic diversity of this plant species in the area (Sarkhosh et al. 2006; Sarkhosh et al. 2011). Pomegranate orchards in most areas of the Middle East are typically composed of a mix of different cultivars, with recently a tendency to increase the genotypic diversity, especially in Iran which is one of the largest producers of this fruit in the world (Sarkhosh et al. 2011; Shakeri 2004; Sobhani et al. 2015). Fruit peel cracking is a very common phenomenon in pomegranate and is considered as a major disorder of the fruits (Galindo et al. 2014; Hoseini et al. 2014; Khalil and Aly 2013; Saei et al. 2014; Shakeri 2004). Recent studies have shown that pomegranate cultivars differ in their susceptibility to fruit cracking (Saei et al. 2014; Yuan et al. 2010). Pomegranate cultivars also exhibit different susceptibility to the carob moth (Moawad et al. 2011; Sobhani et al. 2015). Whether susceptibility of the cultivars to carob moth infestation is related to fruit cracking has been poorly studied. In this study, we monitored seasonal patterns of carob moth infestation and fruit cracking in 10 pomegranate cultivars in the field and determined seasonal variation in association patterns of these two common phenomena in pomegranate. These patterns provide insight into the adaptation of carob moth to pomegranate cultivars in the Middle East, and practical information for management of the pest.

### Material and methods

Field experiments were conducted in a pomegranate orchard located in the Research Station of the College of Agriculture and Natural Resources, University of Tehran, Alborz Province, Iran (35°46'34''N, 50°55'46''E and 1254 m elevation) during the cropping season of 2013 and 2014. In this orchard, 10 high-yield Iranian pomegranate cultivars were planted (cultivars specified in Figure 1), so that all cultivars were grown under the same environmental conditions, and all were of the same age (8 years old in 2013). There were no pesticide treatments in the study area during the experimental period. Pomegranate cultivars were planted in a randomized complete block design in 4 blocks, with a total of 40 (4 × 10) plots. Each plot contained 5 trees of the same cultivar.

To determine the development of infestation and fruit cracking, 2 trees in 2013 and 1 tree in 2014 were selected randomly in each plot, and checked weekly for infested and cracked fruits, which thus amounted to a total of 8 and 4 trees per cultivar in 2013 and 2014, respectively. From the start of the season in which signs of carob moth infestation on pomegranate became visible (i.e., late August), referred to as growing season, infested and cracked fruits on and under the trees were recorded, marked, and left in the orchard. At the end of the season, when the fruits had developed to marketable stage (i.e., in mid-October), all unmarked fruits were harvested and taken to the laboratory where the number of larvae in each fruit was determined.

To determine survival rates of carob moth larvae under simulated winter conditions, 46 cracked-infested and 46 uncracked-infested fruits were randomly picked from the orchard at harvest time (October 2014), and kept in a fridge at 8 °C and 80% RH for 100 days, after which the fruits were dissected and numbers of dead and alive larvae were recorded.

A general linear model (GLM) was used to determine differences among cultivars in terms of the percentage of total fruit infestation, percentage of total fruit cracking, the number of fruits per tree, and the number of larvae per infested fruit. Differences between means were

determined using the Tukey's HSD test with a 95 % confidence interval. To stabilize the variance, percentages of fruit cracking were first arcsine  $\sqrt{X}$  transformed (where  $X$  is the fruit cracking rate), whereas percentages of fruit infestation were square root transformed. Differences between cracked and uncracked fruits in terms of the percentage of infestation and the number of larvae per infested fruit were also determined using GLM. Replicate blocks were included as a fixed factor in the models. Correlation analyses were performed between percentage of total fruit infestation, percentage of total fruit cracking, number of fruits per tree, and number of larvae per infested fruit. The survival rate of carob moth larvae in cracked and uncracked fruits after 100 days at 8 °C was also analyzed using GLM. All analyses were conducted in R version 3.2.3 (R Core Team. 2015).

## Results

Average number of fruits per tree differed significantly between the two growing seasons;  $83.61 \pm 6.43$  (mean  $\pm$  standard error) fruits in 2013 versus  $20.10 \pm 1.88$  in 2014 ( $F = 75.66$ ;  $df = 1, 54$ ;  $P < 0001$ ). The number of fruits per tree was also significantly different across cultivars in 2013, but not in 2014 (Table 1). In both years, the cultivars differed further in terms of percentage of total fruit cracking and percentage of total fruit infestation (Table 1, Figure 1).

**Table 1.** General Linear Model (GLM) analysis of the variation in pomegranate fruit infestation, fruit cracking, number of fruits per tree, and number of carob moth larvae per infested fruit in different pomegranate cultivars in 2013 and 2014. Replicate blocks were included as a fixed factor

		Cultivar			Block			Cracking status of fruit		
		df	F	P	df	F	P	df	F	P
2013	Total fruit infestation (%)	9, 27	19.72	<b>&lt;0.001</b>	3, 27	3.72	<b>0.023</b>	-	-	-
	Total fruit cracking (%)	9, 27	8.48	<b>&lt;0.001</b>	3, 27	4.16	<b>0.015</b>	-	-	-
	Number of fruits per tree	9, 27	6.77	<b>&lt;0.001</b>	3, 27	1.64	0.202	-	-	-
	Number of larvae per infested fruit	8, 32	2.68	<b>0.022</b>	3, 32	2.19	0.108	1, 32	31.15	<b>&lt;0.001</b>
	Infestation (in cracked/uncracked fruits) (%)	-	-	-	3, 74	0.36	0.780	1, 74	29.71	<b>&lt;0.001</b>
2014	Total fruit infestation (%)	6, 18	6.01	<b>0.001</b>	3, 18	1.85	0.175	-	-	-
	Total fruit cracking (%)	6, 18	3.06	<b>0.003</b>	3, 18	1.46	0.259	-	-	-
	Number of fruits per tree	6, 18	1.15	0.373	3, 18	1.61	0.222	-	-	-
	Number of larvae per infested fruit	6, 14	7.28	0.066	3, 14	4.44	0.126	1, 14	47.25	<b>0.006</b>
	Infestation (in cracked/uncracked fruits) (%)	-	-	-	3, 43	0.65	0.585	1, 43	29.61	<b>&lt;0.001</b>

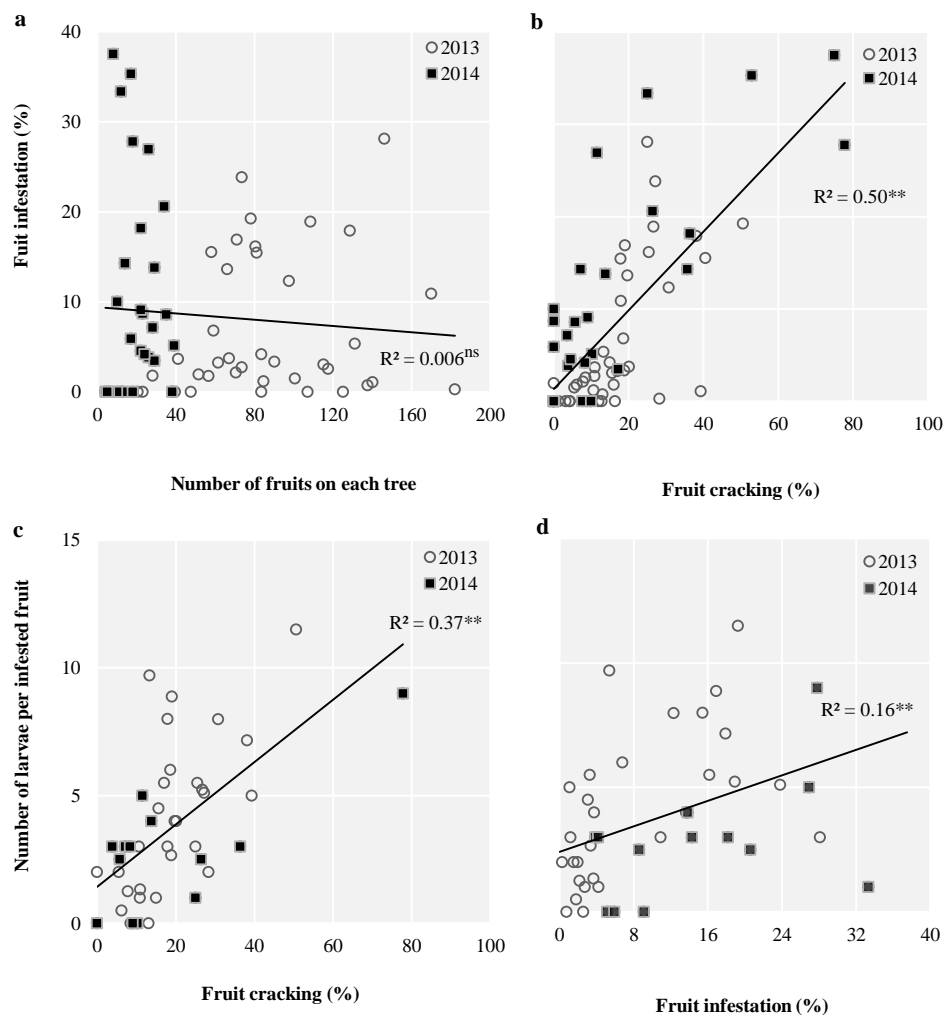
Significant  $P$ -values are highlighted in gray

Fruit infestation was significantly correlated with fruit cracking and the number of insect larvae per infested fruit, but fruit infestation and fruit cracking were not correlated with the number of fruits per tree (Table 2, Figure 2). In both years, the number of larvae per infested fruit and total infestation were significantly higher in cracked fruits than in uncracked fruits (Table 1, Figure 3).

# Seasonal pattern of carob moth infestation

Cultivar	Total fruit cracking (%)		Total fruit infestation (%)	Fruit cracking (%)								Infestation of cracked fruits (%)								Infestation of uncracked fruits (%)								
				Aug. W3	Aug. W4	Sep. W1	Sep. W2	Sep. W3	Sep. W4	Oct. W1	Oct. W2	Aug. W3	Aug. W4	Sep. W1	Sep. W2	Sep. W3	Sep. W4	Oct. W1	Oct. W2	Aug. W3	Aug. W4	Sep. W1	Sep. W2	Sep. W3	Sep. W4	Oct. W1	Oct. W2	
2013	A (Tabestaneh-Torsh)	34.34	18.55	A	0.87	0.16	3.79	4.81	7.43	3.76	7.73	13	100	100	63.1	53.8	76.2	52.5	26.9	31.4	0	0	0	0.42	0.41	0	0.83	2.17
	B (Alak-Torsh)	27.39	20.07	A	0	0.59	4.09	4.73	2.91	3.8	6.88	8.3	66.7	83.3	90.5			52.2	38.4	33.6	0	0.12	0.19	0.21	0.73	1.89	3.2	
	C (Poust-Siah-Shirin)	24.18	0.34	D	0	0	0	0	0	0.12	6.36	19.1						0	0	0.61	0	0	0	0	0	0.07	0.15	
	D (Poust-Sefid-Shirin)	19.62	7.16	Bc	0	0.07	0.63	0.56	0.66	0.69	8.31	9.99		100	87.5	0	77.8	26.3	17.1	0	0	0	0	0.08	0.17	0.73	1.39	
	E (Agha Mohammadali)	16.31	10.88	Ab	0	0	0	1.53	1.89	3.51	5.11	5.71			56.7	75	58.2	29.3	38.6	0	0	0	0.1	0.16	0.34	0.56	0.79	
	F (Alak-Shirin)	16.30	2.19	Cd	0	0	0.15	0.3	0.65	2.55	6.84	6.61		0	0	50	25	6.25	8.57	0	0	0	0	0	0	0.22	0.47	
	G (Malas-Torsh)	14.51	2.93	Bcd	0	0	0.09	1.1	2.55	4.75	3.32	3.61		0	0	31.3	12.1	13.3	37.3	0	0	0	0	0	0.1	0.24	0.37	
	H (Shirin-Saveh)	7.45	1.86	Cd	0	0.15	0.15	0.68	0.9	1.2	1.97	2.72		0	0	0	0	12.5	29.2	0	0	0	0	0	0	0	0.78	
	I (Poust-Sefid-Torsh)	6.91	1.93	Cd	0.18	0	0.66	0.32	1.93	0.74	1.63	1.71	0		0	0	0	0	0	50	0	0	0	0	0	0.49	0.38	
	J (Poust-Sefid-Bihasteh)	4.90	0.00	D	0	0	0	1.22	0	0.64	1.59	1.54						0	0	0	0	0	0	0	0	0	0	
2014	A (Tabestaneh-Torsh)	44.49	26.07	A	1.39	0.96	8.67	8.33	10.2	9.09	18.4		100	100	100	62.5	61.1	33.3	7.14	0	0	0	0	0	1.09	1.09	2.17	
	B (Alak-Torsh)	26.26	18.48	Ab	0	0.74	4.02	1.52	3.57	14.9	11			100	100	100		16.7	16.7	0	0	0	0	2.01	4.17	10.1	1.09	
	D (Poust-Sefid-Shirin)	15.67	7.68	Abc	0	1.14	0	0	3.24	5.55	7.53					25	25	16.7	0	0	0	0	0	0	0	1.47	4.29	
	E (Agha Mohammadali)	12.62	16.49	Ab	0	2.08	2	4.06	0	0	5.25			100	50	100			66.7	0	0	0	1.79	0	0	2.5	3.78	
	H (Shirin-Saveh)	5.45	4.74	Bc	0	0	0.64	0	0	0	3.9	0.96			100			0	100	0	0	0	0	0	2.5	0.71		
	J (Poust-Sefid-Bihasteh)	4.58	1.04	C	0	0	0	0	0	2.5	2.08	0						0	0	0	0	0	0	0	0	1.14		
	I (Poust-Sefid-Torsh)	1.43	3.61	Bc	0	0	0	0	0	0.71	0	0.74				0	0	0	0	0	0	0	0	0	0	3.74		

**Figure 1.** Average percentages of total fruit infestation and total fruit cracking, and averages of the percentages of weekly fruit cracking and weekly fruit infestation by carob moth in cracked and uncracked fruits (i.e., number of cracked/uncracked fruits infested divided by total number of cracked/uncracked fruits) in different pomegranate cultivars during the two growing seasons of 2013 and 2014. Means with different letters (a-d) are significantly different ( $\alpha = 0.05$ ). The gradation of black/gray depicts the averages from high (black) to low (light gray). Blanks indicate no infested and cracked fruit. W= week.



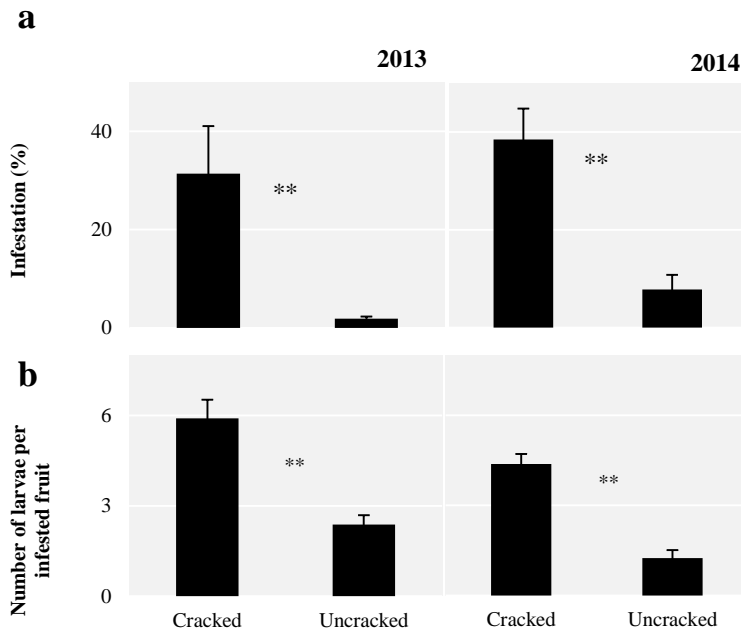
**Figure 2.** Correlations between a) number of fruits on each pomegranate tree and percentage of pomegranate fruit infestation, b) percentage of fruit cracking and percentage of fruit infestation, c) percentage of fruit cracking and number of carob moth larvae per infested fruit, and d) percentage of fruit infestation and the number of carob moth larvae per infested fruit. \*\* indicates a significant correlation at 0.01 confidence level; ns = non-significant.

**Table 2.** Pearson correlation coefficients between the number of pomegranate fruits per tree, percentage of total fruit infestation, percentage of total fruit cracking, and the number of carob moth larvae per infested fruit

	Number of fruits per tree	Total fruit infestation (%)	Total fruit cracking (%)
Total fruit infestation (%)	$r = -0.08$ ns		
Total fruit cracking (%)	$r = 0.16$ ns	$r = 0.71^{**}$	
Number of larvae per infested fruit	$r = 0.14$ ns	$r = 0.39^{**}$	$r = 0.60^{**}$

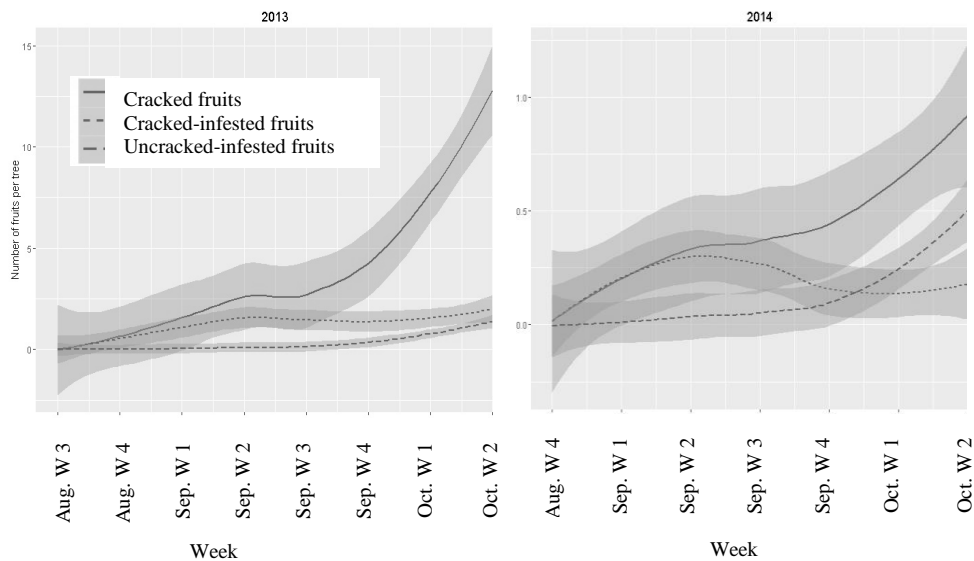
\*\* indicates a significant correlation at 0.01 confidence level; ns = non-significant.

The number of larvae per infested fruit differed significantly among the cultivars in 2013, whereas it was marginally insignificant in 2014 ( $P = 0.06$ ; Table 1), which is likely due to the low number of total fruits in that year.



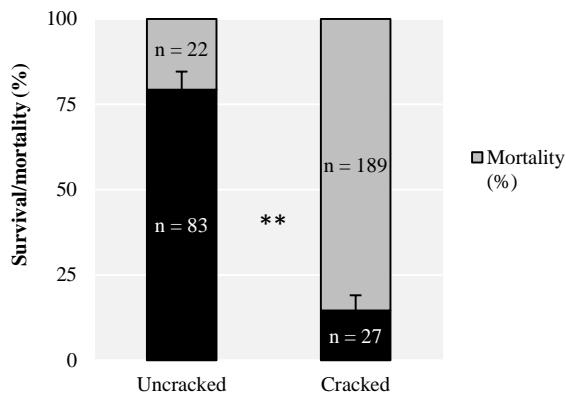
**Figure 3.** Average (+ SE) percentage of infestation (a) and number of carob moth larvae per infested fruit (b) in cracked and uncracked fruits. \*\* indicates significant difference at 0.01 confidence level.

In both years, at the start of the growing season the most susceptible pomegranate cultivars were A (Tabestaneh-Torsh) and B (Alak-Torsh), both in terms of infestation and fruit cracking (Figure 1). At the end of season, all of the cultivars showed infestation, except cultivar J (Poust-Sefid-Bihasteh) in 2013. Cracked fruits were mostly infested early in the season, while uncracked fruits were primarily infested late in the season (Figure 1). Throughout the growing season in both years, the number of cracked-infested fruits did not increase, but fruit cracking did increase dramatically, as well as infestation of uncracked fruits (Figures 1 and 4).



**Figure 4.** Progress of fruit cracking and infestation by carob moth of cracked and uncracked pomegranate fruits in the experimental orchard during the cropping seasons of 2013 and 2014. Solid lines are fitted to the number of fruits per tree and the shaded areas represent 95% confidence intervals.

Larval survival under simulated winter conditions differed significantly between cracked and uncracked fruits, both in terms of percentage survivors ( $F = 87.99$ ;  $df = 1, 90$ ;  $P < 0.0001$ ) and the number of survivors ( $= 1.8$  in uncracked versus  $0.58$  in cracked fruits) ( $F = 24.66$ ;  $df = 1, 90$ ;  $P < 0.0001$ ) (Figure 5).



**Figure 5.** Average (+ SE) percentage of carob moth larvae survival after 100 days at 8 °C in cracked and uncracked pomegranate fruits. N = number of larvae that survived or died. \*\* indicates significant difference at 0.01 confidence level.

## Discussion

Field monitoring during the growing season of two years in a pomegranate orchard with 10 different cultivars showed that fruit infestation by the carob moth is significantly affected by the level of fruit cracking. Although in total only ~ 20% of the fruits were cracked in a year, total infestation rate was 5 to 15 times higher in cracked fruits than in uncracked fruits. We also found that different pomegranate cultivars are differentially susceptible to fruit cracking, confirming previous studies (Saei et al. 2014; Yuan et al. 2010), as well as to fruit infestation by the carob moth (Moawad et al. 2011; Sobhani et al. 2015). As the number of larvae per cracked-infested fruit was higher than that in uncracked fruit, more eggs were likely laid on cracked than on uncracked fruits, or more larvae can survive in cracked fruits. Female carob moths normally lay egg(s) inside the pomegranate crown (calyx) and also in the cracks of the fruit (Shakeri 2004; Talaiee et al. 2010). To get inside the fruit, carob moth larvae need to make a hole and pass the fruit peel inside the crown. Crack on fruit likely eases the access of larvae into the fruit. In our field observations, we found that first instars and most second instars cannot enter the fruit through the crown, while in cracked fruits first instars are found deep inside the fruits. Furthermore, we usually observed larvae in different stages in a fruit, suggesting that females oviposited on already infested fruits.

Even though the fruit quantity differed significantly across the cultivars and between the two study years, this did not affect the patterns of fruit infestation and fruit cracking. In both years, infestation started on the highest cracking-susceptible cultivars. At this point almost all of the cracked fruits were infested by the carob moth. However, towards the end of the growing season, infestation in uncracked fruits increased even though many more cracked-uninfested fruits were available than in early and mid season, so that also the more fruit-cracking resistant cultivars became infested. Cold winters have been reported to cause high mortality in overwintering populations of carob moth (Mehrnejad 2002; Shakeri 1993). Under simulated winter conditions, we found that > 3 times more carob moth larvae survived in uncracked than in cracked fruits. Thus, the increased infestation in uncracked fruits and in cracking-resistant cultivars at the end of season may be related to the survival of insect larvae in uncracked fruits, which better protect the carob moth larvae against unfavorable winter conditions.

Natural enemies may also affect seasonal pattern of infestation of pomegranate by carob moth. At the start of the season, ovipositing females and their offspring are probably more protected from predators and parasitoids in the cracks than in the crown where common predators, such as spiders, lady beetles, true bugs, the green lacewing, as well as parasitoid wasps are generally present (Mehrazin et al. 2016; Shakeri 2004). However, carob moths are reported to be attacked by larval parasitoids mostly at the end of the season (Kishani-Farahani et al. 2010; Mehrazin et al. 2016; Sobhani et al. 2015). Cracks on fruit peel can make carob moth larvae inside more vulnerable to parasitism by facilitating the access of ovipositing female parasitoids to the inside of the fruit, which may explain the finding of larvae mostly in uncracked fruits in the late season.

Since cracked pomegranates were much more susceptible to carob moth infestation than uncracked fruits, agricultural control of fruit cracking is likely to suppress the carob moth population below economic injury levels. Control of fruit cracking may be feasible by using resistant cultivars or through recommended horticultural operations and treatments e.g., management of irrigation and pruning, and application of gibberellic acid and benzyladenine (Galindo et al. 2014; Khalil and Aly 2013; Saei et al. 2014). However, as discussed above, uncracked fruits appear to play an important role in the establishment and growth of the insect

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population in the beginning of the next growing season. Even though collecting and destroying infested pomegranates at the end of the cropping season was already recommended for the control of the carob moth in the mid seventies (Kashkuli and Eghtedar 1975; also see Shakeri 2004), this method has recently been reported to have important negative effects, in terms of population sizes and species diversity, on larval parasitoids of carob moths (Kishani-Farahani et al. 2012). Cracked fruits likely host higher species diversity and larger sizes of larval parasitoids, as cracks on the fruit peel most likely facilitate the access of these natural enemies to the carob moth larvae inside at the end of season. Thus, destroying only uncracked infested fruits after harvest appears to be a good alternative pest control method, as this will conserve populations of natural enemies.

Another lesson that can be learned from the observed seasonal pattern of pomegranate fruit infestation by the carob moth is that for IPM programs it seems better not to grow cracking-susceptible and cracking-resistant pomegranate cultivars in the same area, as cracked and uncracked fruits differently sustain the pest population in different seasons. This is important in the Middle East, especially in Iran, where pomegranate orchards are typically composed of a mix of cultivars that differ in cracking level.



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# Field attraction of carob moth to host plants and conspecific females

Seyed Ali Hosseini, Seyed Hossein Goldansaz, Steph B.J. Menken, Michiel  
van Wijk, Peter Roessingh, and Astrid T. Groot

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**Abstract,**

The carob moth, *Ectomyelois ceratoniae* (Zeller), is the most devastating pest of pomegranate in the Middle East. An efficient sex pheromone attractant is still missing for the management of this pest, because the major pheromone component is unstable. Host plant volatiles attract herbivore insects and have shown to have good potential to be exploited as alternatives or supplements to sex pheromones. To explore this possibility in carob moth, we assessed the attraction of moths to the volatiles of mature pistachio and different fruit stages of pomegranate, alone and in combination with virgin females, using sticky delta traps in pomegranate orchards. Traps baited with mature pomegranates, whether uncracked or cracked, infested or uninfested, caught significantly larger numbers of male and both mated and virgin female carob moths than unbaited traps. Traps baited with headspace extract of cracked pomegranate only caught mated females, while mature pistachio only attracted males. Pomegranate flowers, unripe pomegranate and headspace extract of pistachio did not attract moths. Traps baited with cracked fruit caught more mated females than traps baited with uncracked fruit. Males were attracted similarly to traps baited with cracked-infested pomegranate as to traps baited with virgin females alone. Interestingly, the combination of cracked pomegranate and virgin female enhanced the attraction of virgin females. Together, our results show that volatiles from cracked pomegranates alone or in combination with female sex pheromone have great potential for application in pest management programs of carob moth.

**Introduction**

Over the past decades, and mostly driven by the strict regulations imposed on the use of chemical pesticides worldwide (e.g., EU 2009), semiochemical-based behavioral manipulation methods have been increasingly implemented in Integrated Pest Management (IPM) as an efficient environmentally friendly strategy. For moths, many of which are agricultural pests, long-range female sex pheromones are now widely used to monitor and suppress insect populations through mass trapping and mating disruption (reviewed by Witzgall et al. 2010). However, female sex pheromones only target adult males, while females play a prominent role in population dynamics of insects (Caswell 2001). Therefore behavioral manipulation methods such as monitoring, attract-and-kill, and host finding disruption will be more efficient if they target not only males, but also females. Semiochemicals that would affect both sexes may be good alternatives or supplements to long-range sex pheromones, especially when the sex pheromones are difficult to produce, unstable, and/or inefficient.

In moths, host plant volatiles are involved in host seeking and oviposition behavior of females (Gothilf et al. 1975, Cossé et al. 1994, Landolt and Guédot 2008). Both males and females may also use plant volatiles to find a mate (Coracini et al. 2004, Landolt and Guédot 2008) and adult feeding sites (Lin and Phelan 1991, Tingle and Mitchell 1992, Landolt and Guédot 2008). Moreover, host plant volatiles have been shown to interact with sex pheromones by inducing sex pheromone release of female moths and affecting olfactory perception of the males (McNeil and Delisle 1989, Landolt and Phillips 1997, Groot and Visser 2001, Pregitzer et al. 2012). There are many examples of host plant volatiles that synergize responses of male moths to female sex pheromone and thus enhance their efficiency in pest management (Ochieng et al. 2002, Yang et al. 2004, Schmidt-Buesser et al. 2009, von Arx et al. 2012). Importantly, plant volatiles have great potential for use in IPM compared to

pheromones because plant volatiles are often simple compounds, commercially available and cheap.

A drawback of host plant volatiles, however, is that they may be less attractive than pheromones, as they have to compete with volatiles of the host plants in the field. This drawback may be overcome by using the stimuli at specific times, i.e., when or where the host plant is absent, e.g., before fruiting time in fruit pest, or to attract the pest away from the agricultural area. Another drawback is that individual plant volatiles are generally not specific, and many are shared among a large group of plant species. As behavioral responses of pest insects to host plants are likely encoded by a volatile blend, it is essential to determine which host plants or plant parts are the most attractive sources for the pest, and which volatile blends most attract the insects to their hosts.

The carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae), is a polyphagous destructive pest worldwide, attacking different fruits before and after harvest. This insect is recognized as the most economically important pest of date (*Phoenix dactylifera*) in the United States (Nay et al. 2006, Vetter et al. 2006) and a key pest of almond (*Prunus dulcis*) in Australia (Madge 2014), and frequently infests stored fruits and nuts in Europe (Carter 1984). In the Middle East, *E. ceratoniae* is the most damaging pest of pomegranate, *Punica granatum* L. (Lythraceae), in almost all pomegranate production areas, causing 30 - 80 % yield loss (Kashkuli and Eghtedar 1975, Shakeri 2004, Sobhani et al. 2015). Larvae feed on internal parts of the fruit, resulting in contamination with saprophytic fungi, which makes the fruit unfit for human consumption and for the food processing industries, and thus unmarketable (Shakeri 2004). Since egg-laying and larval development occurs within the fruits, commercial insecticides are not efficient and thus used against this pest. An efficient sex pheromone attractant is still missing for carob moth management. The major female sex pheromone component [(Z,E)-9,11,13-tetradecatrienal] is unstable (Baker et al. 1991), so that an alternative stable mimic of this component, viz., (Z,E)-7,9,11-dodecatrienyl formate, is currently used in commercial sex pheromone lures. However, these lures are not very attractive in pomegranate orchards (Avand-Faghih et al. 2012, Dhoubi et al. 2016). This situation prompted us to explore the potential of host-plant volatiles for IPM against the carob moth.

Pomegranate continuously flowers during the growing season, so that different phenological stages are present to different extents at the same time. Signs of carob moth infestation become visible from the mid-growing season of the pomegranate, i.e., when most fruits are at a mature size (Hoseini et al. 2014, Sobhani et al. 2015). In this period, peels of many pomegranate fruits are cracking (Shakeri 2004, Galindo et al. 2014, Saei et al. 2014, Hoseini et al. 2014), a phenomenon that is common throughout the world.

As a first step in the exploitation of host plant volatiles in the management of carob moth, we determined the attraction of male, mated female, and virgin female moths to different phenological stages of the host fruit and their headspace extracts using sticky delta traps in the field. We further investigated whether the combination of pomegranate with virgin females enhances the attraction of male and female moths. Subsequently, attraction of carob moths to flowers, unripe fruits, mature uncracked fruits, and mature cracked fruits was tested. As pistachio is the other main host of carob moth in the Middle East (Dhoubi 1982, Gothilf 1984, Mehrnejad 1992), we also tested the attraction of carob moth to mature pistachio fruits.

## Material and methods

**Insects.** Carob moths were reared from larvae that were collected throughout the growing seasons of 2014 from a commercial pomegranate orchard located in Chandab, Iran (35°25'13''N, 51°56'04''E and 1141 m elevation). Insect larvae were maintained on a wheat bran diet [wheat bran (300 g), sugar (50 g), water (120 ml), glycerol (130 ml) and yeast (9 g)], and adults were fed with a 10% honey-water solution. The rearing room was kept at 25±1 °C, 60% RH, and a light–dark cycle of 16L:8D.

**Plants.** Pomegranate samples were picked from the cultivar Galu-Barik from the same orchard as the insects, whereas the pistachio cultivar Akbari was obtained from a commercial pistachio orchard in Qazvin, Iran (35°54'07''N, 50°03'06''E and 1201 m elevation). Pomegranate materials used in the insect traps were picked early on the same day of the experiment. Pistachio samples were picked from the orchard 1-2 days prior to the experiment, as this orchard was located ~ 250 km from the experimental orchard, and kept in a refrigerator at 6 °C and 90 % RH until used in the experiments.

**Trapping experiments.** Trapping experiments were conducted in the pomegranate orchard mentioned above during the pest activity season in the area, i.e., from mid July to mid October (Table 1). For all experiments, green delta traps (280 × 200 × 120 mm l × b × h with sticky bottom; Russell IPM, Deeside, UK) were used. Baits were placed in the middle of the sticky bottom of the traps. Traps were placed between pomegranate trees (outside of the plant canopy) on a rope at a height of 160-220 cm, with at least 10 m between adjacent traps. Treatments (baits) were applied in a complete randomized block design (see below). Different treatments were tested at different times, depending on the availability of the plant materials as well as on the results of previous experiments (see Table 1). Traps were checked weekly, at which time the sticky bottoms and the baits were replaced, and treatments were re-randomized within each block. Sticky bottoms that had caught moths were taken to the lab, and kept in the rearing room for two days. After two days, trapped moths were sexed and counted, and the mating status of the females was determined by checking the color of the eggs that trapped females inadvertently laid on sticky bottoms, which is possible because oviposited fertilized eggs become pinkish red within 48 h (Alrubeai 1987).

**Table 1.** Overview of experiments, with different baits (pomegranate and pistachio plant materials and volatiles, and female sex pheromone) tested in sticky delta traps, to evaluate carob moth attraction in the field

		Treatment	Setup date	No. replicates
Experiment 1	Pomegranate	a (1) Flowers, (2) immature fruit	21-Jul-2014	14
		b (1) Cracked fruit and (2) cut fruit	13-Aug-2014	20
		c (1) Uncracked fruit, (2) cut fruit, and (3) cut infested fruit	20-Aug-2014	12
		d (1) Uncracked fruit and (2) cracked fruit	09-Oct-2014	20
	Pistachio	e (1) Mature fruit	13-Aug-2014	14
Experiment 2	Headspace extracts	a (1) Cracked pomegranate and (2) pistachio	19-Sep-2013	12
		b (1) Cracked pomegranate	09-Oct-2014	20
Experiment 3	Pomegranate and virgin female	a (1) Cracked infested fruit, (2) virgin female, and (3) cracked infested fruit + virgin female	10-Sep-2014	12
		b (1) Cracked fruit, (2) virgin female, and (3) cracked fruit + virgin female	25-Sep-2014	20

### Experiment 1. Attraction to host plant materials

To determine the most attractive plant parts to the carob moth, we tested eight different plant materials in five subsequent experiments [experiments (Exp.) 1a-1e; Table 1]. The plant materials tested were: 1) fruitful flowers (i.e., flowers that turn to fruits) of pomegranate, referred to as pomegranate flowers, 2) healthy (i.e., uncracked and uninfested by carob moth) immature pomegranate fruits, referred to as immature pomegranate, 3) healthy mature

pomegranate, referred to as uncracked pomegranate, 4) naturally cracked, mature pomegranate, referred to as cracked pomegranate, 5) naturally cracked, mature pomegranate infested by carob moth, referred to as cracked infested pomegranate, 6) mechanically cut (see next paragraph) mature pomegranate, referred to as cut pomegranate, 7) mechanically cut mature pomegranate infested by carob moth, referred to as cut-infested pomegranate, and 8) mature fresh pistachio, referred to as pistachio. Per trap, we used either two flowers of pomegranate, two immature pomegranates of 3-4 cm diameter, one mature pomegranate (uncracked, cracked, cut, infested, or uninfested) of 7-9 cm diameter, or three pistachios, which were 20-25 mm in length. Cracked-infested fruits were naturally cracked fruits on the pomegranate trees with early signs of infestation by carob moths but without visible mold infection, and the cracks of the cracked fruits tested were not wider than 2.5 cm.

To check whether mechanical damage or mechanical damage plus an artificial infestation in pomegranate fruits makes these fruits as attractive as naturally cracked or cracked infested fruits, cut and cut-infested fruits were also tested. To make a mechanically cut fruit, an incision was made on the peel of a healthy mature fruit from crown to base on both sides, after which the fruit was split in half along the incision, such that the two halves were still attached at the base while creating a maximum 2-cm-wide gap at the crown of the fruit. To make a mechanically cracked-infested fruit, 5 third-instar carob moths were placed in the mechanical gaps early in the day and larval settlement in the fruit was checked before using them in the traps. In all experiments with plant materials, unbaited traps were used as control.

### ***Experiment 2. Attraction to the headspace extracts of host fruits***

To determine whether the volatiles from cracked pomegranate and pistachio were attractive for carob moths, headspace extracts were tested in sticky traps (Exp. 2a and 2b, Table 1). Volatiles emanating from 2 kg of cracked pomegranate (i.e., naturally cracked mature pomegranate) and 1 kg of pistachio (i.e., mature fresh pistachio), placed in a 4-l glass jar, were collected separately as follows. A charcoal-filtered air stream was pulled over the enclosed fruits, and headspace volatiles were collected on an adsorbent trap, containing 50 mg of Tenax-GR 60/80 (Restek, Bellefonte, PA, USA) in a 60-mm-long  $\times$  6-mm-diameter glass tube. For collection of control samples, a charcoal-filtered air stream was pulled through the empty glass jar. Tenax traps were thermally conditioned at 200 °C for 30 min under a stream of nitrogen before use. The air flow in the headspace collection system was 0.5 l/min, and each collection session lasted for 22 h. The Tenax traps were extracted with 2 ml of n-hexane (Merck, Darmstadt, Germany). Samples were sealed and stored at -20 °C until use in the traps. One ml of each headspace extract was pipetted in a gray rubber septum (Pherobank, Wijk bij Duurstede, the Netherlands) and left to dry in the lab. The septa were filled with the extracts and sealed in glass vials 3-4 h before use in the field. Only one dispenser was used per trap.

### ***Experiment 3. Attraction to a combination of virgin females and host fruits***

To determine whether host fruits interact with the female sex pheromone in the attraction of carob moth, we baited traps with combinations of pomegranate fruits and living virgin females as pheromone source (Exp. 3a and 3b; Table 1). The experiment consisted of four treatments: 1) one 2-3 day-old virgin female, with a small piece of cotton wool soaked in tap water for feeding, in a cylindrical cage (2 cm height  $\times$  2.7 cm diameter) covered by gauze, referred to as virgin female, 2) one virgin female plus one cracked pomegranate, 3) one cracked pomegranate, and 4) a cage with cotton-water but without insect and fruit (control).



**Statistical analysis.** The different trapping experiments (Table 2) were analyzed separately. A generalized linear model (GLM) with a negative binomial error distribution using the Simultaneous Inference Procedure described by Hothorn et al. (2008) was used to compare treatments in each experiment. Treatments were compared to each other using a Turkey's test and treatments were compared to the control using Dunnett's test. To analyze single and interaction effects of cracked pomegranate and virgin female carob moth in total (male + female) moth catch in experiment 3b, a two-by-two factorial analysis was performed using GLM. Correlation analysis was performed between the numbers of trapped males and trapped mated and virgin females in all experiments. All analyses were conducted in R version 3.2.3 (R Core Team 2015).

## Results

Effects of different treatments (baits) in different experiments on the catch of male, mated female, and virgin female carob moths are shown in Table 2.

**Table 2.** Statistical significance as indicated by GLM in each of the trapping experiments. Each experiment contains a set of unbaited traps as control

Pom = pomegranate, HE = headspace extract, fem = female.

Experiment (treatment)	Male			Mated female			Virgin female		
	$\chi^2$	Df	P	$\chi^2$	df	P	$\chi^2$	df	P
1a (Flowers pom and immature pom)	2.19	2	0.333	2.19	2	0.333	2.19	2	0.333
1b (Cracked pom and cut pom)	0.46	1	0.493	0.32	1	0.566	0.02	1	0.865
1c (Uncracked pom, cut pom, and cut infested pom)	19.31	3	< 0.001**	30.78	3	< 0.001**	5.38	3	0.145
1d (Uncracked pom, cracked pom)	47.95	2	< 0.001**	84.56	2	< 0.001**	19.3	2	< 0.001**
1e (Mature pistachio)	3.92	1	0.047*	1.04	1	0.306	0.33	1	0.56
2a (Cracked pom HE and pistachio HE)	2	2	0.367	7.98	2	0.018*	0.47	2	0.79
2b (Cracked pom HE)	1.84	1	0.174	9.03	1	< 0.001**	0.33	1	0.56
3a (Cracked infested pom, virgin fem, and cracked infested pom + virgin fem)	63	3	< 0.001**	35.9	3	< 0.001**	17.6	3	< 0.001**
3b (Cracked fruit, virgin fem and cracked fruit + virgin fem)	77.45	3	< 0.001**	101.9	3	< 0.001**	35.52	3	< 0.001**

**Mature pomegranates are attractive to males as well as mated and virgin females.** Numbers of male, virgin female, and mated female carob moths trapped by mature pomegranates, whether uncracked (Exp. 1d; Dunnett's post hoc test, male:  $P < 0.001$ , mated female:  $P < 0.001$ , virgin female:  $P = 0.01$ ), cracked infested (Exp. 3a; Dunnett's post hoc test, male:  $P < 0.001$ , mated female:  $P = 0.005$ , virgin female:  $P = 0.04$ ), or cracked uninfested (Exp. 1d; Dunnett's post hoc test, male:  $P < 0.001$ , mated female:  $P < 0.001$ , virgin female:  $P = 0.008$ ) were significantly higher than in unbaited control traps. Traps baited with pomegranate flowers (Exp. 1a; Dunnett's post hoc test, male:  $P = 0.33$ , mated female:  $P = 0.33$ , virgin female:  $P = 0.33$ ), unripe pomegranate (Exp. 1a; Dunnett's post hoc test, male:  $P = 0.33$ , mated female:  $P = 0.33$ , virgin female:  $P = 0.33$ ), and headspace extract of pistachio (Exp. 2a; Dunnett's post hoc test, male:  $P = 0.99$ , mated female:  $P = 0.99$ , virgin female:  $P = 0.78$ ) did not catch numbers of moths that were different from the control traps (Figures 1, 2, and 3).

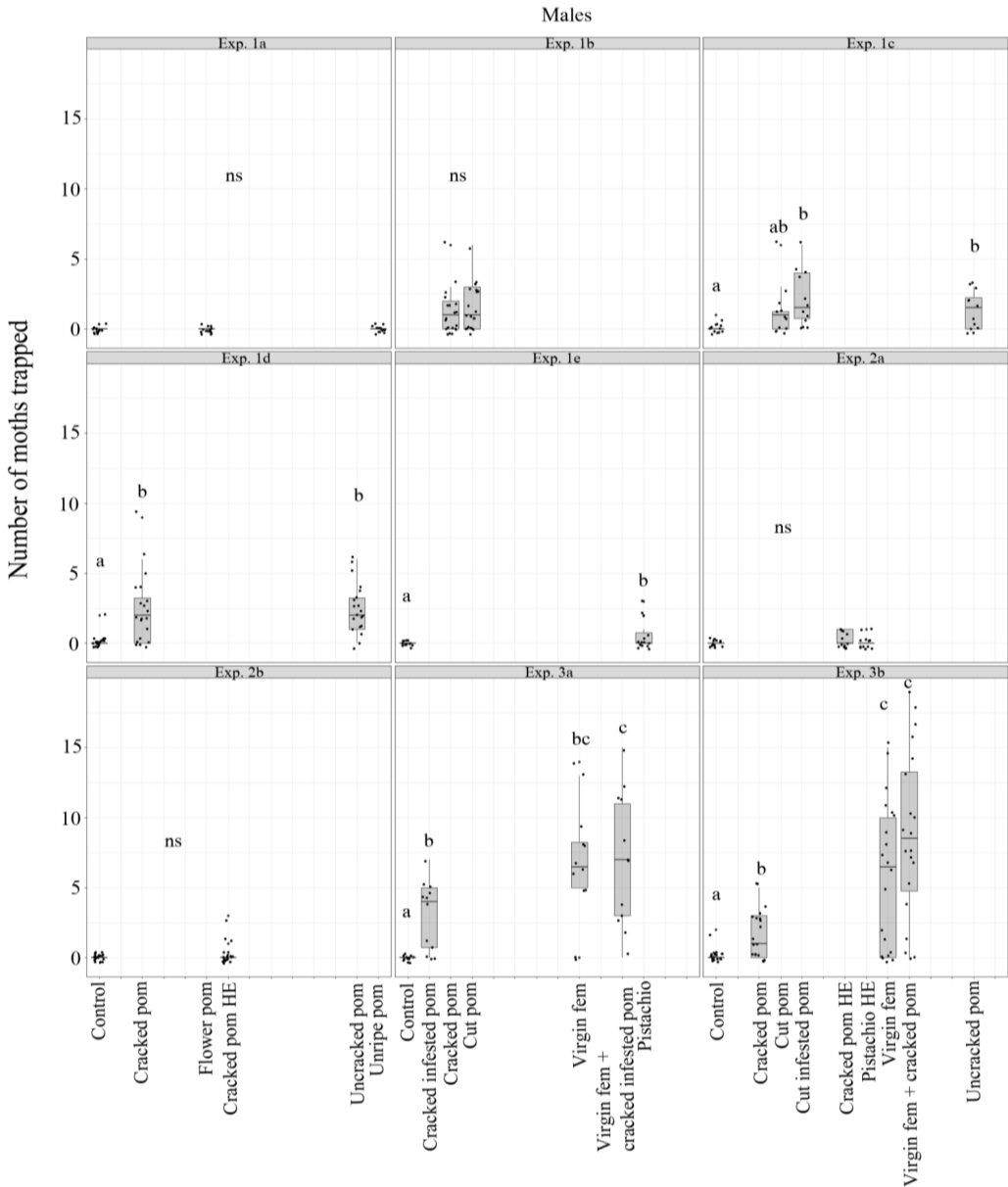
**Mature pistachio only attracts males, while headspace extract of pomegranate only attracts mated females.** When traps were baited with mature pistachio (Exp. 1e), only the number of trapped males was higher than in control traps, whereas the number of trapped mated and virgin females did not differ from control (Table 2; Figures 1, 2, and 3). In both Exp. 2a (Dunnett's post hoc test,  $P = 0.05$ ) and Exp. 2b (Table 2), only mated females were significantly more trapped in the traps baited with the headspace extract of cracked pomegranate than in controls. Traps baited with the headspace extract of cracked pomegranate

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did caught similar numbers of males and virgin females as control traps in both Exp. 2a (Dunnett's post hoc test, males:  $P = 0.32$ , virgin females:  $P = 0.78$ ) and Exp. 2b (Table 2; Figures 1, 2, and 3).

***Mechanical damage on the peel increases attraction of mated females to mature fruit.***

Compared to uncracked mature pomegranates, both cut (Exp. 1c; Tukey's post hoc test,  $P = 0.02$ ) and cracked (Exp. 1d; Tukey's post hoc test,  $P < 0.001$ ) pomegranates trapped significantly more mated females. Cracked and cut pomegranates trapped similar numbers of mated females, as well as males and virgin females (Exp. 1b; Table 2).



**Figure 1.** Number of male carob moths trapped per week in Experiments 1a to 3b (see Table 2). Within each panel trap catch results with different letters are significantly different at  $\alpha = 0.05$ . Pom = pomegranate, HE = headspace extract, fem = female. The bottom and top of the boxplots depict the first and third quartiles. The central line shows the median, and the whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range from the box.

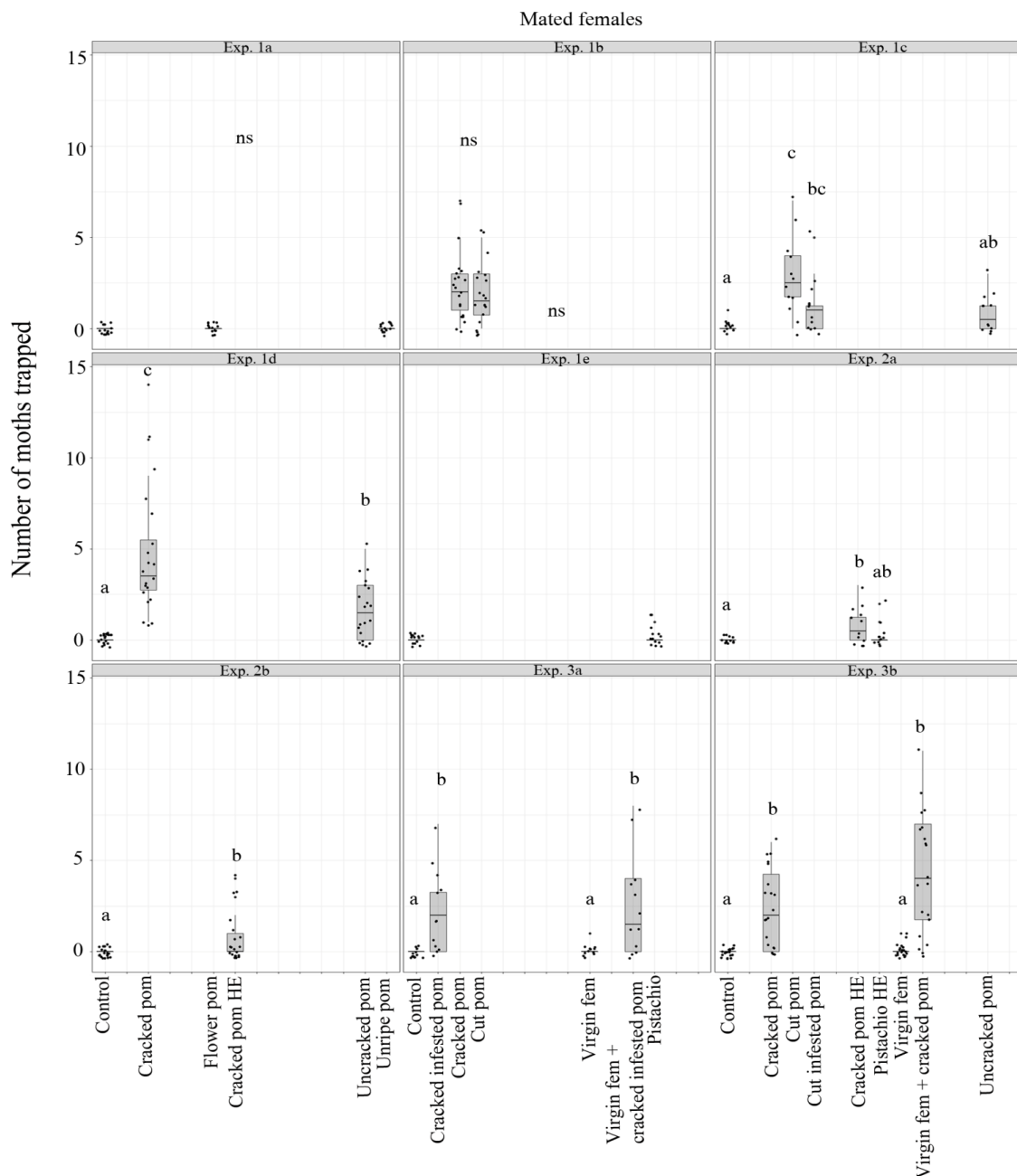
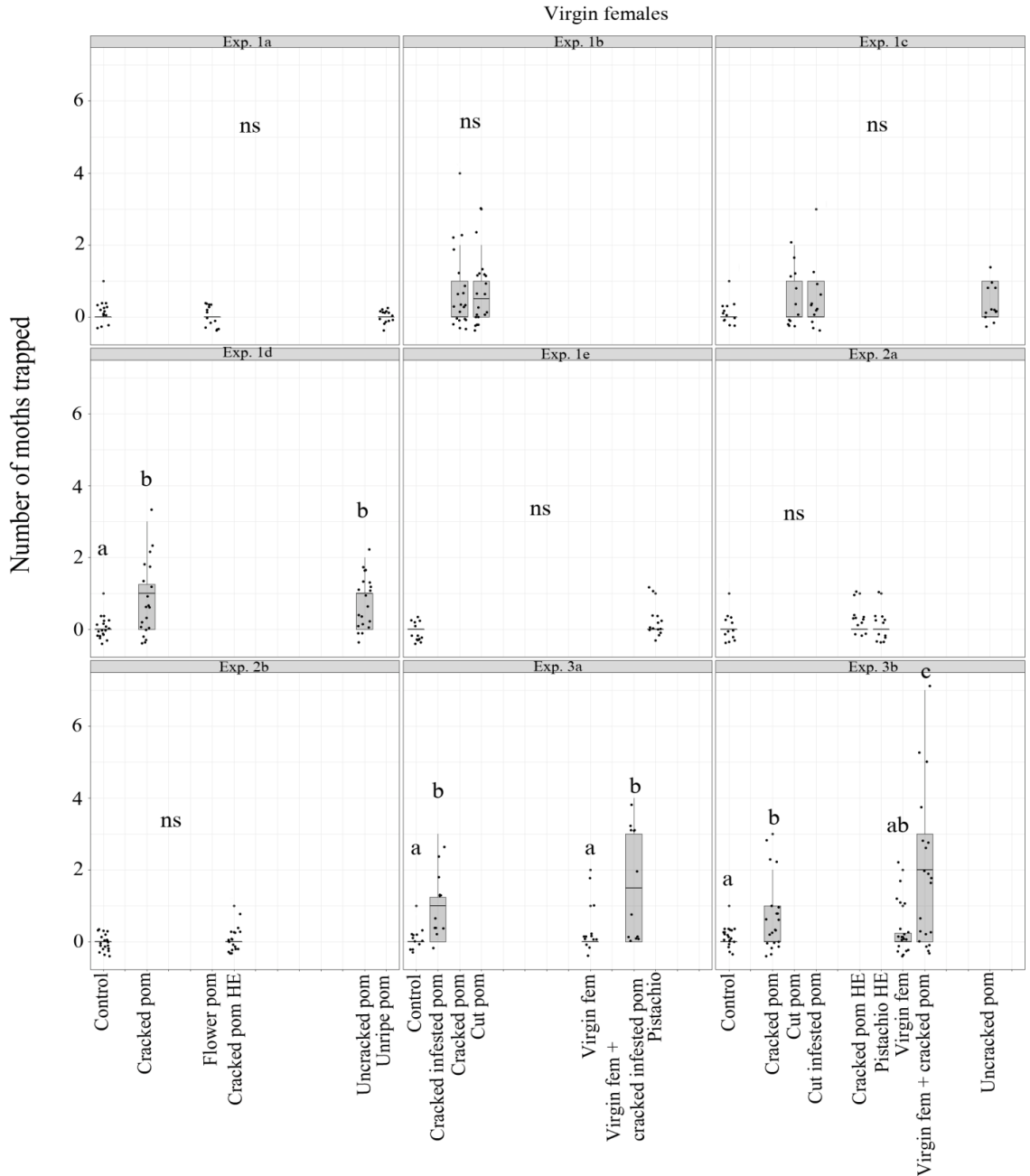
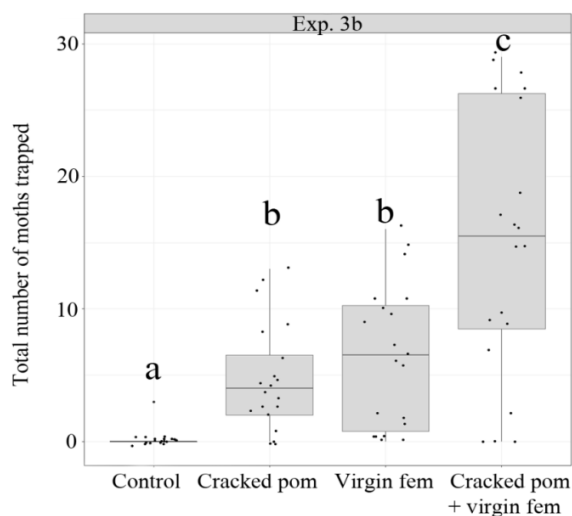


Figure 2. . Number of mated female carob moths trapped per week in Experiments 1a to 3b (see Table 2). Within each panel trap catch results with different letters are significantly different at  $\alpha = 0.05$ . Pom = pomegranate, HE = headspace extract, fem = female. The bottom and top of the boxplots depict the first and third quartiles. The central line shows the median, and the whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range from the box.



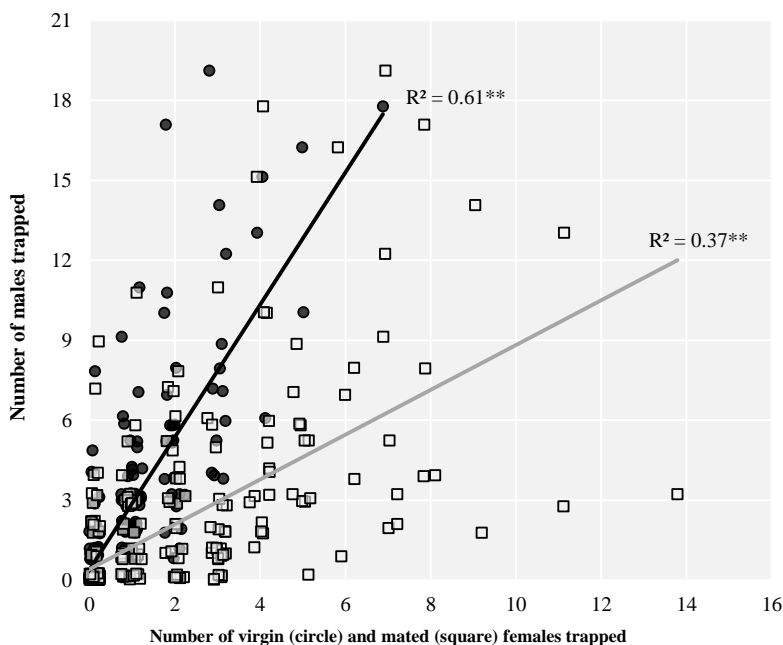
**Figure 3.** Number of virgin female carob moths trapped per week in Experiments 1a to 3b (see Table 2). Within each panel trap catch results with different letters are significantly different at  $\alpha = 0.05$ . Pom = pomegranate, HE = headspace extract, fem = female. The bottom and top of the boxplots depict the first and third quartiles. The central line shows the median, and the whiskers extend to the most extreme data point which is no more than 1.5 times the interquartile range from the box.

**Combination of pomegranates and virgin females increases attraction of virgin females and total moth catch.** The combination of cracked pomegranate and virgin female significantly increased virgin female trap catch compared to cracked pomegranate only (Exp. 3b; Tukey's post hoc test,  $P = 0.04$ ), whereas traps baited with a virgin female did not catch numbers of conspecific virgin females that were more than control traps (Exp. 3b; Dunnett's post hoc test,  $P = 0.27$ ). A significant interaction was observed between cracked pomegranate and virgin female ( $\chi^2 = 14.2$ , d.f. = 1,  $P = <0.0001$ ) in total moth catch, so that total number of moth trapped in the traps baited with the combination of these baits was significantly higher than that of the traps baited with each one alone (i.e., virgin female only (Exp. 3b; Tukey's post hoc test,  $P = 0.03$ ), and cracked pomegranate only (Exp. 3b; Tukey's post hoc test,  $P = 0.002$ )) (Figure 4; Exp. 3b). Traps baited with a cracked or cracked-infested pomegranate plus a virgin female (Exp. 3a and 3b) did not increase male catch compared to the traps baited with a virgin female only (Tukey's post hoc test, Exp. 3a:  $P = 0.99$ , Exp. 3b:  $P = 0.62$ ). Cracked infested pomegranate attracted the same number of males as virgin female only (Exp. 3a, Tukey's post hoc test,  $P = 0.06$ ). Traps baited with a virgin female only did not catch mated females (Dunnett's post hoc test, Exp. 3a:  $P = 0.99$ , Exp. 3b:  $P = 0.78$ ). Combination of cracked or cracked infested pomegranates with a virgin female did not increase mated female catch compared to cracked or cracked infested only (Tukey's post hoc test, Exp. 3a:  $P = 0.99$ , Exp. 3b:  $P = 0.16$ ) (Figure 2).



**Figure 4.** Total (male + female) number of carob moths trapped per trap per week in the traps baited with different baits in Experiment 3b. Treatments with different letters are significantly different at  $\alpha = 0.05$ . Pom = pomegranate, and fem = female.

Significant correlations were observed between the numbers of males and virgin females ( $n = 422$ ;  $r = 0.655$ ,  $P < 0.0001$ ) and males with mated females ( $n = 422$ ;  $r = 0.457$ ,  $P < 0.0001$ ) trapped in all experiments (Figure 5).



**Figure 5.** Correlation between the numbers of virgin (black circles; black line) and mated (blank squares; gray line) female carob moths and the number of male carob moths trapped per week in the experiments. \*\*,  $P < 0.001$ .

## Discussion

Our study provides the first experimental evidence of attraction of adult carob moths to host fruit, as well as to volatile extracts of pomegranate in the field. Previous research had shown behavioral responses of female carob moths to volatiles of carob (*Ceratonia siliqua*) (Gothilf et al. 1975) and date (Cossé et al. 1994) under laboratory conditions. We also demonstrated that male and female carob moths discriminate between different phenological stages of pomegranate.

Before discussing our results in more detail, we find it important to point out that trapped moths may have affected subsequent catches, because in delta traps each moth caught could be a new odor source that may attract other moths, e.g., trapped females could become sex pheromone sources for males and vice versa, as in moths attraction of females to male pheromone has been observed as well (Landolt and Heath 1989). We indeed found strong correlations between the number of males and both the number of virgin and mated females trapped in the various experiments. However, as we do not know which sex was trapped first, we find it more accurate to not take this interaction into account. Below, we discuss different hypotheses of why different treatments were attractive or unattractive for males and/or females.

### 1. Cracked and infested fruits serve as mating site

Similar numbers of males were caught in the traps baited with a virgin female and the traps baited with carob moth-infested fruits. Carob moth larvae develop entirely in one and the same fruit, in which they also pupate (Al-Izzi et al. 1985, Shakeri 2004) and from which the adult emerges. Carob moth females can mate in the night of emergence, so the high attraction of

males to the infested fruits is likely a response to female emergence sites. Interestingly, we found that the presence of a virgin female next to the fruit enhances the attraction of conspecific virgin females, which are likely searching for a calling or mating site. Females can probably reduce the cost of calling by being close to other females (Harari et al. 2011).

### **2. Cracked pomegranate serves as the best oviposition site for mated females**

Mated females preferred both cracked and cut pomegranates over uncracked pomegranates. Headspace extract of cracked pomegranate also significantly attracted mated female moths but not virgin females and males. This is likely due to the fact that gravid females use cracked fruits for oviposition, because cracks on fruit peel can facilitate larval penetration of the fruit. Female carob moths oviposit inside the pomegranate crown (calyx) and in cracks on the fruit (Shakeri 2004, Talaiee et al. 2010). In our field observations, we found that first and many second instars cannot enter the fruit, while in cracked fruits first instars are, almost without exception, found deep inside the fruits. Ovipositing eggs in the cracks may also decrease the risk of predation, as common predators such as spiders and lady beetles are generally present in the fruit crown (Shakeri 2004, Mehrazin et al. 2016). Female carob moths may thus likely increase their fitness by choosing cracked fruits for egg laying. This idea is supported by the findings of Talaiee et al. (2010), who found more carob moth eggs in cracked fruits than on uncracked fruits, as well as by our previous study, in which we showed significantly higher larval population size and infestation rates in cracked than in uncracked pomegranates (Hoseini et al. 2014).

### **3. Microbial volatiles attract carob moths**

Previous research has shown that mated carob moth females prefer injured and mold-infected carob (Gothilf et al. 1975) and date fruit (Cossé et al. 1994) for oviposition. Gothilf et al. (1975) further demonstrated that fungal growth on injured carobs leads to the production of some alcohols that stimulate oviposition by female carob moths. Since cracked pomegranates are usually infected with fungi (Shakeri 2004), a similar mechanism may have been involved in the attraction of mated females to cracked and cut pomegranate in our study. Also, mechanically damaged fruit is likely quickly infected with fungi, which could explain the observation that mated females were similarly attracted to naturally cracked/cracked-infested and mechanically cut/cut-infested fruits.

### **4. Mature pomegranates serve as adult feeding site for male and female carob moths**

Mature pomegranates, whether cracked/cut or uncracked and infested or uninfested, were attractive to males, mated females, and also virgin females. This suggests that adult moths use the volatile blend that emanates from mature pomegranates to locate adult feeding sites. Carob moths feed on sugar and are attracted to ethyl alcohol (Gothilf et al. 1975, Cossé et al. 1994), which is a volatile product of sugar fermentation by bacteria and other microbes and is found in sweet baits (Utrio and Eriksson 1977). Both males and females of other polyphagous pyralid moths are attracted to water (Ryne et al. 2002), as well as chocolate odor (Olsson et al. 2005), and these components are now used in the management of some of these moths (Anderbrant et al. 2009). Therefore, we hypothesize that ripe fruit may provide a source of water and sugar for carob moth adults, and responses to the volatiles of these fruits may be a strategy to locate adult food.

### **5. Pomegranate is a better oviposition substrate than pistachio**

We did not find an attraction of female carob moths to pistachio or its headspace extract, which suggests that female carob moths prefer pomegranate to pistachio for oviposition, as we conducted our experiments in a pomegranate orchard. Such a preference also appears from



the observation that both pistachio and pomegranate are widely grown in the Middle East, but the carob moth has not been reported as an economic pest of pistachio, while it causes heavy losses in pomegranate orchards (Kashkuli and Eghtedar 1975, Shakeri 2004, Sobhani et al. 2015).

## **6. Pomegranate flowers and immature fruits are not oviposition sites for females**

As we found that pomegranate flowers and immature fruits were not attractive to carob moths, while the insects were actively attacking the mature fruits of the orchard at the same time, flowers and immature fruits do not seem to be suitable oviposition sites for female carob moths. It has been reported that carob moths attack pomegranate starting at mid-season when most fruits are mature (Sobhani 2011, Hoseini et al. 2014, Sobhani et al. 2015). Previous studies also did not find carob moth eggs or larvae on pomegranate flowers and immature fruits earlier in the season (Talaiee et al. 2010, Sobhani 2011). However, it is not clear whether this is due to a lack of synchrony between carob moth emergence and pomegranate flowering and early fruiting, or whether the insect is not attracted by flowers and young fruits.

## **Possible use of pomegranate volatiles in IPM against carob moth**

As we found that cracked pomegranate are very attractive to mated females, alone or in combination with female sex pheromone lures, volatiles from this source may be exploited in behavioral manipulation methods as part of integrated pest management programs of the carob moth. Field evidence of attraction of carob moth to host plant materials and their headspace extracts warrants efforts to identify host plant volatiles or volatile blends for trapping both male and female moths. Behavioral manipulation methods to be exploited include monitoring, mass trapping, and push-pull and attract-and-kill.

**Monitoring.** Sex pheromone lures of the carob moth are relatively expensive to make, and as attractant they are not very efficient in pomegranate orchards (Avand-Faghih et al. 2012, Dhouibi et al. 2016), attracting an average of 9 males per week at most (Dhouibi et al. 2016). In traps baited with cracked pomegranate only, we trapped similar numbers of moth per week, but mostly females. We thus show that traps baited with pomegranate fruit or their volatile extracts can be used at least to monitor female populations of the carob moth. This can be especially useful to time the augmentative release of, for example, trichogrammatid egg parasitoids (Moezipour 2006, Poorjavad et al. 2011) and the larval parasitoid *Bracon hebetor* (Saadat et al. 2016) in biological control programs of carob moth.

**Mass trapping.** As we found that the combination of volatiles of cracked pomegranates and virgin females enhances attraction of female carob moths and increases total (i.e., male and female) moth catch, mass trapping female carob moths might be achieved by using the combination of volatiles of cracked pomegranate with female sex pheromone lures when pomegranates of the orchards are not yet mature and cracked, i.e., earlier in the season during flowering/early fruiting stages of the plants, when the fruits do not crack yet (Hoseini et al. 2014).

**Push-pull and attract-and-kill.** The most effective behavioral manipulation in IPM of the carob moth may be achieved by the so-called push-pull strategy, which involves the behavioral manipulation of insects via the integration of stimuli that make one source (the crop) unattractive or unsuitable (push) for the pest insect, while luring them toward an attractive source (pull), from where the pest insects are subsequently removed (Cook et al. 2007). A carob moth repellent, such as the essential oils of *Ferula assafoetida* L. (Peyrovi et al. 2011), could be used on pomegranate to push the moths away, while traps baited with

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cracked-mature pomegranate volatiles, or better, with a combination of pomegranate volatiles and female sex pheromone lures, could be used to pull the female moths to a removable source.

In conclusion, we have identified sources of attractive volatiles to which both carob moth males and females are attracted. The next step will be to identify which of these volatiles can be used for the development of attractive lures. Meanwhile, delta traps with live virgin females and cracked pomegranate may be used as attractive lures, which are likely to be sufficiently effective to monitor moth populations and possibly even to be used in push-pull and/or attract-and-kill strategies.

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# Electrophysiological and behavioral responses of the carob moth, *Ectomyelois ceratoniae*, to pomegranate and pistachio

Seyed Ali Hosseini, Seyed Hossein Goldansaz, Sybille Lorenz, Steph B.J. Menken, Astrid T. Groot\* and Peter Roessingh\*

\* Contributed equally to this study

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### Abstract

Carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae), is the most important insect pest of pomegranate in the Middle East. We previously showed that attraction to and infestation of pomegranates in the field is correlated with fruit phenology. In this study, antennal and behavioral responses of carob moths to volatiles of different phenological stages of pomegranate and ripe pistachio were determined under laboratory conditions. Using coupled gas chromatography-electroantennography and gas chromatography-mass spectrometry,  $\beta$ -caryophyllene was identified from the headspace extracts of pomegranate and pistachio as a compound that consistently elicited the strongest antennal responses from female carob moths. Significant variation in the amount of this compound was found between different plant parts tested. In the wind tunnel, males, virgin females, and gravid females responded similarly to the host fruits. Responses were negatively correlated with the amount of  $\beta$ -caryophyllene in head space extracts. Mature pomegranates (both cracked and uncracked) and pistachio contained the lowest amount of the compound and significantly activated and attracted the moths. Pomegranate flowers and unripe fruits contained the highest amounts of  $\beta$ -caryophyllene and did not attract the insects. Cracked pomegranate elicited the highest rate of moth landing. Carob moths also flew upwind to the headspace extract of cracked pomegranate and pistachio.

### Introduction

In most insects, olfaction is of great importance in localizing and recognizing food plants and mates (Dethier 1947, Bernays and Chapman 1994, Hansson and Stensmyr 2011). In moths, sexual communication has well been studied, and sex pheromones of more than 1600 species of Lepidoptera have been identified (see Pherobase.net). The field of chemical ecology partly derives its motivation and justification from the prospect of applying sex pheromones in pest management strategies including monitoring, attract-and-kill, and mating disruption [reviewed by Witzgall et al. (2010)]. However, female sex pheromones only affect males, while females play the most prominent role in population dynamics of insects (Caswell 2001). Behavioral manipulation methods will thus be more efficient if they target not only males but also females. Both female and male moths perceive host plant volatiles via specialized olfactory receptor neurons, and use them to identify adult food sources, rendezvous sites for mating, or larval food plants from the background chemical environment (Ramaswamy 1988, Menken et al. 1992, Isman 1992, Honda 1995, Coracini et al. 2004, Landolt and Guédot 2008, Hansson and Stensmyr 2011). Moreover, host plant volatiles have been shown to interact with sex pheromones by, e.g., inducing sex pheromone release by female moths or affecting pheromone perception by male insects (McNeil and Delisle 1989, Landolt and Phillips 1997, Groot and Visser 2001, Pregitzer et al. 2012). Thus, in pest management host plant volatiles may serve as alternatives or supplements to the commonly used long-range sex pheromones, especially in cases where sex pheromones are difficult to produce, unstable, or inefficient.

Phytophagous insects synchronize their life cycle with growth stages and phenology of host plants to enhance fitness (Kooi et al. 1991, Schoonhoven et al. 2005). Synchronization can be achieved when insects respond to signals that are specific to different phenological stages or parts of the host plant (Proffitt et al. 2007). Phenological stage is known to affect insect attraction and oviposition behavior via different chemical cues (Van Rensburg et al. 1988, Ramaswamy 1988, Sosa 1988, Spangler and Calvin 2000, Smyth et al. 2003, Tasin et

al. 2005). Different plant parts of the same phenological stage may also emit distinctly different volatile blends (Bengtsson et al. 2001, Tasin et al. 2005, Vallat and Dorn 2005). Individual plant volatiles are not always specific, and in fact many of them are common to most plants species. Host attraction is likely encoded by a specific blend of host-plant volatiles, comprising both attractants and repellents. At high concentrations, an attractive host plant volatile may become unattractive or even repellent to the herbivorous insect (Finch 1978, Hern and Dorn 1999, Mewis et al. 2002, Cha et al. 2011). Males, virgin females, and gravid females may respond differently to host-plant volatiles, as the threshold and ultimate outcome of the response of insects to plant volatiles is determined by the integration of external stimuli with the internal physiological state of the insect (Miller and Strickler 1984, Hern and Dorn 1999, Mechaber et al. 2002, Masante-Roca et al. 2007). The search for proper plant volatiles that could be used for the behavioral manipulation of pest insects commences with determining which host plants or plant parts are the most attractive sources, and for which sex, followed by identification of the volatiles that are emanating from the plant materials to which the insect is attracted.

The carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae), is a destructive polyphagous pest worldwide that attacks the fruits of a variety of commercially important plants before and after harvest. It is recognized as the most important pest in the date industry in the United States (Nay et al. 2006, Vetter et al. 2006), a key pest of almond in Australia (Madge 2014) and frequently a problem in stored fruits and nuts in Europe (Carter 1984). In the Middle East, *E. ceratoniae* is the most damaging pest of pomegranate, *Punica granatum* L. (Lythraceae), in almost all pomegranate production areas and can cause 30 - 80% yield loss (Kashkuli and Eghtedar 1975, Shakeri 2004, Sobhani et al. 2015). Pistachio [*Pistachia vera* L. (Anacardiaceae)] is another major host of this insect in the Middle East (Dhouibi 1982, Gothilf 1984, Mehrnejad 2002). *Ectomyelois ceratoniae* has 4-5 generations per year on pomegranate in the field in the Middle East (Al-Izzi et al. 1985). Larvae feed on the internal parts of the fruit, resulting in contamination with saprophytic fungi, which makes the fruit unmarketable and unfit for human consumption or food processing industries. Since egg-laying and larval feeding occur within the fruits and are thus hidden from the outside world, commercial insecticides are not efficient and thus not used against this pest insect. In addition, an efficient sex pheromone attractant is still lacking in carob moth pest management. The major female sex pheromone component [(Z,E)-9,11,13-tetradecatrienal] is unstable (Baker et al. 1991), which is the reason that a stable mimic of this component, viz., (Z,E)-7,9,11-dodecatrienyl formate, is used in commercial sex pheromone lures. However, these lures are not very attractive in pomegranate orchards (Avand-Faghhi et al. 2012, Dhouibi et al. 2016).

Pomegranate continuously flowers during the growing season and different phenological stages, from fresh flowers to mature fruits, are present when carob moths are active. In the Middle East, carob moth activity starts in mid-season (July-August) when most pomegranate fruits are mature (Hoseini et al. 2014, Sobhani et al. 2015) and peels of many fruits are cracking (Shakeri 2004, Galindo et al. 2014, Saei et al. 2014). Previously, we showed that delta sticky traps baited with pomegranate fruits of different stages of fruit ripening caught male and female carob moths in the field (Hosseini et al. 2016). In this study, we investigate the attraction of male, mated female, and virgin female carob moths in a standardized setting (a wind tunnel) to determine which host-derived signals mediate adult carob moths host location behavior. In addition, we screened headspace extracts of pomegranates and pistachio for bioactive compounds, using coupled gas chromatography-electroantennography (GC-EAD) and gas chromatography-mass spectrometry (GC-MS).

## Material and methods

**Insects.** Carob moths were reared from larvae, which were collected regularly throughout the season (from July to October) during 2013 and 2014 from commercial pomegranate orchards located in Chandab, Iran (35°25'13''N, 51°56'04''E and 1141 m elevation). The larvae were reared on a wheat bran diet [wheat bran (300 g), sugar (50 g), water (120 ml), glycerol (130 ml), and yeast (9 g)], and adults were fed with a 10% (w/v) honey–water solution. The rearing room was kept at  $25 \pm 1$  °C, 60–65% RH, and a light–dark cycle of 16 L: 8 D. The insects used in wind tunnel experiments had been reared for at most two generations on the diet. For the electrophysiological experiments conducted at the University of Amsterdam, larvae were reared under the same temperature and RH conditions, but with 14 L: 10 D light–dark cycle.

**Plants.** Pomegranate samples were obtained from cultivar Galu-Barik and picked from the same orchard as the insects in Chandab. Pistachio fruits were collected from cultivar Akbari, grown in a commercial pistachio orchard in Qazvin, Iran (35°54'07''N, 50°03'06''E and 1201 m elevation). Plant parts that were tested in the wind tunnel included 1) fertilized pomegranate flowers, referred to as pomegranate flowers, 2) uncracked and uninfested immature pomegranate fruits, referred to as immature pomegranate, 3) healthy mature pomegranate fruits, referred to as healthy/uncracked pomegranate, 4) naturally cracked mature pomegranate fruits, referred to as cracked pomegranate, 5) naturally cracked mature pomegranate fruits with early signs of infestation by carob moth but without visible mold infection, referred to as cracked-infested pomegranate, and 6) mature fresh uninfested pistachio fruits, referred to as pistachio. Plant materials were picked 1–2 days before each experiment and kept in a refrigerator at 5 °C and 90% RH prior to the tests.

In the wind tunnel experiments, we used as a test stimulus either two pomegranate flowers, two immature pomegranates of 3–4 cm diameter, one mature pomegranate (including cracked, uncracked, infested, and uninfested mature fruits) of 7–9 cm diameter, or three pistachios, which were 20–25 mm in length. The cracks of the cracked fruits were not wider than 2 cm.

**Collection of plant volatiles.** Headspace volatiles of the various plant parts were collected for behavioral, electrophysiological, and chemical analysis. Two kg of pomegranate fruits, 300–400 g of pomegranate flowers, or 1 kg of pistachio fruit were placed in a 4-l glass jar. A charcoal-filtered air stream was pulled through the jar using a vacuum pump, and the headspace was collected on an adsorbent trap, containing 50 mg of Tenax-GR 60/80 (Restek, Bellefonte, PA, USA) in a 60-mm-long  $\times$  6-mm-diameter glass tube. Control samples were collected using an empty glass jar. Tenax traps were thermally conditioned at 200 °C for 30 min under a stream of N<sub>2</sub> before use. The air flow in the headspace collection system was 0.5 l/min, and each collection session lasted for 22 h. Tenax traps were extracted with 2 ml of n-hexane (Merck, Darmstadt, Germany). Samples were sealed and stored at –20 °C prior to the experiments and chemical analysis.

**Wind tunnel bioassay.** Attraction of male, mated female, and virgin female carob moths to different odour sources was determined using a plexiglas wind tunnel with a flight section of  $120 \times 50 \times 50$  cm<sup>3</sup> ( $l \times w \times h$ ), placed in a walk-in climate chamber, at  $23 \pm 1$  °C and 50–60% RH. Air was blown by a fan at 50 cm/sec through a charcoal filter into the tunnel. The flight section was lit diffusely from the sides, yielding a light intensity of  $< 1$  lx along the wind tunnel floor.

Test insects were placed individually in cylindrical, transparent plastic cups with  $8 \times 7 \times 5$  cm (height  $\times$  top  $\times$  base). We used 2- to 3-day-old virgin females and males, and 4- to 5-day-old mated females. The containers with moths were placed in the wind tunnel at least 1 h before testing to let them adjust to wind tunnel conditions. Each insect was tested for 15 min. All experiments were conducted 1-4 h into the scotophase. During this period, behavioral responses of 4-10 moths to one odour stimulus were tested.

The container with a test moth was positioned horizontally on a glass platform at 20 cm high and 100 cm downwind from the odour source. The upwind end of the container was open, whereas at the downwind side several pores (smaller than the insect) allowed air to pass through the container. Odour stimuli were placed on a similar glass platform at 20 cm height and 20 cm from the tunnel's upwind end. Half a ml of headspace extract was pipetted on a rubber septum dispenser (Pherobank, Wijk bij Duurstede, the Netherlands) and left to dry in the lab for 30 min. The dispensers were charged immediately before the start of the experiment in the wind tunnel and sealed in 14-ml vials until use. One dispenser was used per tested insect. Two types of controls were used, a blank platform (blank) and dispensers filled with 0.5 ml of the control samples (control).

The following sequence of behaviors was recorded for each test insect: take off, upwind flight to 50 cm of the odour source, upwind flight to 7 cm of the odour source, and landing. Moths that landed on a wall for  $> 10$  s and moths that did not take off within 10 min from the start of the experiment were discarded and removed from the wind tunnel.

***Coupled gas chromatography-electroantennogram recordings (GC-EAD).*** To determine which volatile compound(s) in the plant extracts were detected by the carob moth, GC-EAD experiments were conducted. Whole 2- to 3-day-old female carob moths were mounted in a plastic pipet tip and the antenna was immobilized with a small strip of parafilm pressing the antennal base against the head. Electrical contact was made using silver wires inserted in glass micro-electrodes (GC150TF-10; Warner Instruments, Hamden, CT, USA) with insect Ringer's solution (9 g NaCl, 0.42 g KCl, 0.33 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , and 0.20 g  $\text{NaHCO}_3$  per liter). The recording electrode was inserted at the base of the antenna, and the reference electrode made contact with the antennal uncut tip. The amplitude of the EAD was measured with an IDAC-4 amplifier (Syntech, Kirchzarten, Germany) equipped with a high impedance ( $> 10^9$  Ohm) head stage and captured with GC-EAD/2014 software (Syntech). A Carlo Erba GC8000 gas chromatograph, equipped with a septumless *instant* Connect Cold-On-Column injection module (Global Analyser Solutions, Breda, the Netherlands), was coupled to the EAG setup to deliver odour stimuli. A 2-m-long, 0.53-mm internal diameter retention gap (Phenomenex, Utrecht, the Netherlands) was connected with a glass pressfit (Techrom, Purmerend, the Netherlands) to a 30-m EC-5 column (5% phenyl, 95% methylpolysiloxane) with 0.25 mm inner diameter and a 0.25- $\mu\text{m}$  film (Fisher Scientific Pittsburgh, PA, USA). The effluent from this column was split using a 0.25-mm glass Deans switch (Techrom). The switch was controlled with  $\text{N}_2$  and the pressure on both control inputs (30 kPa) was kept equal to create a 1:1 split ratio. The helium pressure over the analytical column was increased by the same 30 kPa to maintain an optimal linear gas flow ( $\sim 25$  cm/sec) in the column. The two outlets of the Deans switch were connected to sections of a 80-cm  $\times$  0.25-mm-diameter deactivated capillary column (Phenomenex), one going to the standard flame ionization detector (FID) set at 250  $^\circ\text{C}$ , the other leaving the GC oven via a 30-cm-long heated transfer-line (Syntech) set to 200  $^\circ\text{C}$  and transferred to the antenna. The capillary emerging from the transfer-line protruded through a small hole 0.5 mm into a 1-cm-wide, L-shaped glass tube (Syntech) carrying a charcoal filtered and humidified clean air flow of 2 l/min to the preparation that

was placed directly in front of the outlet. The oven temperature program started at 60 °C (with secondary cooling on). The secondary cooling was switched off after 7 seconds and after 1 minute, temperature went up with 10 °C/min to 240 °C. From each headspace collection sample, a sub-sample of 50 µl was reduced to 1 µl under a stream of N<sub>2</sub> and injected in the GC.

**Coupled gas chromatography-mass spectrometry (GC-MS).** The structure of the most active compound in the extract of cracked mature pomegranate was further investigated using an Autospec double focusing sector field mass spectrometer (Waters, Manchester, UK) connected to a gas chromatography HP6890 (Agilent, Santa Clara, CA, USA) equipped with a ZB5HT column (30 m x 0.25 mm x 0.25 µm; Phenomenex). The injector temperature was set at 230 °C and the oven programmed at 60 °C for 3 min, 10 °C/min to 180 °C, 5 °C/min to 250 °C, 20 °C/min to 280 °C, and kept for 3 min. Helium was used as a carrier gas with a flow rate of 1 ml/min. Two µl of extract was injected splitless and the peak of interest was tentatively identified as β-caryophyllene via NIST (Mass Spectral Library 2008) search. A β-caryophyllene standard (Sigma, Dorset, UK) showed the same RT and EI mass spectrum as the peak of the active compound when run under the same conditions. Identification was further confirmed by comparing the retention times of the β-caryophyllene, using the above described GC-EAD setup. The biological activity of the identified compound was verified using electroantennogram (EAG) recordings with an authentic standard (-)-trans-caryophyllene from Sigma.

**Caryophyllene identification and quantification.** To test the presence of β-caryophyllene in the other headspace extracts, the retention time of the authentic compound was compared to the retention time of peaks in the samples, using the GC of the GC-EAD setup mentioned above and in addition on an Agilent 7890A gas chromatograph (Agilent Technologies) equipped with an Agilent DB-WAXetr (extended temperature range) column of 30 m × 0.25 mm × 0.25 µm coupled with a flame ionization detector (FID) at 250 °C. The oven program for the 7890A was as follows: 60 °C (hold for 2 min) to 180 °C (30 °C/min), followed by an increase of temperature to 230 °C (5 °C/min). Between samples, the column was heated to 245 °C (20 °C/min) and stayed at that temperature for 15 min. The extracts were injected with an Agilent 7693A Automatic Liquid Sampler in splitless mode.

To quantify the amount of β-caryophyllene in the headspace extracts, three samples (replicates) of each plant material were analyzed using the Agilent 7890A GC. Each sample was a mixture of 5 rounds of 22 h of odour collection, extracted with 2 ml of hexane (in total 5 × 2 = 10 ml). Plant material was replaced each round. Ten µl of each sample was mixed with 10 µl of n-hexane containing 40 ng pentadecane as internal standard. The mixture was reduced to 3 µl under a gentle stream of N<sub>2</sub> and injected, after which the ratio of β-caryophyllene in each sample was calculated relative to the internal standard and also relative to a constant peak at retention time 6.99 that originated from the solvent and was present in all samples and controls.

**Statistical analysis.** Differences in overall behavioral performance in the wind tunnel assays between different treatments (stimulus) and different moths (males, mated females, and virgin females) were determined by Kaplan-Meier survival analysis, using the Log Rank (Mantel-Cox), Breslow (Generalized Wilcoxon), and Tarone-Ware tests. Differences between different treatments in terms of the number of moths that reached different behavioral steps (take off, flight to 50 cm upwind, flight to 7 cm upwind, and landing) were determined using a generalized linear model (GLM) with binomial distribution, in which treatment and moth type

were the explanatory variables. A GLM was also performed with the amount of  $\beta$ -caryophyllene as explanatory variable. Differences between means were determined using the Tukey's HSD test at 95% confidence level. Pearson's correlation analysis was performed between the average amount of  $\beta$ -caryophyllene in each plant material and the percentage of carob moths that responded to that plant source. All analyzes were performed in R, version 3.3.1 (R Core Team (2016)).

## Results

**Wind tunnel experiments.** Carob moths were significantly attracted to the plant materials and headspace extracts tested, irrespective of sex or mating status of the females, as shown by the Kaplan-Meier survival analysis (Table 1; Figure 1). Sex and mating status of the females were not significant sources of variation in the behavioral responses (Tables 1 and 2).

**Table 1.** Kaplan-Meier survival analysis of behavioral performances between different treatments (plant stimuli) and moth types (i.e., males, mated females, and virgin females) in wind tunnel experiments

Statistical test	Plant material						Headspace collection					
	Treatment			Moth type			Treatment			Moth type		
	$\chi^2$	df	P	$\chi^2$	df	P	$\chi^2$	df	P	$\chi^2$	df	P
Log Rank (Mantel-Cox)	61.812	6	<0.0001	3.101	2	0.212	10.389	2	0.006	1.341	2	0.511
Breslow (Generalized Wilcoxon)	40.668	6	<0.0001	0.605	2	0.739	9.329	2	0.009	0.598	2	0.742
Tarone-Ware	50.07	6	<0.0001	1.447	2	0.485	9.982	2	0.007	0.821	2	0.663

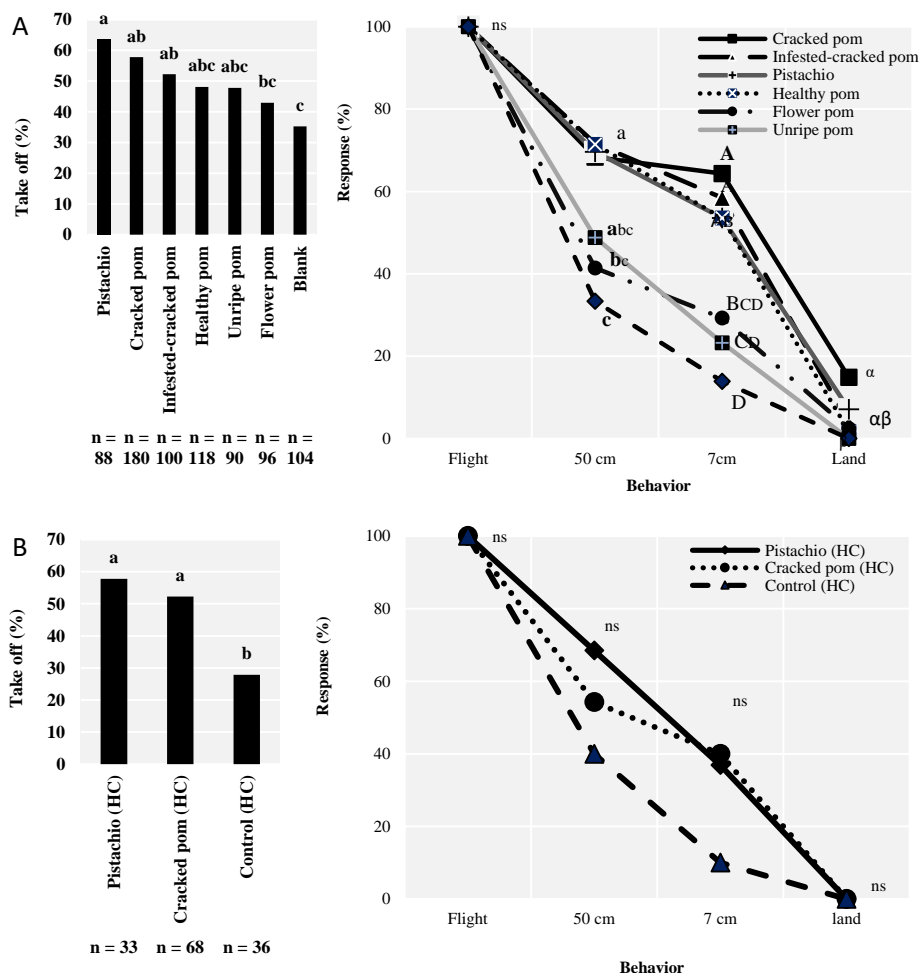
Significant *P*-values are highlighted in gray.

When comparing the behavioral elements separately, take-off was mostly initiated by pistachio, cracked pomegranate, cracked infested pomegranate, and the headspace extracts tested (Figure 1). The other pomegranate samples (uncracked fruit, unripe fruit, and flowers) did not elicit a behavioral response. Upwind flight to within 50 cm and 7 cm of the odour sources was mostly towards cracked, cracked-infested, and uncracked pomegranates as well as to pistachio, but not to pomegranate flowers and unripe fruits. Finally, only cracked pomegranates elicited significant landing responses (Figure 1A).

**Table 2.** GLM analysis of the behavioral responses of carob moth to different stages of pomegranate fruit and mature pistachio as well as their headspace extracts, tested in the wind tunnel. Treatment (stimulus) and moth type (males, mated females, and virgin females) are tested as explanatory variables. The behaviors recorded include take off, upwind flight sustained to 50 cm of odour source, upwind flight to 7 cm of odour source, and landing

Behavioral response	Plant material						Headspace collection					
	Treatment			Moth type			Treatment			Moth type		
	$\chi^2$	df	P	$\chi^2$	df	P	$\chi^2$	df	P	$\chi^2$	df	P
Take off	21.54	6	0.001	0.79	2	0.67	7.14	2	0.02	0.73	2	0.69
50 cm	27.31	6	<0.0001	0.63	2	0.72	1.44	2	0.48	1.15	2	0.56
7 cm	39.41	6	<0.0001	0.54	2	0.76	2.7	2	0.25	3.09	2	0.21
Landing	24.18	6	<0.0001	3.16	2	0.2						

Significant *P*-values are highlighted in gray.

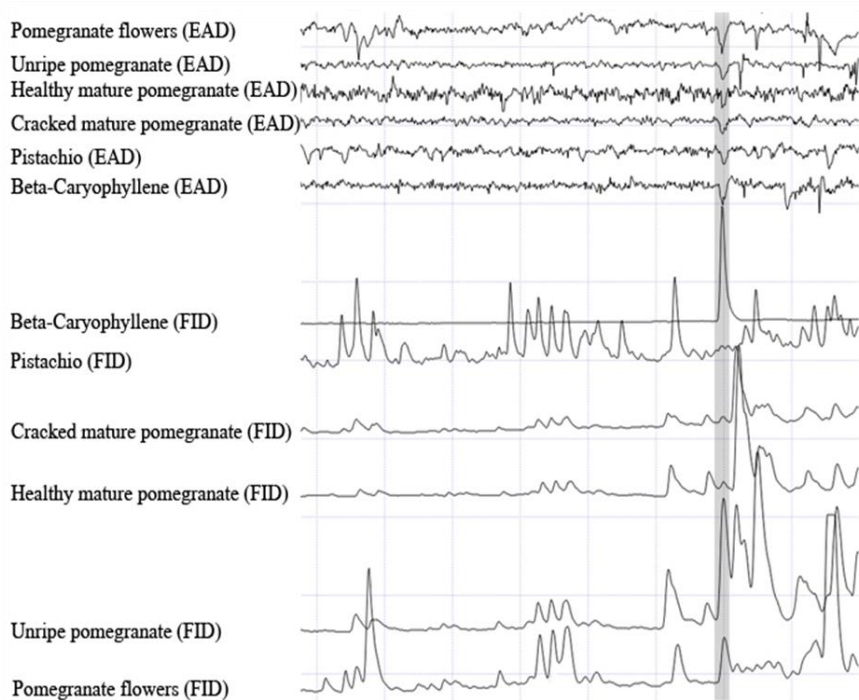


**Figure 1.** Behavioral responses of carob moths to a series of (A) plant materials (pomegranate and pistachio) and (B) headspace collections (HC) (pomegranate and pistachio) in the wind tunnel. Different letters within each behavioral step indicate significant differences ( $P < 0.05$ ).

**Electrophysiological and chemical analysis.** Analyses of headspace extracts of cracked pomegranate volatiles with GC-EAD and GC-MS led to the identification of  $\beta$ -caryophyllene as the electrophysiologically most active compound. Carob moth showed significant EAG response to the authentic  $\beta$ -caryophyllene (Figure 2). Analysis of headspace extracts by GC-EAD and single GC showed that  $\beta$ -caryophyllene was present in the volatile blend of all pomegranate samples as well as in that of pistachio (Figure 2 and 3). The absolute amount of  $\beta$ -caryophyllene differed significantly between the different headspace collections ( $F = 522.99$ ,  $df = 4, 10$ ;  $P < 0.0001$ ) (Figure S1). Also, the relative amount of  $\beta$ -caryophyllene to the constant peak, retention time: 6.99, was significantly different among different plant materials tested ( $F = 264.11$ ,  $df = 4, 10$ ;  $P < 0.0001$ ). Unripe pomegranate and pomegranate flowers contained relatively high amounts of the compound, while the extracts of pistachio and ripe pomegranate, whether cracked or uncracked, contained low amounts of  $\beta$ -

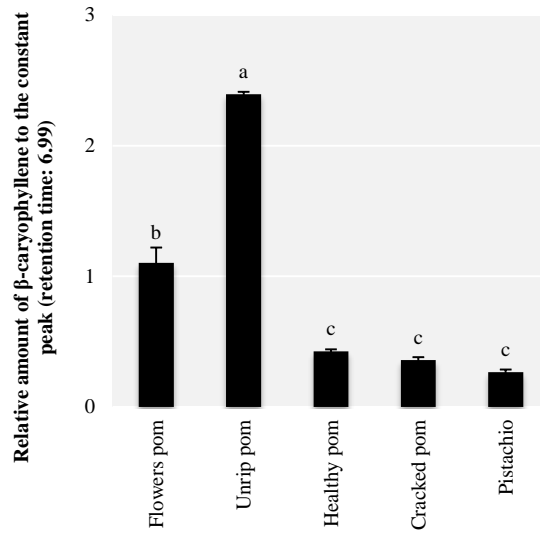


caryophyllene (Figure 3). In addition, significant negative correlations were observed between the ratio of  $\beta$ -caryophyllene and the following behavioral performances of moths in the wind tunnel: take off ( $r = -0.55$ ;  $P = 0.031$ ), upwind flight to 50 cm ( $r = -0.74$ ;  $P = 0.002$ ), upwind flight to 7 cm ( $r = -0.88$ ;  $P < 0.0001$ ), and landing on the source ( $r = -0.61$ ;  $P < 0.01$ ) (Figure 4).

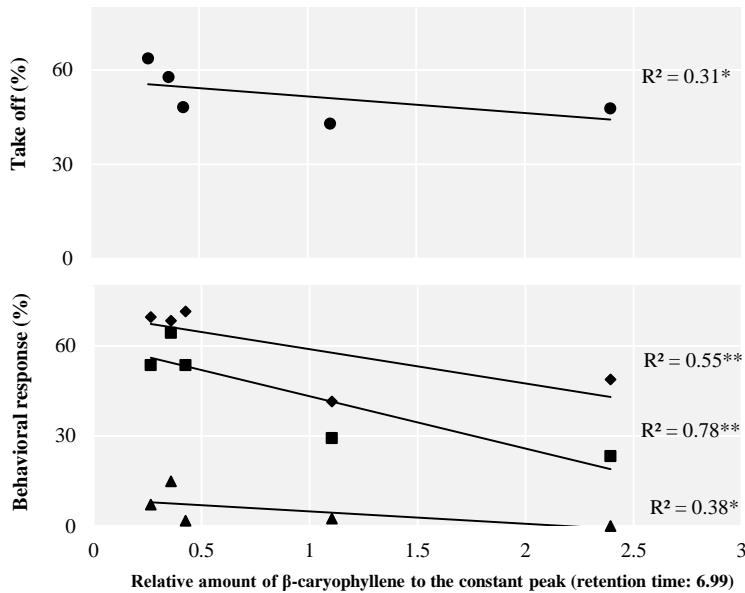


**Figure 2.** Representative GC-EAD responses of female carob moths to headspace extracts of different phenological stages of pomegranate fruit and mature pistachio as well as to  $\beta$ -caryophyllene (authentic compound). Gray color highlights GC-EAD antennal responses of  $\beta$ -caryophyllene.





**Figure 3.** Average (+ SE) relative amounts of  $\beta$ -caryophyllene to the constant peak of the solvent (retention time: 6.99) in pomegranate and pistachio materials tested. Means with different letters are significantly different ( $\alpha = 0.05$ ). Three samples were analyzed per plant.



**Figure 4.** Pearson's correlations between behavioral responses of carob moth to a series of plant materials (pomegranate flowers, unripe pomegranate, ripe cracked and uncracked pomegranate, and ripe pistachio) and average amount of  $\beta$ -caryophyllene (relative to the constant peak with retention time of 6.99) in their headspace extracts. \* $P < 0.05$ , \*\* $P < 0.01$ .

## Discussion

Attraction of carob moths to different phenological stages of pomegranate in the wind tunnel was similar to the attraction found in the field (chapter 3), but pistachio and its headspace extract did attract female carob moths in the wind tunnel but not in the field. This difference may be due to the fact that we tested the plant materials in the wind tunnel under no choice conditions, while in the field we tested the plant materials in a pomegranate orchard where pomegranate was likely preferred over pistachio by carob moth. Since pistachio is a host plant of carob moth (Dhouibi 1982, Mehrnejad 1992, Shakeri 2004), the attraction in the wind tunnel was to be expected.

Behavioral performances of male, mated female, and virgin female carob moths were similar in the wind tunnel, i.e., all life stages responded similarly to the various plant materials and headspace collections. Mated female moths are expected to be highly attracted to their host plants for oviposition (Cossé et al. 1994, Yan et al. 1999, Landolt and Guédot 2008). However, males and virgin females may also respond to plant volatiles to increase their chances of finding a mate (Coracini et al. 2004, Landolt and Guédot 2008, Bakker et al. 2008). In addition, both males and females may be attracted to host plants in species that use host plants as adult feeding sites (Lin and Phelan 1991, Tingle and Mitchell 1992, Landolt and Guédot 2008).

***Is  $\beta$ -caryophyllene a kairomone for carob moth?*** We identified  $\beta$ -caryophyllene as an electrophysiologically active compound in carob moth females. It was the most active compound in different phenological stages of pomegranate as well as in ripe pistachio.  $\beta$ -Caryophyllene is a sesquiterpene that is widely distributed in the essential oils of a wide variety of higher plants; it was also identified in pomegranate (Melgarejo et al. 2011, Vázquez-Araújo et al. 2011) and pistachio (Bachrouch et al. 2010). As a semiochemical,  $\beta$ -caryophyllene has been identified in > 60 arthropod species (see Pherobase.com).  $\beta$ -Caryophyllene has been shown to attract males and females of moth species, including the European grapevine moth (*Lobesia botrana*) (Tasin et al. 2006, Tasin et al. 2007, von Arx et al. 2012) and the tobacco budworm (*Helicoverpa assulta*) (Sun et al. 2012). This compound might also be an attractant for the carob moth. However, we found a significant negative correlation between the amount of  $\beta$ -caryophyllene in different plant materials and the attraction of carob moths in the wind tunnel, indicating that high concentrations of this sesquiterpene is probably not attractive to the carob moth or even may repel it. Previously, Cha et al. (2011) also found that doubling the amount of  $\beta$ -caryophyllene significantly reduces the attraction of female grape berry moth, *Paralobesia viteana*. Concentrations of  $\beta$ -caryophyllene in the headspace of the unattractive hosts, unripe pomegranate and pomegranate flowers, were respectively 5 and 2 times higher than in attractive fruit stages (Figure 3), which may explain the lack of attraction of carob moths to these plant sources in the wind tunnel and in the field.

***Geographic variation in host plant adaptation of carob moths.*** Carob moths are polyphagous insects, but their main host plant is different in different parts of its distribution area, even though other favorable host plants are also present. For example, the main host plants of carob moth, pomegranate and date fruit, are widely grown in North America and Middle East, but in North America the insect is the main economic pest of date fruit (Nay et al. 2006, Vetter et al. 2006) and not common on pomegranate (see ipm.ucanr.edu). In contrast, in the Middle East the carob moth is the most important pest of pomegranate (Kashkuli and Eghtedar 1975, Dhouibi 1982, Shakeri 2004, Hoseini et al. 2014, Sobhani et al. 2015), but has not yet been reported from date fruits, while this is a very common crop in the area. Previous studies have

identified  $\beta$ -caryophyllene from pomegranate (Melgarejo et al. 2011, Vázquez-Araújo et al. 2011) and pistachio (Bachrouch et al. 2010), but not from healthy date fruit (Guido et al. 2011) or fungus-infected date fruit (Cossé et al. 1994) in the United States. In fact,  $\beta$ -caryophyllene has never been reported from any date fruit. Possibly, in the Middle East adaptation of carob moths to pomegranate has resulted in selection of high sensitivity to  $\beta$ -caryophyllene, which is present in the same relative amounts in both pistachio and the most attractive pomegranate samples. It will be interesting to determine whether American carob moths and Middle-Eastern carob moths are differentially attracted to  $\beta$ -caryophyllene.

It is important to note that the ability to detect a plant compound at the antennal level does not necessarily mean that the compound is associated with host plant recognition. Although we found a (negative) correlation between the relative amount of  $\beta$ -caryophyllene and attraction, the evidence is still indirect. Further laboratory behavioral assays and field studies are required to test the attraction of different amounts of  $\beta$ -caryophyllene. Nevertheless, our results indicate that  $\beta$ -caryophyllene has potential for implementation in integrated pest management (IPM) of the carob moth.  $\beta$ -Caryophyllene has already been successfully used to trap *L. botrana* in field cages when used in a blend containing synthetic plant compounds (Anfora et al. 2009). Under field conditions, it has also been used in attractant blends with other synthetic plant volatiles to trap the emerald ash borer, *Agrilus planipennis* (Crook et al. 2008), and corn rootworm beetles (Hammack 2001). If low amounts of  $\beta$ -caryophyllene are attractive to carob moths as well, it may be used alone or in combination with the long-range sex pheromone in monitoring or attract-and-kill methods in IPM. In addition, as we found high amounts of  $\beta$ -caryophyllene in immature pomegranate fruits, which are not attractive to carob moths, spraying high concentrations of this compound on mature pomegranate fruits may reduce the infestation of ripe fruits.

In conclusion, with our wind tunnel experiments we identified attractive and unattractive host plant sources for male and female carob moths. The attractive host plant sources contained low amounts of electrophysiologically active  $\beta$ -caryophyllene, while the unattractive host plant sources contained high amounts of this compound. Our results indicate that the identification of host plant odours that are attractive to both males and females can be a fruitful route to further develop sustainable pest management strategies.

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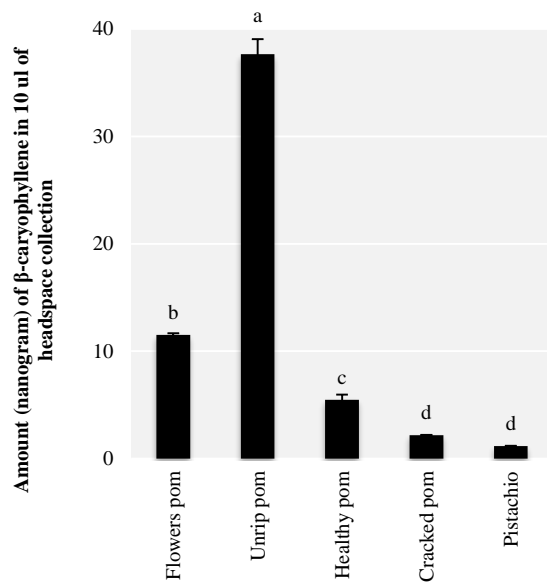


Figure S1 (supplement). Average (+ SE) amount (nanogram) of  $\beta$ -caryophyllene in 10  $\mu$ l of headspace extract of different pomegranate and pistachio materials, calculated based on the amount of internal standard. Means with different letters are significantly different ( $\alpha = 0.05$ ). Three samples were analyzed per plant.

## 5

# Experimental evidence for chemical mate guarding in a moth

Seyed Ali Hosseini, Michiel van Wijk, Gao Ke, Seyed Hossein Goldansaz,  
Coby Schal, Astrid T. Groot

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### Abstract

In polyandrous species, males seek to maximize their reproductive output by monopolizing their mate. Often the male transfers substances to the female that suppress her sexual receptivity or antagonize the behavior of competing males; both are usually transferred in seminal fluids and represent forms of chemical mate guarding. In moths, more long-range female sex pheromones have been identified than in any other animal group, and males often display with close-range sex pheromones, yet odor-based post-copulatory mate guarding has not been described in moths so far. We tested the hypothesis that the male sex pheromone in the noctuid moth *Heliothis virescens* perfumes the female and functions as an anti-aphrodisiac. Indeed, virgin females perfumed with male pheromone extract, or with its main component, mated significantly less than control virgin females, and this effect persisted for two successive nights. This chemical mate guarding strategy was disadvantageous for *H. virescens* females, because the reproductive output of twice-mated females was significantly higher than that of once-mated females. Since the female and male sex pheromones are biosynthetically related in this and other moth species, chemical mate guarding may also impose selection pressure on the long-range female sex pheromone channel and consequently affect the evolution of sexual communication.

### INTRODUCTION

In many animal species, females become unreceptive after mating. This sexual refractiveness may be generated by the females themselves, e.g. through reduced emission of aphrodisiacs so that her attractiveness is reduced <sup>1,2</sup>, or through anti-aphrodisiacs that are transferred from males to females during copulation. Within the framework of sexual selection, anti-aphrodisiacs can be receptivity-inhibiting “matedness factors” that are transferred by the male in the seminal fluid and affect the female’s physiology, e.g. <sup>3,4-6</sup>, or they can be odor-based and thus perceived through the chemosensory organs of nearby males <sup>4</sup>.

Odor-based anti-aphrodisiacs that are deposited on the abdomen of the female have been found in both vertebrates and invertebrates <sup>7-9</sup>. Interestingly, in night-active moths where female-produced sex pheromones have been identified for > 1600 species (see Pherobase.com), odor-based anti-aphrodisiacs have not been described to date. Males of some species produce a close-range sex pheromone that is emitted from their so-called hairpencils, i.e. long hairs surrounding their aedeagus <sup>10</sup>, whose function is still poorly understood. A function in antagonizing approaching competing males during courtship has been suggested, as for instance the hairpencils of the noctuid moth *Heliothis virescens* contain 16-carbon acetate esters, which are known repel competing males <sup>11,12</sup>.

The benefits to the signaling mated male are obvious, as prolonged non-receptivity of the female reduces sperm competition and increases the number of offspring sired by him. Receivers of this signal can be the mated female as well as competing males. The benefit for the mated female has generally been assumed to be reduced male harassment, as females are often presumed to not gain reproductive benefit from multiple matings <sup>13,14</sup>. However, the generality of this assumption is questionable, as females may mate multiply not only to increase the genetic variation of their offspring, but also to increase the females’ reproductive output <sup>15,16</sup>. Additionally, in moths, male harassment is unlikely to be significant, because females attract males only when they actively emit a long-range sex pheromone during

“calling”, the active extrusion of the sex pheromone gland <sup>17</sup>. Hence, odor-based anti-aphrodisiacs may not be of any discernible benefits to female moths, and may even come at a cost when females do gain reproductive benefit from multiple matings.

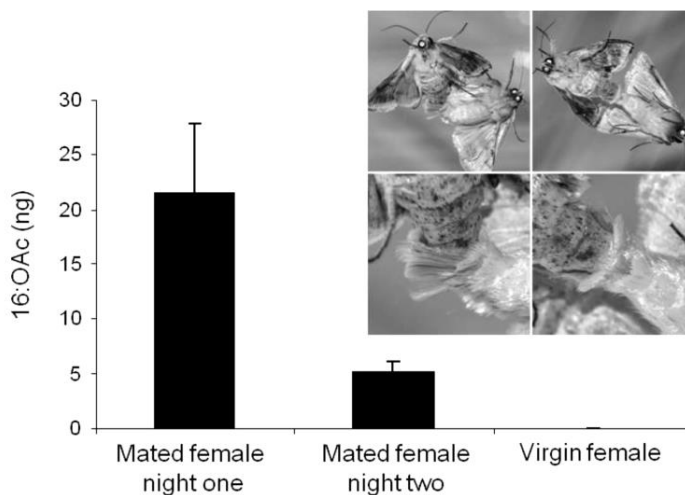
Receivers can also be competing males, for whom the anti-aphrodisiac pheromone may be informative to distinguish virgin from mated females. This would be advantageous for receiving males if females become unreceptive after mating, so that males don't waste energy courting unreceptive females, and/or if the chance of fertilizing a large proportion of eggs is significantly higher in virgin than in mated females. For example, in the moth *H. virescens*, females oviposit ~ half their eggs after the first mating and fewer eggs in subsequent nights <sup>18</sup>.

In this study, we test the hypothesis that the male sex pheromone in *H. virescens* is deposited onto the female and acts as a persistent odor-based anti-aphrodisiac. This perfuming can be regarded as post-copulatory chemical mate guarding, because the anti-aphrodisiac hairpencil compounds maximize the male's paternity but reduce the female's overall fecundity.

### RESULTS AND DISCUSSION

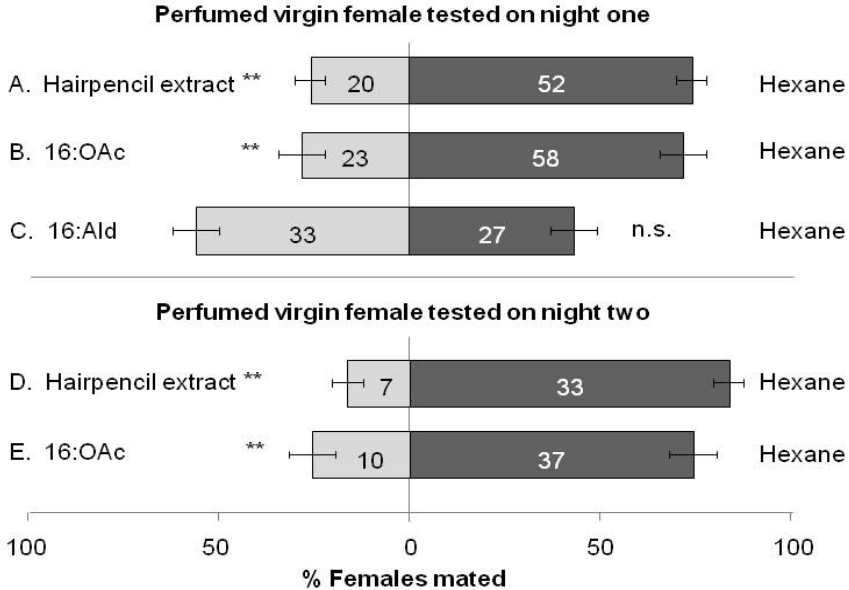
***Males, but not females, choose virgin partners.*** We first determined whether males and females prefer to mate with virgin partners, and found that virgin *H. virescens* females mated equally frequently with virgin and mated males (15 virgin vs 15 mated males). In contrast, virgin males mated significantly more often with virgin than with mated females (52 virgin vs 20 mated females,  $\chi^2 = 10.554$ , d.f. = 1,  $P = 0.001$ ). It is important to note that both virgin and previously mated females exhibited calling behavior, indicating sexual receptivity. These results show that females do not prefer to mate with virgin or mated males, but males prefer to mate with virgin females.

***Females are perfumed by males.*** Females extracted immediately after mating (night one), or in the second night after mating (night two), contained appreciable amounts of the major male hairpencil compound (16:OAc), whereas virgin females did not (Figure 1). Thus, during the 2-3 h copulation, the male “perfumes” the pheromone onto the female's abdomen by embracing the terminal segments of the female's abdomen with his hairpencils (see photos in Figure 1).

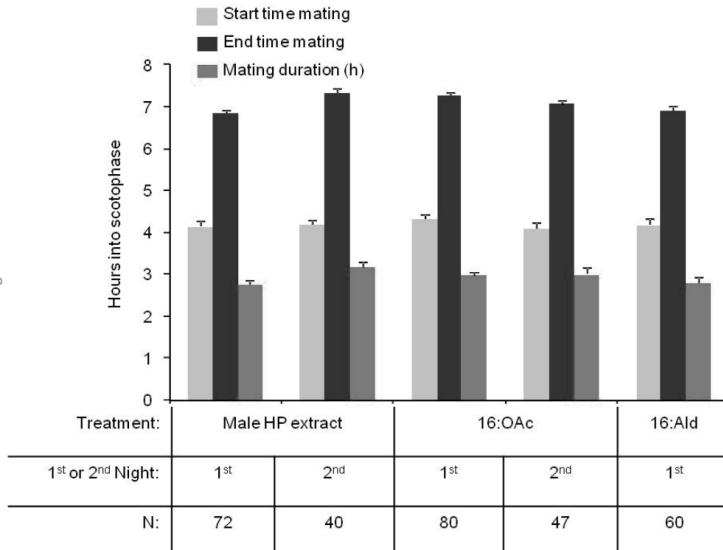


**Figure 1.** Average amount of 16:OAc ( $\pm$  SEM) extracted from one female thorax and abdomen. Females were extracted immediately after mating (night one,  $n=13$ ), 24–25 h after mating (night two,  $n=15$ ), or as virgins ( $n=18$ ). Insert pictures: During mating (A and C), the male (on the right) envelops the terminal end of the female's abdomen with his hairpencils (B and D).

**Males choose unperfumed females.** When we applied crude pheromone extract of the male hairpencils or its main pheromone component to the abdomen of virgin females, female mating chances significantly reduced (Figure 2). This was true both for females that were tested directly after perfuming (night one) (hairpencil extract  $\chi^2 = 14.732$ , d.f. = 1,  $P = 0.0001$ ; 16:OAc  $\chi^2 = 15.633$ , d.f. = 1,  $P < 0.0001$ ), as well as for females perfumed in the previous night (tested in night two; hairpencil extract  $\chi^2 = 18.354$ , d.f. = 1,  $P < 0.0001$ ; 16:OAc  $\chi^2 = 16.502$ , d.f. = 1,  $P < 0.0001$ ). Virgin females perfumed with 100 ng of the non-pheromonal compound 16:Ald were mated as often as hexane-perfumed females ( $\chi^2 = 0.601$ , d.f. = 1,  $P = 0.4382$ ) (Figure 2). The start time and duration of mating did not differ between the different groups; start time mating (d.f. = 3, 282;  $F = 0.36$ ,  $P = 0.77$ ), end time mating (d.f. = 3, 282;  $F = 0.74$ ,  $P = 0.52$ ) and mating duration (d.f. = 3, 282;  $F = 0.25$ ,  $P = 0.85$ ; Figure 3). These data suggest that the antiaphrodisiac has quantitative effects, causing a decline in mate-finding by male, but does not appear to change elements of the copulation once it is initiated. Together, these results clearly show that the close-range male sex pheromone emitted from the hairpencils of the noctuid moth *Heliothis virescens* acts as a persistent odor-based anti-aphrodisiac pheromone.



**Figure 2.** Male mate choice ( $\pm$  SEM) for virgin females perfumed with male hairpencil extract (A and D), the main male pheromone compound (16:OAc, B and E), or a compound found in the female pheromone gland (16:Ald, C). Numbers in bars are number of matings. Since we did not find differences between control females and females treated with 16:Ald, we did not test these females the second night. \*\*  $P \leq 0.0001$ , ns: not significant.



**Figure 3.** Average start and end time of all matings and the average mating duration ( $\pm$  SEM) of the differently treated females, showing that there were no significant differences among the groups (see text for details).

**Female fecundity is negatively affected by male perfuming.** Twice-mated females tended to have a higher lifetime fecundity than once-mated females ( $P = 0.052$ ), while the mean number of eggs per day was significantly higher ( $P = 0.012$ ). The longevity of females and the percent

hatched eggs were not affected by the number of matings (Table 1). The male strategy of perfuming females with an odor-based anti-aphrodisiac thus represents a form of chemical mate guarding, similar to what has been described in other insects e.g. <sup>19</sup> and primates <sup>20</sup>.

**Table 1.** Fecundity and longevity ( $\pm$  SEM) of once-mated versus twice-mated females.

	Once-mated females (n = 21)	Twice-mated females (n = 11)	F-value	P-value
Lifetime fecundity	440 $\pm$ 47	543 $\pm$ 63	4.016	0.051
Mean # eggs /day	30 $\pm$ 2	44 $\pm$ 5	7.254	0.012
% Hatched eggs*	48.7	46.2	-	0.696*
Longevity	14.81 $\pm$ 1.07	12.55 $\pm$ 1.15	1.768	0.194

The twice-mated females mated on consecutive nights. Fecundity was measured by counting the number of hatching larvae of each female during her life, longevity was determined by checking each female every day until death, mean # eggs /day is the total number of eggs per female oviposited divided by longevity, and % hatched eggs is the median fraction hatched eggs of all eggs that were oviposited. All females were kept in separate transparent beakers and sugar water was replaced every other day. \*Not normally distributed, and thus tested with a Mann-Whitney-U test.

To gain insight into the evolution of this anti-aphrodisiac pheromone, its effects on the fitness of the mated male and the main receivers – competing males and the mated female – need to be determined. In *H. virescens* the male appears to invest heavily in his mate: males mate only once per night, one mating lasts  $\sim$  3 h, during which he transfers a spermatophore that is  $\sim$  5-10 % of his body mass <sup>21</sup>. Perfuming the female significantly reduces the probability that she will remate (Figure 2). Because there is no last-male sperm precedence in *H. virescens* <sup>22</sup>, the perfuming strategy minimizes sperm competition and protects the male's investment. The anti-aphrodisiac pheromone may also benefit competing males, because females oviposit  $\sim$  50% of their eggs after the first mating and significantly fewer eggs on each subsequent night <sup>18</sup>. The anti-aphrodisiac pheromone may guide the competing male's decision to abandon the calling mated female, or to pursue her but adjust his investment in her <sup>23</sup>.

In contrast, *H. virescens* females are negatively affected by the anti-aphrodisiac marker. The anti-aphrodisiac pheromone reduced subsequent matings and lessened female fecundity, as twice-mated females oviposited more eggs than once-mated females. Females normally re-mate every night (11 out of 21 in this study) or every other night <sup>21,24</sup>. Therefore, there may be selection pressure on females to accept males with less pheromone or less saturated pheromone to minimize the chemical mate guarding effects, as unsaturated compounds are more volatile. Recently, *Drosophila melanogaster* females were found to actively eject the male pheromone a few hours after copulation, resulting in increased attractiveness and remating<sup>6</sup>. Such active process is unlikely to occur in moths, as this pheromone is deposited on the outside of abdomen and we never observed females to actively rub (wash) her abdomen after mating. Whether and how male mating success varies with the quantity or quality of pheromone he produces remains to be tested.

Interestingly, in *H. virescens* the female long-range sex pheromone and the male close-range courtship pheromone are biosynthetically related <sup>25</sup>, which introduces the possibility that selection forces that shape the pheromone of one sex affect the pheromone composition of the opposite sex. If females choose to mate with males with less saturated (and thus more unsaturated) pheromone compounds, to increase the volatility and thus decrease the duration of chemical mate guarding, then there would be selection for a decreased ratio saturated-to-unsaturated compounds. Typical *H. virescens* females produce a high ratio and are attractive to males, whereas females with a lower ratio are less attractive <sup>26</sup>. So far, the evolution of moth sex pheromones has been regarded as determined by selection pressures acting only on the female sex pheromones (e.g. <sup>27,28</sup>). As these are important species-



recognition cues that minimize cross-species communication interference<sup>11,29,30</sup>, moth pheromones are generally thought to be under stabilizing selection, which makes it hard to envision how these sexual communication systems can evolve<sup>29,31,32</sup>. Now that we show that the close-range male sex pheromone can also serve in both intersexual and intrasexual conflict, these additional selection pressures may help explain the enormous diversity of moth pheromone blends<sup>33</sup>.

In conclusion, our study shows the presence of a persistent odor-based anti-aphrodisiac in a noctuid moth, which mediates male-male competition and negatively impacts female fecundity. The major male pheromone component alone is sufficient to impose substantial chemical mate guarding of the mated female. It remains to be determined whether females choose males depending on the quantity and/or quality of their close-range pheromone.

## METHODS

*Heliothis virescens* was obtained from North Carolina State University laboratory colonies, and has been reared at the University of Amsterdam since 2011 in a climate chamber at 25 °C, 60% relative humidity and a light–dark cycle of 10 L: 14 D with lights off at 11:00. Larvae were reared singly in plastic cups (37 ml, Solo, Lake Forest, Illinois) on artificial pinto bean diet<sup>34</sup>. Emerged adults were checked daily and provided with a 10% sucrose solution. For all experiments 2–5 day old adult moths were used.

***Do males and females choose virgin partners?*** We determined whether virgin *H. virescens* males and/or females choose to mate with virgin or mated partners in 30 x 30 x 30 cm mesh cages. To obtain mated individuals, one virgin male and female were paired in transparent plastic beakers (473 ml, Solo, Lake Forest, Illinois) containing a piece of cotton soaked in sugar-water. The pairs were observed every 30 min during the scotophase. After each mating pair separated, the male and female were placed in separate beakers until the next scotophase. In the choice experiments, one virgin male or female was offered a virgin and a mated mating partner. To distinguish between virgin and mated individuals, one of the two was marked randomly with a black marker.

***Are females perfumed by males?*** To determine whether females are perfumed by the male pheromone during copulation, we immersed the abdomen and thorax of a mated female directly after mating for 30 min in 150 µl hexane, containing 200 ng pentadecane as internal standard. All extracts were kept at -20°C prior to chemical analysis and analyzed individually on an Agilent 7890 Gas Chromatograph (GC), as described in<sup>35</sup>.

***Do males recognize perfumed females?*** Male choice experiments were conducted with virgin females in the same cages as described above. 30–60 Minutes before the start of the scotophase, the abdomen of each virgin female was treated with 3 µl of hexane (control) or 3 µl hairpencil extract, using a 10 µl Hamilton syringe (Reno, Nevada). Females were marked by clipping the tip of either the right or left forewing. Matings were recorded every 30 min throughout the scotophase. To assess the persistence of perfuming until the second night, when females normally resume calling, virgin females were also perfumed 6–7 h into scotophase of night one (the time that matings would generally end), and placed in cages without a male. 30–60 Min before the next scotophase (night two), virgin males were added to the cages and experiments were conducted as above. Females were perfumed with a) male pheromone extract or b) male pheromone compounds, as described below.

**a) Male pheromone extract.** Hairpencils of 2–3-day-old virgin males were extracted from 30 males 4–5 h after the start of the scotophase by immersing them in 1 ml hexane. 15 Min after the last hairpencils had been placed in the vial, all hairpencils were removed from the extraction vial and the extract was kept at -20°C prior to the behavioral experiments and chemical analysis. The extract was reduced to 90 µl under a gentle flow of nitrogen, so that 3 µl represented one male equivalent.

**b) Male pheromone compound.** As male hairpencils of *H. virescens* contain ~ 200 ng hexadecyl acetate (16:OAc) as the main component <sup>36,37</sup>, and male approach and landing was significantly affected by exposure to 100 ng 16:OAc <sup>38</sup>, abdomens of virgin females were perfumed with 100 ng 16:OAc. To ensure that our findings could be attributed to this male pheromone compound and not to any odor that changes the female's odor, we also perfumed virgin females with 100 ng hexadecanal (16:Ald). This compound is present in the female sex pheromone gland <sup>39,40</sup> and on the male's tarsi <sup>41</sup> and perceived by both males and females <sup>42</sup>, but it has not been shown to affect male attraction <sup>43</sup>.

**Is females fecundity affected by the number of matings?** To quantify fecundity effects of a second mating on female fitness, virgin females and males were paired in the same 473 ml beakers, as described above, containing a piece of cotton soaked in sugar-water. The pair was observed throughout the scotophase, and the start time and duration of copulations were scored. Some of the mated females were paired with a new virgin male the next night and confirmed to mate again with observations throughout the scotophase. For each female, the number of oviposited eggs and hatching larvae were determined daily until death.

Statistical analysis was conducted in R-studio (0.98.490). All mate choice experiments were analyzed using GLM with a binomial error distribution. The perfumed or control females were treated as binary response variables, while the dates of the experiment were fixed effects. Cages without matings were excluded from the analysis (n=6). To determine differences in the reproductive output of females, lifetime fecundity (number of hatching larvae) and lifespan, we first assessed normality of the data, using a Shapiro-Wilk's normality test. Since we did not find significant deviations from a normal distribution for the reproductive output ( $W = 0.979$ ,  $P = 0.7699$ ), lifetime fecundity ( $W = 0.9598$ ,  $P = 0.2712$ ) and mean number of eggs/day ( $W = 0.97563$ ,  $P = 0.6663$ ), we used a one-way ANOVA to compare these measures between once- and twice-mated females. As the fraction of hatched eggs did deviate significantly from normality, these data were analyzed with a Mann-Whitney U test.

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## 6

# General discussion

Over the past decades, development and application of semiochemical-based behavioral manipulation methods as an efficient environmentally friendly strategy in Integrated Pest Management (IPM) have seen a steady growth in most parts of the world, mostly due to strict regulations imposed on the use of chemical pesticides (e.g., EU 2009; see <http://eur-lex.europa.eu>). However, these methods are not yet available for all pest insects. In moths, long-range female sex pheromones are now widely used to monitor insect populations and to suppress them through mass trapping or mating disruption (reviewed by Witzgall et al. 2010). As female sex pheromones only affect adult males and mating-disruption is not efficient at high population densities, and as females play the main role in the population dynamics of insects (Caswell 2001), a behavioral manipulation method that concerns females will be more efficient than one only directed at males. However, it would be best to manipulate the behaviors of both sexes, as males also contribute to population dynamics (Rankin and Kokko 2007). Semiochemicals that affect the behavior of females or both sexes may serve as alternatives or supplements to the long-range sex pheromones, especially when the sex pheromones are difficult to produce, unstable, or inefficient. In moths, semiochemicals affecting behaviors of females, of both sexes, and of immature stages, have been identified in a number of species and can potentially be used in IPM. Such semiochemicals include host plant volatiles (Menken et al. 1992, Landolt and Guédot 2008, Hansson and Stensmyr 2011, Piesik et al. 2013, Lee et al. 2016), male pheromones (Birch et al. 1990, Hillier and Vickers 2004, Hillier et al. 2007), host marking pheromones (Huth and Pellmyr 1999), aggregation pheromones (Jumeau et al. 2005), and alarm pheromones (Teerling et al. 1993, MacDonald et al. 2002, de Bruijn et al. 2016).

There are three principal elements of behavioral manipulation in pest management: (1) a behavior that is related to pest damage, (2) a stimulus that manipulates that behavior, and (3) a method that utilizes the stimulus against the pest (Foster and Harris 1997). In this thesis, I investigated host finding and mating behaviors of two important moth pests, the carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae), and the tobacco budworm, *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae).

In the carob moth management system, an efficient sex pheromone attractant is still missing. As the major female sex pheromone component of the insect [(Z,E)-9,11,13-tetradecatrienal] is unstable, an alternative stable mimic of this component [(Z,E)-7,9,11-dodecatrienyl formate] is used in commercial sex pheromone lures, but these lures are not very attractive in pomegranate orchards (Avand-Faghih et al. 2012, Dhouibi et al. 2016). In search of a suitable host plant volatile-based attractant semiochemical for the carob moth, I investigated the relations between host-fruit phenology and fruit infestation as well as the pest host location behavior both in the field in pomegranate orchards of Iran and in the wind tunnel.

In addition to host plant volatiles, close-range male sex pheromones have good potential to be used in behavioral manipulation strategies, as such pheromones may affect behaviors of both males (as an inhibitor to conspecific males) and females (as an aphrodisiac). The male pheromone of *H. virescens* has already been identified to serve as aphrodisiac to females (Hillier and Vickers 2004). I therefore tested the hypothesis that the male-specific pheromone of the tobacco budworm inhibits conspecific males from mating.

## 1. Host plant volatile-based attractants for use in IPM against the carob moth

An efficient host plant volatile-based attractant can make a great contribution to IPM programs by targeting adult females (e.g., in monitoring and attract-and-kill methods). There are also quite a few examples of host plant volatiles that synergize responses of male moths to female sex pheromone and thus enhance the efficiency of IPMs that combine sex pheromones with

plant volatiles (Ochieng et al. 2002, Yang et al. 2004, Schmidt-Buesser et al. 2009, von Arx et al. 2012). Further advantages of plant volatiles are that they are often simple and cheap chemicals, and commercially available.

However, compared to pheromones, host plant volatiles may be less effective as they have to compete with the volatiles of the target plant for attraction. This drawback may be overcome by using the stimuli at the right time or place, i.e., using when or where the host plant is absent, for example before plant fruiting time in fruit pests or outside the agricultural area. Since individual plant volatiles are not always specific, in fact many of them are common to most plants species, it is essential to accurately understand insect host searching behaviors when considering the exploitation of host plant volatiles for IPM, i.e., to determine which host plant(s) at which phenological stage(s) or which plant part(s) are the most attractive sources to the pest and through which volatiles and in which ratios the insect is attracted to the host plant source.

### **1.1. The carob moth-pomegranate association**

In Chapter 2, it is shown that different pomegranate cultivars differed significantly in terms of fruit cracking and fruit infestation by the carob moth, and there was a strong positive correlation between the susceptibility of cultivars to fruit cracking and the level of fruit infestation. Although ~ 20% of the fruits were cracked in a year, infestation rates of cracked fruits were ~ 15- and ~ 5-fold higher than uncracked fruits in the 2 years of study. The number of larvae per infested-cracked fruit was also significantly higher than in uncracked fruit, indicating that female carob moths lay more eggs on cracked than on uncracked fruits.

In Chapter 3 and 4, it is shown that adult carob moths are attracted to volatiles of mature pomegranates as well as their headspace extracts, both under field and under wind tunnel conditions. Male and female carob moths discriminated between different phenological stages of pomegranate fruit. Mature cracked pomegranate appeared to be the most attractive fruiting stage to gravid female carob moths. In both field and wind tunnel experiments, mature pomegranate was behaviorally active and attractive to male as well as to virgin and mated female carob moths, irrespective of whether the fruits were uncracked or cracked, infested or uninfested. Also, headspace extract of cracked pomegranate was attractive to males and females. In contrast, pomegranate flowers and immature fruits did not attract carob moths in both field and wind tunnel. The field experiments further showed that the combination of cracked pomegranate fruits and a virgin female in a trap enhanced the attraction of virgin female carob moths and total (male + female) moth catches in the traps.

### **1.2. $\beta$ -Caryophyllene, a biologically active compound to the carob moth**

In Chapter 4,  $\beta$ -caryophyllene was identified as the most electrophysiologically active compound in carob moth females; it was found in the headspace extracts of pomegranate and pistachio fruits.  $\beta$ -Caryophyllene is a sesquiterpene widely distributed in essential oils of a great variety of higher plants. It has already been identified to function as a semiochemical in > 60 arthropod species (see Pherobase.com), and in line with this it was the most electrophysiologically active compound of flowers, unripe fruit, and mature cracked/uncracked fruit of pomegranate as well as mature pistachio. Strikingly, we found a significant negative correlation between the amount of  $\beta$ -caryophyllene in different plant materials and the level of attraction of carob moths in the wind tunnel, suggesting that high concentrations of this compound are not attractive to the carob moth.



In moths,  $\beta$ -caryophyllene alone or in blends with other components has been shown to attract males and females of pest species such as the European grapevine moth, *Lobesia botrana* (Denis & Schiffermüller) (Lepidoptera: Tortricidae) (Tasin et al. 2006, Tasin et al. 2007, von Arx et al. 2012), and the tobacco budworm, *Helicoverpa assulta* (Guenée) (Lepidoptera: Noctuidae) (Sun et al. 2012).  $\beta$ -Caryophyllene is a good example of an attractive host plant volatile that becomes unattractive or even repellent to an herbivorous insect at high concentrations of the compound (Finch 1978, Hern and Dorn 1999, Mewis et al. 2002). Doubling the amount of  $\beta$ -caryophyllene in a blend of host plant components, while keeping the concentration of the other components constant, significantly reduced the attraction of female grape berry moth, *Paralobesia viteana* (Clemens) (Lepidoptera: Tortricidae) (Cha et al. 2011), a situation that seems comparable to that of the carob moth.  $\beta$ -Caryophyllene has been successfully used to trap *L. botrana* in field cages when used in a blend containing synthetic plant components (Anfora et al. 2009). Under field conditions, this plant compound has also been used in attractant blends with other synthetic plant odor blends to trap the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) (Crook et al. 2008), and corn rootworm beetles of the genus *Diabrotica* (Hammack 2001).  $\beta$ -Caryophyllene also seems to be a promising tool to use in IPM strategies against the carob moth, as will be specified below.

### 1.3. Potential use of pomegranate volatiles (total blends and $\beta$ -caryophyllene) in IPM control of the carob moth

Similar to sex pheromones, host plant-derived semiochemicals may be used to monitor insect populations as well as to directly control insect pests, e.g., in mass trapping and attract-and-kill approaches. Host plant volatiles may also be used to disrupt host-finding behavior of pest insects.

#### 1.3.1. Monitoring

Sex pheromone lures of the carob moth are relatively expensive to make and, as mentioned above, are not very efficient in pomegranate orchards (Avand-Faghih et al. 2012, Dhouibi et al. 2016), attracting an average of 9 males per week at most (Dhouibi et al. 2016). In traps baited with cracked pomegranate only, we trapped similar numbers of moth per week, but mostly females. We thus show that pomegranate fruit baits or their volatile extracts can be used to monitor the entire carob moth population. Monitoring pest insects with plant volatiles is already a management practice in quite a few moth species. In a study by Sussenbach and Fiedler (1999), for example, 119 species of noctuid moths have been monitored using traps baited with red wine. Different fruit baits have also been used to trap the fruit-piercing moth *Eudocima phalonia* (L.) (Lepidoptera: Erebididae) (Reddy et al. 2007).

Some biological control methods have been developed against carob moths in pomegranate cultures, including the augmentative release of mass-reared trichogrammatid egg parasitoids (Moezipour 2006, Poorjavad et al. 2011) and larval parasitoids, e.g., *Bracon hebetor* Say (Hymenoptera: Braconidae) (Saadat et al. 2016), as well as spraying with *Bacillus* spp. formulations (Harpaz and Wysoki 1984, Mnif et al. 2013). However, these methods are not yet effective enough to keep the pest populations below economic threshold levels. This may be because of the application of the biocontrol agents at inappropriate times, i.e., when their target hosts are absent or its population density is too low. A monitoring method, in combination with temperature-dependent development models to effectively predict emergence of specific growing stages of the pest, as has been developed in some other pest species (Knight and Croft 1991, Witzgall et al. 2008, Jones et al. 2013), may accordingly improve the efficiency of current biological control methods against the carob moth. Host

plant volatile-based traps can give important information on population dynamics of gravid female carob moths and help to optimize the timing of augmentative release of the trichogrammatid egg parasitoids. The emergence of larvae of carob moth which are targeted by the larval parasitoids and *Bacillus* spp., can also be predicted by monitoring the female population.

### 1.3.2. Mass trapping

Field experiments showed that the traps baited with pomegranate and pistachio as well as their headspace extracts do not mass trap carob moth. However, we did show that the combination of the volatiles of cracked pomegranate and virgin females enhances attraction of female carob moths and increases total catch. Mass trapping will be much more efficient if females rather than only males (by e.g., female sex pheromones) are targeted, because the entire offspring of every mated female that can be trapped before oviposition is eliminated from the next generation. It will thus be interesting to determine whether mass trapping female carob moths can be achieved by using the combination of the volatiles of cracked pomegranate with female sex pheromone lures when pomegranates of the orchards are not yet mature and cracked, i.e., early in the season during flowering/early fruiting stages of the plants, when the fruits do not crack (Hoseini et al. 2014).

### 1.3.3. Disruption of host finding

Disruption of the host plant finding process can be achieved by disrupting the chemical communication between host plant and insect through repelling host-searching insects from the host plant that is normally attractive. Field and wind tunnel experiments clearly indicate that pomegranate flowers and immature fruits are not attractive to carob moth. Furthermore, a negative correlation exists between the amount of  $\beta$ -caryophyllene in the fruit blend and the attraction of the carob moth to different fruit types. We thus hypothesized that ripe pomegranate fruits will not be infested by carob moths if they can be successfully manipulated to smell like immature fruits. As we found high amounts of  $\beta$ -caryophyllene in the immature fruits, spraying high concentrations of this compound on mature pomegranate fruits may reduce infestation of ripe fruits.

From the perspective of human health, application of  $\beta$ -caryophyllene does not seem to be problematic. On the contrary, it may have positive effects on humans as  $\beta$ -caryophyllene has been reported to act as an analgesic (Fiorenzani et al. 2014), anti-carcinogenic (Legault and Pichette 2007), and anti-depressant (Bahi et al. 2014). However, as this compound functions as a semiochemical in many insect species, side effects of its application in pomegranate orchards on non-target insects are to be expected and should carefully be assessed. For instance,  $\beta$ -caryophyllene may be an attractant for - besides pests such as aphids and mites - the moth *Notocelia punicana* Kuznetsov (Lepidoptera: Tortricidae), which infests pomegranate early in the growing season (Shakeri 2004, Alipanah et al. 2004) when the plant is in the flowering and early fruiting stages and produces high amounts of this compound.

### 1.3.4. Push-pull

The most effective behavioral manipulation in IPM may be achieved by the so-called push-pull strategy, which involves the behavioral manipulation of insect pests via the integration of stimuli that act to make the protected resource unattractive or unsuitable to the pests (push) while at the same time luring them toward an attractive source (pull) from where the pests can be subsequently removed (Cook et al. 2007). Insects recognize suitable host plants by using key volatiles that are often present in specific ratios (Bruce et al. 2005). As discussed above, female carob moths may be pushed away from mature pomegranate fruits that are sprayed

with a high concentration of  $\beta$ -caryophyllene. At the same time, females can be pulled towards traps baited with cracked-mature pomegranate volatile-based attractants and removed. We showed that mature pomegranate volatiles significantly enhance male trapping with female sex pheromone. So, a combination of the pomegranate volatiles and female sex pheromone lures may more efficiently be used to remove both males and females in such a push-pull strategy than the standalone application.

#### **1.4. Suggestions for management of carob moth based on the seasonal pattern of fruit infestation**

In Chapters 2, 3 and 4, it was shown that (1) cracked pomegranate is the most attractive type of fruit to gravid female carob moths and the most susceptible pomegranate type to carob moth infestation, (2) cracked fruits are highly infested all over the season and host larger populations of larvae than uncracked fruits, and (3) there is a strong positive correlation between susceptibility of pomegranate cultivars to fruit cracking and level of fruit infestation. Therefore, control of fruit cracking is likely to suppress the pest population during the growing season, and can be deployed using resistant cultivars or recommended horticultural operations and treatments such as management of irrigation and pruning as well as application of gibberellic acid and benzyladenine (Khalil and Aly 2013, Galindo et al. 2014). On the other hand, we found that (1) infestation in uncracked fruits increases during the season so that the highest number of uncracked-infested fruits is recorded at the end of season, (2) the more resistant a cultivar is to fruit cracking, the later in the season it is infested, and (3) the number of carob moth larvae that can survive in uncracked fruits is much higher than in cracked fruits (at least under simulated winter conditions in the laboratory). Higher infestation of uncracked fruits and cracking-resistant cultivars at the end of season may thus be related to the survival of the insect larvae in the following winter season, as carob moths overwinter in their host fruits. Thus, uncracked fruits are likely to harbor a large part of the overwintering generation, which in turn will form the first generation of the following year.

Collecting and destroying all infested pomegranates after harvesting the fruits was already recommended for the control of the carob moth in the mid 1970s (Kashkuli and Eghtedar 1975; also see Shakeri 2004). However, this measure has recently been reported to have important negative effects on population sizes and species diversity of its larval parasitoids (Kishani-Farahani et al. 2012). It is possible that cracked fruits host more larval parasitoids, as cracks on the fruit peel likely facilitate the access of the female parasitoids to the larvae inside. These parasitoids will be better conserved if only uncracked-infested fruits are destroyed after harvest, which are the very fruit types that harbor most carob moth larvae.

From the seasonal patterns of pomegranate fruit infestation by carob moth, the strong suggestion emerges that it is better not to grow cracking-susceptible and cracking-resistant pomegranate cultivars in the same area. That is because cracked and uncracked fruits differently sustain the pest population in different seasons: susceptible cultivars mainly support the insect population during the growing season but the resistant cultivars do so in winter. This is particularly important in the Middle East, and especially in Iran, where pomegranate orchards are typically composed of mixed cultivars that differ in cracking level, with a current tendency to increase the genotype diversity.

## **2. The potential use of male sex pheromone in IPM against *Heliothis virescens***

In the noctuid moth *H. virescens*, the pheromone that is released from male hair pencils acts as an anti-aphrodisiac, i.e., it inhibits conspecific males from mating with females perfumed with the male pheromone (Chapter 5). Not only did the whole extract of the male hairpencil,

perfumed onto virgin females, reduce the female's chances to mate, but also the main component of this extract, 16:OAc, was sufficient to induce this effect. Also, at long-range, acetates in female pheromone of the closely related species *Heliothis subflexa* (Guenée) (Lepidoptera: Noctuidae) have already been shown to be responsible for inhibiting males of *H. virescens* from mating with *H. subflexa* females (Groot et al. 2006, Hillier et al. 2007). Moreover, male pheromone of *H. virescens* has been identified to function as aphrodisiacs for females and reduce their movement and calling behavior (Hillier and Vickers 2004). Therefore, by affecting behaviors of both males and females, the male pheromone has excellent potential for application in behavioral manipulation tactics against the tobacco budworm, including mating suppression and push-pull approaches.

Suppression of mating in pest insects to reduce the pest population below a particular economic threshold is a central tenet of IPM. In moths, mating suppression has so far been achieved through mating disruption by releasing high levels of synthetic female sex pheromone in the crop environment, resulting in mate-searching males failing to find a mate. However, a mating suppression method may be more efficient if besides the mate-searching behaviors of males, calling behaviors of the females can also be manipulated. Even though it does not seem feasible to treat (i.e., to perfume) females in the field, perfuming calling sites of *H. virescens* (e.g., host plants) with male pheromone may delay or even prohibit mating by confusing and repelling males from the area and by reducing calling activities of females.

Male pheromones may also be exploited in a push-pull method. Mating sites of the insect can be perfumed with the pheromone to repel the males but arrest the females (i.e., reduce females' movement and calling activities). At the same time, males may be efficiently trapped in a mass-trapping/attraction-and-kill method outside the area that is treated with male pheromone.

### 3. Conclusions

In my thesis, I have quantified host finding and mating behaviors of two important moth pest species that can be exploited in IPM to reduce their damage, which is the first principal step in the development and implementation of a behavioral manipulation program (Foster and Harris 1997). I showed that fruit cracking makes pomegranate susceptible to carob moth attack and that cracked fruits provide the most attractive cues for mated female carob moths. I also demonstrated the efficiency of the short-range male pheromone of *H. virescens* in inhibiting conspecific males from mating. The second principal step for a behavioral manipulation method to be used in IPM is to identify a stimulus that can manipulate these behaviors. For the carob moths, I found that pieces of pomegranate can simply be used to monitor the pest population, which may be much easier, cheaper, and more effective than the currently used commercial lures. This study further provided evidence for the role of  $\beta$ -caryophyllene in host location by the carob moth. Pursuing behavioral effects of this compound in the field is needed to determine how this compound can be successfully used in the IPM of the carob moth. The last principal element of a behavioral manipulation method is the development of a method that utilizes the stimuli against the pest. In the final chapter of this thesis, I discussed how my findings can be employed in the IPM against the two study pests. Behavioral manipulation appears to have more potential than what has been achieved in IPM. My efforts to improve current and develop new behavioral manipulation methods will hopefully contribute to reduce the problems that carob moth and tobacco budworm cause around the world to economically acceptable levels in an environmentally friendly way.

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## Summary

Behavioral manipulation of insect pest species is an environmentally friendly approach in integrated pest management (IPM). Even though there are quite a few examples of successful application of behavioral manipulation methods in insect pest management programs, such as mating disruption, monitoring, and push-pull, for many pest species efficient methods have yet to be developed. In moths, long-range female sex pheromones have been the basis of behavioral manipulation programs, and sex pheromones of > 1600 species have been identified by now. Female sex pheromones only affect adult males, while females play the main role in insects population dynamics, and thus control measure should be primarily targeted at females. Semiochemicals that would affect the behavior of females or both sexes may thus serve as alternatives or supplements to long-range sex pheromones, especially in cases where the sex pheromones are difficult to produce, unstable, or inefficient. In this thesis, as first steps in creating new, rigorous behavioral manipulation tools, we investigated host finding and mating behaviors of two important insect pests, the carob moth, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae), and the tobacco budworm, *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae).

Carob moth is a devastating pest in high-value crops around the world, including date, almond, pomegranate, and pistachio. In Iran and other parts of the Middle East, carob moth is the most important pest of pomegranate, *Punica granatum* L. (Lythraceae). Unfortunately, an efficient sex pheromone attractant is still missing for this species, as the major pheromone component is unstable. Host plant volatiles attract both male and female herbivorous insects and are known to synergize responses to sex pheromones, and thus have shown good potential to be exploited in IPM. To explore host plant volatiles for their application in IPM programs, we investigated the pomegranate – carob moth interaction in field experiments in pomegranate orchards of Iran and in wind tunnel bioassays.

Pomegranate orchards in the Middle East are typically composed of a mix of different cultivars, which exhibit different sensitivity to infestation by carob moth. In **Chapter 2**, we aimed to determine which factors mostly contribute to pomegranate susceptibility to carob moth by monitoring the progress of fruit infestation in 10 pomegranate cultivars during the growing season of two consecutive years. Overall, levels of infestation in pomegranate fruits were strongly correlated with susceptibility to fruit cracking so that cracked fruits and cracking-susceptible cultivars were most infested. However, this pattern changed during the season. Early in the season, infestation was observed on cracking-susceptible cultivars. At this point almost all cracked fruits were infested. Towards the end of the season, however, infestation in uncracked fruits and cracking-resistant cultivars increased, which seem favorable overwintering sites for carob moth larvae.

In **Chapter 3**, we aimed to assess which volatile sources are most attractive to carob moths by conducting trapping experiments in the pomegranate orchards. Numbers of male and both mated and virgin female carob moths trapped in sticky traps baited with mature pomegranates, whether uncracked or cracked, infested or uninfested, were significantly higher than in unbaited traps. Traps baited with headspace extract of cracked pomegranate only caught mated females and mature pistachio only attracted males. Pomegranate flowers, unripe pomegranate, and headspace extract of pistachio did not attract moths. Furthermore, mated females were more caught by traps baited with cracked fruit than uncracked fruit. And, finally, males were attracted similarly to cracked-infested pomegranate and virgin females alone.

Interestingly, the combination of cracked pomegranate with a virgin female enhanced the attraction of virgin females, and also significantly increased total moth catch.

In **Chapter 4**, we aimed to identify the volatiles in the plant sources that we found in Chapter 3 to be attractive to carob moths. Using coupled gas chromatography-electroantennography (GC-EAD) and GC-mass spectrometry, we identified  $\beta$ -caryophyllene from the headspace extracts of pomegranate and pistachio fruits as the volatile that consistently elicited the strongest antennal responses from female carob moths. Significant variation in the relative amount of this compound was found between different plant parts tested. In the wind tunnel, where males, virgin females, and gravid females responded similarly to the host fruits, responses were negatively correlated with the amount of  $\beta$ -caryophyllene in head space extracts. These results suggest that low amounts of  $\beta$ -caryophyllene increases the attraction of carob moths, while high amounts are not attractive.

Taken together, results show that fruit cracking makes pomegranate susceptible to carob moth attack, that cracked fruits provide the most attractive cues for mated female carob moths, and that  $\beta$ -caryophyllene is involved in the attraction of carob moths to pomegranate fruits. Results further show that volatiles from cracked pomegranates alone or in combination with female sex pheromone have good potential for carob moth management. Traps baited with pieces of pomegranate can simply be used to monitor the pest population. This approach [or method] is much easier, cheaper, and more effective than the currently used commercial lures based on sex pheromones.

Close-range male sex pheromones may affect the behavior of both males (as an inhibitor to conspecific males) and females (as an aphrodisiac). In **Chapter 5**, we tested the hypothesis that the male sex pheromone of *H. virescens* perfumes the female and functions as an anti-aphrodisiac. Indeed, we found that virgin females perfumed with male pheromone extract, or with its main component, mated significantly less than control virgin females, and this effect persisted for two successive nights. This form of chemical mate guarding strategy was disadvantageous for *H. virescens* females, because the reproductive output of twice-mated females was significantly higher than that of once-mated females. Finally, in **Chapter 6**, all findings are discussed in the context of their suitability in future IPM programs that are aimed at controlling the carob moth and the tobacco budworm.

## Samenvatting

Gedragsmanipulatie van plaaginsecten is een milieuvriendelijke manier in de geïntegreerde bestrijding. Hoewel er een aantal succesvolle gedragsmanipulatiemethoden bekend zijn, zoals verstoring van de paring (“mating disruption”), monitoring en afstoten-en-aantrekken (“push-pull”), moet voor een aantal plaaginsecten nog een efficiënte methode ontwikkeld worden. In motten zijn vooral de seksferomonen van vrouwtjes, vluchtige stoffen die mannetjes aantrekken over grotere afstanden, de basis voor succesvolle gedragsmanipulatieprogramma’s; inmiddels is van >1600 soorten motten het seksferomoon geïdentificeerd. Met deze door vrouwtjes geproduceerde seksferomonen kunnen alleen volwassen mannetjes gemanipuleerd worden, terwijl vrouwtjes de belangrijkste rol spelen in de populatiedynamica van insecten, zodat beheersmethoden vooral, of ook, gericht zouden moeten zijn op het wegvangen of manipuleren van vrouwtjes. Naast seksferomonen zouden signaalstoffen van de voedselplanten, die het gedrag van vrouwtjes of van beide seksen beïnvloeden, daarom als alternatieve of toegevoegde methode kunnen dienen, vooral in die gevallen waar de seksferomonen moeilijk te produceren, instabiel, of inefficiënt zijn. In dit proefschrift hebben wij het zoek- en paringsgedrag onderzocht van twee belangrijke plaaginsecten, de johannesbroodboommot, *Ectomyelois ceratoniae* (Zeller) (Lepidoptera: Pyralidae), en de tabaksmot *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae), met als doel om de eerste stappen te zetten in de ontwikkeling van nieuwe, robuuste gedragsmanipulatie-methoden.

De johannesbroodboommot is wereldwijd een verwoestende plaag in hoogwaardige gewassen, zoals dadels, amandelen, granaatappels en pistachenoten. In Iran en andere delen van het Midden-Oosten, is de johannesbroodboom-mot de belangrijkste plaag op granaatappel *Punica granatum* L. (Lythraceae). Helaas ontbreekt nog steeds een efficiënte, op het vrouwlijke seksferomoon gebaseerde, lokstof, omdat de hoofdcomponent van het synthetische seksferomoon instabiel is. Vluchtige stoffen afkomstig van waardplanten zijn aantrekkelijk voor zowel mannetjes als vrouwtjes van herbivore insecten en staan bekend om hun synergistische effect met seksferomonen. Dit maakt dergelijke stoffen potentieel zeer interessant om toegepast te worden tegen plaakinsecten. Om te bepalen hoe waardplantgeuren ingezet kunnen worden in geïntegreerde bestrijdingsprogramma’s, hebben we de interacties tussen granaatappels en johannesbroodboomotten onderzocht door middel van veldexperimenten in granaatappelboomgaarden en in windtunnel experimenten.

Granaatappelboomgaarden in het Midden-Oosten zijn over het algemeen een mengvorm van verschillende cultivars, die verschillen in hun gevoeligheid voor aantasting door johannesbroodboomotten. In Hoofdstuk 2 beschrijf ik hoe we de belangrijkste factoren hebben bepaald die bijdragen tot de gevoeligheid van granaatappels voor deze motten, door de progressie van de aantasting te meten gedurende twee veldseizoenen in 10 granaatappel-cultivars. Over het geheel genomen was de mate van aantasting in granaatappels sterk gecorreleerd met de gevoeligheid van het openbreken van granaatappels, zodanig dat opengebroken vruchten en cultivars met vruchten die gemakkelijk openbreken het meest waren aangetast. Echter, dit patroon veranderde gedurende het seizoen. Vroeg in het seizoen vonden we vooral aantasting in cultivars met vruchten die gemakkelijk breken, maar tegen het einde van het seizoen verhoogde de aantasting in ongebroken vruchten en in cultivars die resistent zijn tegen het openbreken van vruchten, waarschijnlijk doordat ongebroken vruchten betere plaatsen zijn voor de rupsen om in te overwinteren.

In Hoofdstuk 3 beschrijf ik hoe we door middel van experimenten met plakvallen in granaatappel-boomgaarden hebben bepaald welke geurbronnen het meest aantrekkelijk zijn voor johannesbroodboommotten. Het aantal mannetjes en maagdelijke en gepaarde vrouwtjes dat in vallen gevangen werd was significant hoger in vallen met rijpe granaatappels, waarbij het niet uitmaakte of ze ongebroken of gebroken waren en of ze al dan niet aangetast waren, dan in vallen zonder granaatappels. In vallen met geurextract van gebroken granaatappels vingen we alleen gepaarde vrouwtjes en in vallen met rijpe pistachenoten vingen we alleen mannetjes. In vallen met granaatappel-bloemen, onrijpe granaatappels en geurextracten van pistachenoten, vingen we helemaal geen motten. Bovendien werden gepaarde vrouwtjes meer gevangen in vallen met gebroken vruchten dan in vallen met hele vruchten. Tenslotte werden vergelijkbare hoeveelheden mannetjes gevangen in vallen met gebroken vruchten die aangetast waren als in vallen met alleen maagdelijke vrouwtjes. Interessant genoeg was de combinatie van gebroken granaatappels met een maagdelijk vrouwtje het meest aantrekkelijk voor maagdelijke vrouwtjes, en vingen we in deze vallen in totaal ook het meeste aantal motten.

In Hoofdstuk 4 beschrijf ik hoe we de geurstoffen uit de plantenbronnen, die we als aantrekkelijk hadden gevonden in hoofdstuk 3, geïdentificeerd hebben. Door middel van gaschromatografie-electroantennografie (GC-EAD) en GC-massaspectrometrie hebben we  $\beta$ -caryofyleen als de geurstof uit de headspace-(geurbouquet)-extracten van granaatappels en pistachenoten geïdentificeerd, die de sterkste antennale respons gaf in de johannesbroodboommotenvrouwtjes. We vonden ook significante variatie in de relatieve hoeveelheid van deze stof tussen de verschillende plantendelen die we hebben getest. In de windtunnel, waarin mannetjes, maagdelijke en gepaarde vrouwtjes vergelijkbaar reageerden op de plantenvruchten, waren de reacties negatief gecorreleerd met de hoeveelheid  $\beta$ -caryofyleen in de headspace-extracten. Deze resultaten suggereren dat lage hoeveelheden  $\beta$ -caryofyleen de aantrekkelijkheid van johannesbroodboommotten verhoogt, terwijl hoge hoeveelheden juist niet aantrekkelijk zijn.

Samengenomen laten onze resultaten zien dat breuken granaatappels gevoelig maken voor aantasting door de johannesbroodboommot, dat gebroken vruchten de meest aantrekkelijke bron zijn voor gepaarde vrouwtjesmotten en dat  $\beta$ -caryofyleen betrokken is bij het aantrekken van johannesbroodboom-motten in granaatappelboomgaarden. Onze resultaten laten ook zien dat geurstoffen van granaatappels alleen of in combinatie met het vrouwelijke seksferomoon de moeite waard zijn om ingezet te worden tegen johannesbroodboommotten. Plakvallen met stukken granaatappels kunnen eenvoudig gebruikt worden voor het monitoren van de plaagpopulaties. Deze methode is veel makkelijker, goedkoper en effectiever dan de huidige, commerciële seksferomoonvallen.

Naast de vrouwelijke seksferomonen, die mannetjes motten van een afstand aantrekken, produceren mannetjes motten ook seksferomonen; deze feromonen beïnvloeden op korte afstand het gedrag van zowel mannetjes (remt de paringsbereidheid van conspecifieke mannetjes) als vrouwtjes (werkt als een afrodisiacum).. In Hoofdstuk 5 beschrijf ik hoe we de hypothese getest hebben dat het mannelijk seksferomoon van de tabaksmot de vrouwtjes parfumeert en als anti-afrodisiacum functioneert. We vonden inderdaad dat maagdelijke vrouwtjes, die we geparfumeerd hadden met mannelijk feromoonextract of met het hoofdbestanddeel van het mannelijk feromoon, significant minder paarden dan controle vrouwtjes, en dat dit effect twee opeenvolgende nachten aanhield. Deze vorm van chemische bewaking van een paringspartner was ongunstig voor de vrouwtjes, omdat het aantal nakomelingen van vrouwtjes, die tweemaal gepaard hadden, significant hoger was dan die van

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vrouwtjes, die slechts eenmaal hadden gepaard. Ten slotte beschrijf ik in hoofdstuk 6 alle bevindingen in de context van hun bruikbaarheid in toekomstige geïntegreerde bestrijdingsprogramma's die tot doel hebben om johannesbroodboom-mottenplagen en tabaksmottenplagen onder de duim te houden.

## Author contributions

**Chapter 2.** Seasonal pattern of infestation by the carob moth (*Ectomyelois ceratoniae*) in pomegranate cultivars

**Seyed Ali Hosseini, Seyed Hossein Goldansaz, Seyedeh Masoumeh Fotoukkaaii, Steph B.J. Menken, and Astrid T. Groot**

SAH, SHG, and SMF designed and performed the experiments. SAH and ATG analyzed the data and drafted the manuscript. SHG and ATG supervised the project. All authors wrote the manuscript.

**Chapter 3.** Field attraction of carob moth to host plants and conspecific females

**Seyed Ali Hosseini, Seyed Hossein Goldansaz, Steph BJ Menken, Michiel van Wijk, Peter Roessingh, and Astrid T Groot**

SAH and SHG designed and performed the experiments. SAH, ATG, MvW, and SBJM analyzed the data and drafted the manuscript. SHG and ATG supervised the project. All authors wrote the manuscript.

**Chapter 4.** Electrophysiological and behavioral responses of the carob moth, *Ectomyelois ceratoniae*, to pomegranate and pistachio

**Seyed Ali Hosseini, Seyed Hossein Goldansaz, Sybille Lorenz, Steph B.J. Menken, Astrid T. Groot\*, and Peter Roessingh\***

SAH, SHG, PR and ATG designed and performed the experiments. SAH, ATG and PR analyzed the data and drafted the manuscript. SHG, PR and ATG supervised the project. SL conducted gas chromatography-mass spectrometry analysis. All authors wrote the manuscript.

\* Contributed equally to this study.

**Chapter 5.** Experimental evidence for chemical mate guarding in a moth

**Seyed Ali Hosseini, Michiel van Wijk, Gao Ke, Seyed Hossein Goldansaz, Coby Schal, Astrid T. Groot**

SAH and ATG designed the experiments; SAH, GK and MvW performed the experiments; MvW, CS and ATG supervised the experiments and helped with data analyses; all authors wrote the manuscript.

## **Author addresses**

**Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, 1090 GE Amsterdam, the Netherlands.**

Seyedeh Masoumeh Fotoukiai, Astrid T. Groot, Seyed Ali Hosseini, Gao Ke, Steph B.J. Menken, Peter Roessingh, and Michiel van Wijk

**Department of Plant Protection, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran.**

Seyed Hossein Goldansaz and Seyed Ali Hosseini

**Max Planck Institute for Chemical Ecology, Dept. Entomology, Hans Knoell Strasse 8, 07745 Jena.**

Astrid T. Groot

**North Carolina State University, Department of Entomology and Plant Pathology, Raleigh NC, USA**

Astrid T. Groot, Coby Schal, Michiel van Wijk

**Max Planck Institute for Chemical Ecology, Research Group Mass Spectrometry/Proteomics, Hans Knoell Strasse 8, 07745 Jena.**

Sybille Lorenz



## Biography

Seyed Ali Hosseini was born on 24 February 1986, in Abhar, Iran. He obtained his high school diploma in 2003, and after passing an entrance exam, he started a Bachelor's of Agricultural Engineering-Plant Protection degree at the University of Zanjan, Iran, in the same year. In 2007, he started his Masters studies in Agricultural Entomology at Urmia University, Iran, and graduated in 2010. In 2011, Seyed Ali got the first rank of the PhD entrance exam of Iran in Entomology and started his PhD project at the Department of Plant Protection of the University of Tehran under supervision of Dr. Seyed Hossein Goldansaz. In 2015, he was transferred to the Institute for Biodiversity and Ecosystem Dynamics of the University of Amsterdam and continued his PhD project under supervision of Dr. Astrid Groot and Prof. Steph Menken.

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