University of Alberta

Development of a pheromone-based attract and kill formulation with visual cues to target the diurnally active apple clearwing moth, *Synanthedon myopaeformis* (Borkhausen), (Lepidoptera: Sesiidae)

by

Jessica Jen Kwon

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Dedication

This thesis is dedicated to my loving parents, O-Ki and Nok Young Kwon, and partner, Jeremy Katulka. I am overwhelmed with gratitude for your love and tenacious support through these past few years. Mom and Dad, thank you for providing me the opportunity to pursue entomology. Jeremy, thank you for teaching me to be a skeptic.

Abstract

The apple clearwing moth, *Synanthedon myopaeformis* (Borkhausen), (Lepidoptera: Sesiidae) is a serious pest of apple trees in British Columbia (BC) in Canada. Due to its recent introduction to BC and the lack of natural predators, the development of an attract and kill formulation using SPLAT® to control this pest in BC was initiated. SPLAT droplet shape and pheromone dose affected male moth attraction to traps; SPLAT droplets molded into hemispheres baited with 1- or 10 mg of sex pheromone were the most attractive. The addition of codlemone, the sex pheromone of a sympatric species, the codling moth (*Cydia pomonella,* L.) (Lepidoptera: Tortricidae), and the pyrethroid insecticide, cypermethrin, did not affect attraction of male apple clearwing moths to sex pheromone released from SPLAT. The use of visual cues characteristic of female apple clearwing moths enhanced male moth close-range orientation to pheromone-baited SPLAT and increased the number of contacts made to droplets.

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1. Introduction

1.1 Chemical communication in moths

Many organisms use chemical signals to convey information via semiochemical communication (Birch & Haynes, 1982). Semiochemical communication is widely used by moths and butterflies (Hansson, 1995). Semiochemicals can be divided into two broad categories - pheromones that act intraspecifically (Karlson & Lüscher, 1959) and allelochemicals that mediate communication between individuals in different species (Whittaker & Feeny, 1971). Pheromones can induce immediate behavioral changes or initiate developmental processes in the receiver of the signal (Karlson & Lüscher, 1959; Howse, 1998). Insects often rely on these chemical signals that can evoke a behavioral response alone (Birch & Haynes, 1982) or in conjunction with other signals. Allelochemicals are characterized according to which participant in the communication channel (sender or receiver) benefits from the interspecific communication. Chemical communication with allomones benefits the sender, whereas kairomones mediate interspecific communication that benefits the receiver, and synomones provide communication that is advantageous to both parties (Brown et al., 1970; Birch & Haynes, 1982; Howse, 1998).

Sex pheromone-mediated communication between individuals of the same species in the Lepidoptera has been extensively studied (Butenandt et al., 1959; Roelofs & Brown, 1982; Baker, 1989; Hansson, 1995; Roelofs et al., 2002). Moths and butterflies depend on the detection of sex pheromones for mate location and often, the initiation of courtship and copulatory behaviors (Birch &

Haynes, 1982; Baker 1989). In most moths, females are the signalers and they release volatile sex pheromone from a subcuticular gland located at the tip of the abdomen (Schneider, 1992); most basal Lepidoptera and Trichoptera release sex pheromone from sternum V glands (Djernaes, 2013). The highly specific pheromone signal is detected by sensory receptors on the male moth antennae and evokes long-distance upwind-oriented flight by the male responder (Schneider, 1992; Hansson, 1995).

In many moths, adults and larvae are attracted to kairomones emitted by their host plants. While host-plant volatiles can act as feeding attractants for larvae that aid in host location and host recognition (Knight & Light, 2001; Hughes et al., 2003), they can also act as attractants for adult females to find and select suitable oviposition sites (Hughes et al., 2003; Witzgall et al., 2005). As kairomones are known to stimulate mating and oviposition in some moth females, adult males may utilize these compounds to access potential mates (Landolt & Phillips, 1997; Bengtsson et al., 2006; Estrada & Gilbert, 2010; von Arx et al., 2011). Host-plant volatiles can also enhance the attraction of males to sex pheromone (Yang et al., 2004; Li et al., 2012).

1.2 Semiochemical-based management of lepidopteran pests

The reliance of moths on chemical communication for mate and host location makes chemical communication a good target for pest management. Semiochemical-based monitoring (Gu et al., 2013; Williams et al., 2013) and control tactics (Evenden & McClaughlin, 2005; Wins-Purdy et al., 2007;

Kamarudin et al., 2010) have been developed as pest management strategies against moths in a variety of agroecosystems. Monitoring tools such as pheromone- or kairomone-baited traps (McBrien & Judd, 1998; Jones et al., 2009; Miluch et al., 2013) can be used to detect the presence of pests and to establish thresholds to time control treatment applications (Howse, 1998; Gut et al., 2004). Other successful uses of semiochemical-based monitoring include evaluation of the effectiveness of mating disruption (Joshi et al., 2011; Knight & Light, 2012) and assessment of insecticide resistance levels (Bush & Rock, 1993; Shearer & Riedl, 1994) in target pests (Gut et al., 2004).

Semiochemical-based direct control tactics include mating disruption, mass trapping and attract and kill. Mating disruption is a pre-emptive strategy that delays or prevents successful mating through interference with pheromone-based communication and mate-finding behavior (Byers, 2007). The mechanisms by which pheromone-based mating disruption is hypothesized to work include desensitization or confusion, camouflage and false-plume following (Bartell, 1982). The constant exposure to high concentration of pheromone causes adaptation of the antennal receptors in the male and/or habituation of the central nervous system, which can inhibit responses of male moths to natural pheromone sources (Birch & Haynes, 1982; Howse, 1998; Judd et al., 2005). Camouflage is thought to occur when pheromone plumes emitted by calling females become indistinguishable from the synthetic plumes released by dispensers, which renders the male unable to locate wild females (Byers, 2007). False-plume following occurs when the point sources of pheromone outcompete calling females, which results in males that seek out dispensers and reduces mating encounters with female moths (Stelinski et al., 2004). These effects can be classified into non-competitive (desensitization and camouflage) and competitive attraction (false-plume following) mechanisms (Miller et al., 2006). If competitive attraction is the major mating disruption mechanism in a pheromone-treated crop, there is a reduction in the number of male moths that encounter calling females because males orient to attractive pheromone plumes emitted from dispensers (Miller et al., 2006) instead of to females.

Mass trapping is a tactic that directly removes insects from the population through capture in pheromone- or kairomone-baited traps positioned in a cropping system (El-Sayed et al., 2006). Mass trapping is a semiochemical-based approach used in pest management and has been used against other orders of pests, such as Coleoptera (Alpizar et al., 2012; Torres-Vila et al., 2012), Diptera (Noce et al., 2009; Ben Jemaa et al., 2010) and Hemiptera (Branco et al., 2004; Kim, 2012) in agriculture, orchards and forestry (El-Sayed et al., 2006). For

mass trapping to be effective, traps must be baited with highly attractive lures and traps must retain the attracted insects (El-Sayed et al., 2006). Mass trapping of lepidopteran pests with pheromone-baited traps has not been widely adopted because only male moths are removed from the population (Jones, 1998).

Attract and kill is a technique that involves a lure and an affector (El-Sayed et al., 2009). Attract and kill is similar in concept to mass trapping because both methods lure and remove individuals from a population; however, attract and kill removes insects via exposure to a lethal point source, rather than retention in a trap. Attract and kill and mass trapping for moth pests can be advantageous over mating disruption because the target pests are eliminated from the population and cannot mate once sexual communication is uninterrupted. The attractive lure may consist of olfactory cues, such as sex pheromone, visual cues, acoustic cues or a combination of these signals. The attracted target insect is killed by direct contact with the affector via insecticidal poisoning, or indirectly by sterilants or pathogens (El-Sayed et al., 2009). Many commercially available attract and kill formulations for lepidopteran pests incorporate sex pheromone as the lure and pyrethroid insecticides as the affector (Suckling & Brockerhoff, 1999; Charmillot et al., 2000; Evenden & McLaughlin, 2004; Nansen & Phillips, 2004). Moth suppression has been successful in cropping systems that target the citrus leafminer (*Phyllocnistis citrella,* Stainton) (Lepidoptera: Gracillariidae) in Florida citrus production (Stelinski & Czokajlo, 2010), the codling moth (*Cydia pomonella*, L.) (Lepidoptera: Tortricidae) in apple orchards in Syria (Mansour, 2010) and the light brown apple moth (*Epiphyas postvittana*, Walker) (Lepidoptera: Tortricidae) in New Zealand apple orchards (Suckling & Brockerhoff, 1999).

High initial pest populations influence the effectiveness of attract and kill but this tactic remains more cost-effective than mating disruption, since small amounts of pheromone are required. Attract and kill formulations have smaller amounts of insecticide than conventional pesticide sprays which eliminates insecticide residue on crops and the potential for adverse effects on natural predators. Competitive attraction through false-plume following is exploited by attract and kill formulations (Suckling & Brockerhoff, 1999). The success of the attract and kill tactic requires that: (1) the formulation is highly attractive and promotes source contact with the lure by the target insect; (2) the killing agent is in close association with the lure to promote exposure to the affector; (3) the insect acquires a sufficient dose of the affector from the point source; and (4) the mortality or behavior-modifying effects of the formulation significantly reduce the pest population (El-Sayed et al., 2009). As sex pheromones are species-specific signals, attract and kill using sex pheromones provides an environmentally-sound approach to manage target insect pests with minimal insecticidal use (Howse, 1998; Gut et al., 2004).

The deployment of attract and kill formulations varies for pests in different families of insects. Attract and kill or male annihilation techniques that target dipteran pests typically include the use of a food bait or feeding stimulant in conjunction with a killing agent in a bait station. Attractants for the Oriental fruit fly (*Bactrocera dorsalis*, Hendel) (Diptera: Tephritidae) and the melon fly (*B. cucurbitae*, Coquillett) (Diptera: Tephritidae), methyl eugenol and cue-lure, respectively, are used in conjunction with spinosad (Vargas et al., 2008), naled (Vargas et al., 2003), fipronil (Vargas et al., 2005) or other toxicants to provide control of tephritids in Hawaii. These formulations are presented in bucket traps on cotton wicks (Vargas et al., 2000), impregnated into molded paper fiber blocks (Vargas et al., 2005), or released from wax droplets dispensed in the field (Vargas et al., 2008). Attract and kill devices tested against the Mediterranean fruit fly

(*Ceratitis capitata*, Wiedemann) (Diptera: Tephritidae) in citrus plants in Spain include Magnet® MED, a paper envelope impregnated with an insecticide that contains two membranes that each dispense the attractants, trimethylamine and ammonium acetate, and a yellow-colored cylinder prototype that contains a protein bait with cypermethrin as the killing agent (Navarro-Llopis et al., 2013). Attractive visual cues and fruit mimics are added to attract and kill for control of pestiferous flies, especially to target females (Piñero et al., 2009). Fruit mimics are used to control the apple maggot fly (*Rhagoletis pomonella*, Walsh) (Diptera: Tephritidae) by attract and kill in eastern North America (Prokopy et al., 1990).

The use of aggregation pheromones is common in attract and kill systems to control coleopteran pests of both sexes. *Carpophilus* spp. beetles (Coleoptera: Nitidulidae) are serious pests of ripening stone fruits in Australia (James et al., 1997). Attract and kill stations consist of a polystyrene box filled with ripening peaches and peach nectar absorbed into polyacrylamide granules as the co-attractants, a rubber septum baited with aggregation pheromones of the targeted species as the main attractant, and a fipronil spray over the peaches as the killing agent (Hossain et al., 2006). For forest pests, the use of trap trees, trees baited with aggregation pheromone and sprayed with insecticide, have been tested against many bark beetles including *Ips typographus* (L.) (Coleoptera: Curculionidae) in Norway spruce trees (Dedek et al., 1988), *Scolytus multistriatus* (Marsham) (Coleoptera: Curculionidae) in American elm (Lanier & Jones, 1985) and *Dendroctonus brevicomis* (LeConte) (Coleoptera: Curculionidae) in ponderosa pine (Hall et al., 1982). The attracted beetles are killed by insecticidal poisoning and fail to colonize treated trees.

Many attract and kill formulations that target lepidopteran pests incorporate sex pheromones as the lure and a pyrethroid insecticide as the killing agent. Commercially available products for attract and kill present the lure and the affector in an inert matrix such as LastCall™OFM and LastCall™CM (IPM Tech Inc., Portland, OR) that target the Oriental fruit moth (*Grapholia molesta*, Busck) (Lepidoptera: Tortricidae) and the codling moth, respectively (Evenden & McClaughlin, 2005) and MalEx (Alpha Scents, Bridgeport, NY) for control of the citrus leafminer (Stelinski & Czokajlo, 2010). As attract and kill gains popularity due to its environmentally- and economically-sound approach to control crop pests, more experimental products are formulated and tested. Wax or paste-based products include Mesaj CP, a product developed by the Raluca Ripan Chemical Research Institute of Romania to control codling moth (Somsai et al., 2011) and SPLAT® Attract and Kill Grafo+Bona (ISCA Technologies Inc., Riverside, CA) (Pastori et al., 2012), a product tested for the simultaneous control of the Oriental fruit moth and the Brazilian apple leafroller (*Bonagota salubricola*, Meyrick) (Lepidoptera: Tortricidae). Other techniques include sprayable formulations of microencapsulated insecticide with sex pheromones (Kovanci et al., 2011), wax panels that contain insecticide and release sex pheromone from a lure placed in the center of the panel (Campos & Phillips, 2013), and plastic mesh cylinders sprayed with insecticide with a pheromone-baited lure placed inside the cylinder (Campos & Phillips, 2013). The addition of multiple species' pheromones

(Evenden & McClaughlin, 2005), other semiochemicals such as host volatiles and feeding attractants (Knight et al., 2002; Potting & Knight, 2002) and visual components (Piñero et al., 2009) to current attract and kill formulations can improve pest suppression.

SPLAT® (Specialized Pheromone & Lure Application Technology) (ISCA Technologies, Inc., Riverside, CA) is an inert wax matrix used for controlled release of semiochemicals and/or pesticides (ISCA Technologies Inc., 2012). This product is biodegradable, protects the active ingredients from UV exposure and weathering and is easily manipulated to control the release rate of the active ingredients by adjustment of droplet size (ISCA Technologies, 2012). The flowable product allows for a variety of application methods that include hand-applications with spatulas and caulking guns to mechanized applications with backpack sprayers, tractors and aircraft (Stelinski et al., 2010; Teixeira et al., 2010; ISCA Technologies Inc., 2012). Though most commercial products of SPLAT release only semiochemicals for mating disruption (Jenkins & Isaacs, 2008; Teixeira et al., 2010), there is potential for attract and kill technology with SPLAT due to the possibility of the incorporation of insecticide to the matrix (Vargas et al., 2010; ISCA Technologies Inc., 2012; Pastori et al., 2012). The possibility to include other semiochemical attractants, such as host volatiles, and visual cues, by the addition of color to the matrix, makes SPLAT more advantageous over other commercially available attract and kill products that target lepidopteran pests.

1.3 Attract and kill formulations with multiple attractants

Long-distance orientation in most male moths is achieved by the detection of sex pheromones with highly sensitive pheromone sensory neurons (Löfstedt & Kozlov, 1997). Host-plant volatiles can act as attractants for female moths for orientation to plants where mating and oviposition occur (Cha et al., 2008; Beck et al., 2012); male moths may also utilize plant-produced olfactory cues to orient to hosts used as rendezvous sites (von Arx et al., 2011; Beck et al., 2012; Lu et al., 2012). Detection of host-plant volatiles can synergize the response of male moths to sex pheromones (Yang et al., 2004; Li et al., 2012; von Arx et al., 2012).

The attractiveness of attract and kill formulations needs to be optimized as the effectiveness of the formulation relies on the ability of the target insect to orient to and touch the formulation. The duration of contact must be adequate for the insect to acquire enough of the affector to experience the adverse effects of the killing agent (El-Sayed et al., 2009). To improve the efficacy of attract and kill formulations, the simultaneous release of plant volatiles and sex pheromones can enhance male moth response to the formulation and can also attract female moths. The potential for kairomone-based attract and kill formulations against the alfalfa looper (*Autographa californica*, Speyer) (Lepidoptera: Noctuidae) (Camelo et al., 2007) and the cotton bollworm (*Helicoverpa armigera*, Hübner) (Lepidoptera: Noctuidae) (Del Socorro et al., 2010), have been examined. The inclusion of plant volatiles to attract and kill formulations that target codling moth has been modeled; the model demonstrates that the addition of pear ester, the host volatile, can improve the effectiveness of attract and kill in cropping systems through the control of both sexes simultaneously (Knight et al., 2002).

Host-plant volatiles can also act as feeding attractants to both adult male and female moths. Food baits have been widely tested as attractants for noctuid moths (Landolt & Higbee, 2002; Landolt et al., 2007a; Tóth et al., 2010; Landolt et al., 2011) but also for codling moth (Landolt et al., 2007b) and the apple clearwing moth (*Synanthedon myopaeformis,* Borkhausen) (Lepidoptera: Sesiidae) (Aurelian et al., 2012; Eby et al., 2013), which are both serious pests of apple trees in BC. Nectar-feeding noctuid moths are attracted to phenylacetaldehyde, a floral cue used to find suitable nectar sources (Landolt & Higbee, 2002; Landolt et al., 2007a; Tóth et al., 2010; Landolt et al., 2011). Similarly, phenylacetaldehyde attracts nectar-feeding apple clearwing moths (Eby et al., 2013). Alcohols that occur in fermenting molasses, such as isoamyl alcohol in combination with acetic acid are highly attractive to noctuid moths (Landolt & Higbee, 2002; Landolt et al., 2007a; Tóth et al., 2010; Landolt et al., 2011); the latter also acts as a sugar cue for codling moth, even though adult feeding on ripened fruit has not been documented (Landolt et al., 2007b). Pear ester and grape juice release volatiles that are attractive to codling moths and apple clearwing moths, respectively (Landolt et al., 2007b; Aurelian et al., 2012).

Attract and kill formulations can also be enhanced by the combination of multimodal cues to lure the target pest. This approach may be most effective against diurnally active pests because these groups rely less on olfaction for mate finding and utilize visual cues for close-range orientation (Barry & Nielsen, 1984;

Koshio & Hidaka, 1995; Karalius & Būda, 2007; Toshova et al., 2007; KonDo et al., 2012; Monteys et al., 2012). In diurnal moths, mate searching is initiated at a distance through the detection of female-produced sex pheromone but visual cues are often used for close-range mate recognition and the initiation of courtship and copulatory behaviors (Barry & Nielsen, 1984; Karalius & Būda, 2007; Toshova et al., 2007; KonDo et al., 2012). Visual cues, such as UV (Robertson & Monteiro, 2005) and visible wavelength reflectance (Karalius & Būda, 2007), object shape (Gross et al., 1983) and size (Pinzari & Sbordini, 2013), when combined with semiochemicals, can enhance the orientation of male lepidopterans (Raguso & Willis, 2005; Reddy et al., 2009). Trap color is a visual cue that has been manipulated to optimize moth capture in semiochemical-baited traps (Hendricks & Calcote, 1991; Athanassiou et al., 2007; Knight, 2010). Trap color is particularly important for the optimization of trap capture of diurnal moths in the Sesiidae (Childers et al., 1979; Trematerra, 1993; Suckling, 2005; Roubos & Liburd, 2008; Judd & Eby, 2013). Although the inclusion of visual cues, such as color, has not been tested in attract and kill systems that target diurnal moths, it is important in attract and kill for dipteran pests (Prokopy et al., 1990; Piñero et al., 2009). Fruit mimics and colors associated with host plants are often incorporated in attract and kill technologies against tephritid flies (Prokopy et al., 1990), particularly when female flies are targeted (Piñero et al., 2009). The attractiveness of attract and kill formulations can be enhanced by the combination of olfactory cues such as sex pheromones, host-plant volatiles and feeding attractants, and visual cues that are used for close-range recognition of mates and initiation of courtship and copulatory behaviors in diurnally active moths. Visual cues that mimic female peachtree borers (*Synanthedon exitiosa,* Say) (Lepidoptera: Sesiidae) increase male attraction to their sex pheromone and initiate persistent copulatory behaviors similar to those of the apple clearwing moth (Barry, 1978). Trap color also has a significant effect on the attraction of male peachtree borers to pheromone sources (Childers et al., 1979). Apple clearwing moth males can spectrally discriminate between differently colored traps (Judd & Eby, 2013). The exploitation of this close-range behavior to visual cues in attract and kill strategies for diurnal moths may increase the exposure of the target insect to the insecticide and ensure adequate dosing of the toxicant.

1.4 Life history of the apple clearwing moth, *Synanthedon myopaeformis* (Borkhausen)

The apple clearwing moth, *Synanthedon myopaeformis* (Borkhausen), (Lepidoptera: Sesiidae), is a serious pest of apple trees in the Similkameen Valley of BC (Judd et al., 2011). This invasive pest is a native of Eurasia (Zhang, 1994) and Africa (Ateyyat & Al-Antary, 2006). It was first discovered in Canada in an apple orchard in Cawston, BC in 2005 (Philip, 2006) most likely introduced by infested rootstocks. The apple clearwing moth was identified, soon after, in Ontario (Beaton & Carter, 2006). This moth has since spread to the northern regions of the Okanagan Valley in Kelowna, BC, and has been reported as far south as Washington State, USA (LaGasa et al., 2009). Spread of the apple clearwing moth still occurs in Europe, where Denmark confirmed its presence in 2005 (Aachmann-Andersen & Kofoed Nielsen, 2006).

The apple clearwing moth has a 2-year life cycle in interior BC (Judd, 2008), but may be univoltine and even bivoltine in warmer regions of the world (Dickler, 1976; Al-Antary & Ateyyat, 2006). Their common host plant is apple (*Malus* spp.) in the family Rosaceae (Spatenka et al., 1999) though some varieties, such as Mutsu and Marigold in Germany (Dickler, 1976) and Golden Delicious, Delicious and Rennet in Italy (Trematerra, 1993), are more vulnerable to attack than other apple varieties. Other hosts include pears (*Pyrus* spp.), mountain ash (*Sorbus* sp.), hawthorn (*Crataegus* spp.), *Prunus* spp., cherry (*Cerasus* spp.), *Padus* spp. and the common medlar (*Mespilus germanica*) in the Rosaceae family, and the common sea-buckthorn (*Hippophae rhamnoides)* (Elaeagnaceae) (Spatenka et al., 1999).

Female apple clearwing moth lay small, ovoid, brown eggs (Figure 1.1) singly in bark crevices or at injury sites, and burr knots of apple trees (Stüber & Dickler, 1988). Burr knots are common among rootstocks and are caused by the formation of adventitious root primordial. The rootstocks attempt to produce roots above the ground that creates entry points for insect attack and sites for disease infection (Swingle, 1925). Egg hatch occurs between nine and fourteen days after oviposition (Stüber & Dickler, 1988). Early instar larvae feed within the bark and move to the vascular tissues between the bark and cambium as they grow. Larvae are slightly flattened dorsoventrally with a reddish-brown head capsule (Alford, 2007) (Figure 1.2). The larvae go through seven larval instars in ~20 months and generate irregular feeding galleries (Alford, 2007) within the cambium as older larvae. There is no obligate diapause and instars three to seven typically overwinter (Stüber & Dickler, 1988). The developmental threshold is thought to be 10°C (Judd, 2008). In May, seventh instar larvae construct a silken cocoon made of frass and bark fragments in preparation for pupation (Spatenka et al., 1999).

The apple clearwing moth pupates for about one month before the adults eclose in early June. Pupae are light brown with rows of chitinous spines (Figure 1.3). Adults emerge in early June and leave their pupal exuvia on the tree bark (Figure 1.4). Males emerge slightly earlier than females (Stüber & Dickler, 1988; Judd, 2008). Adult apple clearwing moths have characteristic black antennae, a white border around the compound eyes, white labial palps, transparent wings, and a black-blue body with an orange tergite on the fourth abdominal segment (Spatenka et al., 1999; Lastuvka & Lastuvka, 2001; Alford, 2007) (Figure 1.5). The females are slightly larger and wider and have an orange sternite on the fourth segment of the abdomen (Spatenka et al., 1999). Males are white between sternites four and six (Spatenka et al., 1999). Both sexes of moths have a fan-shaped tuft of scales on the last abdominal segment (Lastuvka & Lastuvka, 2001). Peak adult activity occurs in mid-July (Judd, 2008) between 1200-1430 h (Judd & Eby, 2013).

A sex attractant for the apple clearwing moth was identified as (3*Z*,13*Z*)-octadecadienyl acetate ((3*Z*,13*Z*)-18:OAc) (Voerman et al., 1978) and confirmed as the main sex pheromone component released by adult female moths (Judd et al., 2011). This pheromone is used to monitor pest populations of the apple clearwing moth (Judd, 2008) and other sesiid borers such as the Japanese persimmon tree borer (*Synanthedon tenuis,* Butler) (Naka et al., 2013), the lilac borer (*Podosesia syringae,* Harris) (Nielsen & Purrington, 1978), the peachtree borer (Tumlinson et al., 1974) and the dogwood borer (*Synanthedon scitula,* Harris) (Nielsen et al., 1975). Detection of the sex pheromone by male apple clearwing moths initiates long-distance orientation to calling females (Stüber & Dickler, 1987) with the highest response to sex pheromone between 1200 and 1430 h (Judd & Eby, 2013). Once in the vicinity of the female, the male moth hovers directly above her and attacks the orange tergite with the terminal end of his abdomen and tries to reach the ovipositor with his extended valvae (Stüber & Dickler, 1987). Males repeat this behavior until the ovipositor is successfully clasped and copulation takes place (Stüber & Dickler, 1987). Copulation occurs for up to 1.5 h with the male turned 180° away from the female (Stüber & Dickler, 1987) (Figure 1.6). Similar mating behavior occurs in the peachtree borer (Barry, 1978) and the lilac borer (Nielsen et al., 1975).

1.5 Pest status of the apple clearwing moth

The apple clearwing moth was originally labeled as a pest of secondary importance, but the cultural practice of grafting dwarf rootstocks to apple trees has enabled this moth to reach serious pest status around the world (Trematerra, 1993; Balázs et al., 1995; Ateyyat & Al-Antary, 2006; Kutinkova et al., 2006). The introduction of lesions and burr knots near graft unions and pruning cuts create preferred sites for oviposition and ideal developmental sites for their cambium-feeding larvae (Dickler, 1976). Although larval feeding does not directly damage the apple fruit, a reduction in apple yield occurs in heavily infested trees which results in serious economic loss (Dickler, 1976; Judd, 2008). Other important pests that belong to this family include the peach tree borer (Smith, 1951; Shapiro-Ilan et al., 2009), the raspberry crown borer (*Pennisetia marginata,* Harris) (McKern et al., 2007), the lilac borer (Appleby, 1973; Aurelian et al., 2008) and the dogwood borer (Bergh & Leskey, 2003).

The larval stage of the apple clearwing moth is well protected under the bark, which makes direct control of the feeding larvae difficult. The use of *Metarhizium brunneum* (Petch) and *Beauvaria bassiana* (Balsamo) Vuillemin in biological control (Cossentine et al., 2010) for North America populations is still being formulated and tested. Tree trunk coatings of insecticidal paint, cheesecloth wrappings around the trunk and the use of wire have also been used to control larvae by insecticidal poisoning, prevention of oviposition on burr knots, and mechanical removal of larvae, respectively (Ciglar & Masten, 1977; Ateyyat & Al-Antary, 2006; Erler, 2010). Various semiochemical-based control strategies that target adults, such as mass trapping (Trematerra, 1993; Bosch et al., 2001; Aurelian et al., 2012) and mating disruption (Stüber & Dickler, 1987; Kyparissoudas & Tsourgianni, 1993), have been tested. Mass trapping of apple clearwing moth males with sex pheromone produced low levels of pest control in Italy over two years (Trematerra, 1993). Male trap capture was reduced by 58-65% in two orchards, which does not reach the trapping efficiency of 80-95%

proposed by Knipling and McGuire (1966). Apple orchards in Spain produced inconsistent results in two orchards over three years where male trap capture was reduced by 93.6% in a heavily infested orchard but only 51.9% where initial pest populations were low (Bosch et al., 2001). The use of grape juice baits for mass trapping both sexes of apple clearwing moths effectively caught high numbers of male and female moths (Aurelian et al., 2012). Due to the labor-intensive maintenance involved with juice traps, the identification of attractive volatiles from grape juice for use in mass trapping should be considered. Phenylacetaldehyde, a volatile released by showy milkweed, *Asclepias speciosa* (Torrey), (Apocynaceae), a common nectar source for apple clearwing moth adults, is also attractive to both sexes of apple clearwing moths and has the potential to be used as a bait in mass trapping or monitoring (Eby et al., 2013). Mating disruption efforts in apple orchards in Greece (Kyparissoudas & Tsourgianni, 1993) and Germany (Stüber & Dickler, 1987) over three years reached a degree of confusion of almost 100%, with a reduction in mated females by 72.86 and 72.8%, respectively. Though these may provide some level of pest control, these strategies can also be labor- and cost-intensive.

Attract and kill may provide a more economical approach to control the apple clearwing moth in BC with minimal insecticide use. The identification of the main sex pheromone component of the apple clearwing moth and the ability of male moths to detect the spectral reflectance of colored objects makes this moth pest a good candidate for attract and kill with SPLAT. As a result of the long hydrocarbon chain of the main sex pheromone component, the pheromone is less volatile and can potentially last longer in the field than other non-Sesiid sex pheromones. Although the sex attractant, (3*Z*,13*Z*)-18:OAc, is detected by other diurnal sesiids, populations of other borer pests are low in apple orchards in the Similkameen Valley in BC and were rarely caught in pheromone-baited traps (personal observation). Since half of the apple production in the Similkameen Valley is grown organically, the use of insecticides to control the apple clearwing moth is problematic. Apple clearwing moth control must also be compatible with the area-wide program for the control of the codling moth (Judd & Gardiner, 2005). The minimal use of insecticide in attract and kill and the relatively specific nature of the attractant can provide apple growers an environmentally-sound control tactic for apple clearwing moth control that will not interfere with control programs for the codling moth. Though the SIR program effectively suppresses wild populations of codling moth, the addition of codlemone (8*E*,10*E*-dodecadienol), the sex pheromone of the codling moth (Roelofs et al., 1971), to the attract and kill formulation can provide multispecies control in apple orchards and provide a better level of codling moth control in urban areas.

1.6 Research objectives

In an effort to develop an effective product used for attract and kill against the apple clearwing moth in BC, the following research objectives were developed:

(1) enhance the attractiveness of pheromone-baited SPLAT through testing the effect of sex pheromone dose, SPLAT droplet size and droplet shape on male apple clearwing moth attraction (Chapter 2)

(2) identify the effects of codlemone and cypermethrin in the SPLAT formulation on male apple clearwing moth attraction to sex pheromone (Chapter 2)

(3) determine if male apple clearwing moths discriminate among variously colored SPLAT droplets in the presence of sex pheromone (Chapter 3)

(4) determine if visual cues can enhance male apple clearwing moth orientation to and contact with pheromone-baited SPLAT (Chapter 3)

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Figures

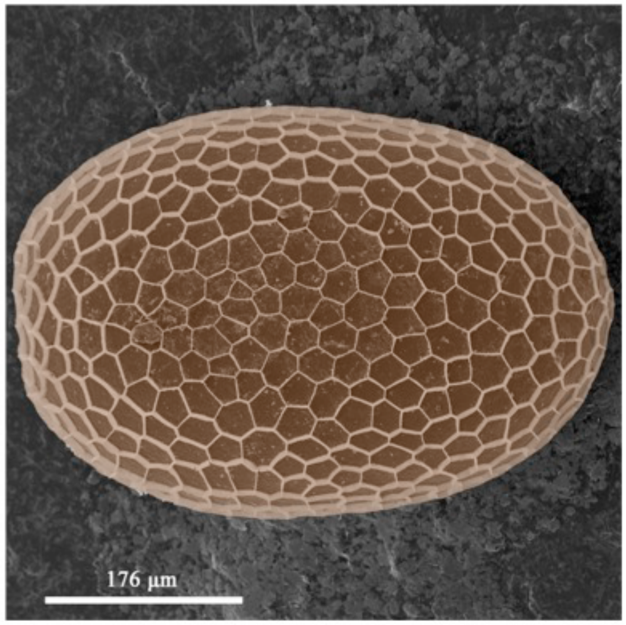

Figure 1.1 Scanning electron micrograph of an artificially colored apple clearwing moth egg (Photo credit: Michael Weis)

Figure 1.2 Apple clearwing moth larva in an apple tree trunk (Photo credit: Marker Gardiner)

Figure 1.3 Apple clearwing moth pupae and cocoons (Photo credit: Mark Gardiner)

Figure 1.4 Pupal exuvia of apple clearwing moth on apple tree bark

Figure 1.5 Nectar-feeding adult apple clearwing moth

Figure 1.6 Apple clearing moths in copulation. Note the wider body of the female (above) compared to the male (below). The male has white sternites.

2. Development of a pheromone-based attract and kill formulation to target the apple clearwing moth, *Synanthedon myopaeformis*

2.1 Abstract

Synanthedon myopaeformis, (Borkhausen) (Lepidoptera: Sesiidae), is a diurnally active clearwing moth and a serious invasive pest of apple trees in British Columbia (BC), Canada. Properties of a pheromone-based attract and kill formulation (SPLAT®) were tested to target the apple clearwing moth. In trapping experiments, male moths are more attracted to sex pheromone released from the SPLAT matrix than from rubber septum lures when pheromone was applied topically. The shape but not the size of SPLAT droplets used to release pheromone influenced male moth attraction. Incorporation of the killing agent, cypermethrin, into SPLAT did not alter the attractiveness of the pheromone-based attract and kill formulation. Addition of codlemone, the sex pheromone of a sympatric pest species, *Cydia pomonella* (L.)*,* (Lepidoptera: Tortricidae), did not affect apple clearwing moth trap capture suggesting that one attract and kill formulation could be developed to control both species.

2.2 Introduction

The apple clearwing moth, *Synanthedon myopaeformis* (Borkhausen), (Lepidoptera: Sesiidae), is a serious pest of apple trees in Eurasia (Zhang, 1994) and Africa (Ateyyat & Al-Antary, 2006) that was introduced to the Similkameen Valley of BC in 2005 (Judd et al., 2011) most likely via infested rootstock (Philip, 2006). The presence of this diurnally active moth has also been confirmed in Ontario, Canada (Beaton & Carter, 2006) and in Washington State, USA (LaGasa et al., 2006). The identification of the sex pheromone of the apple clearwing moth as (3*Z*,13*Z*)-octadecadienyl acetate ((3*Z*,13*Z*)-18:OAc) (Voerman et al., 1978; Judd et al., 2011), has prompted research into the development of pheromone-based control strategies. Pheromone-based control tactics such as mating disruption (Stüber & Dickler, 1987; Kyparissoudas & Tsourgianni, 1993) and mass trapping (Trematerra, 1993; Aurelian et al., 2012), have been tested that target the adult stage of the apple clearwing moth, as the damaging larval stage is protected under the bark (Dickler, 1976).

Mating disruption is achieved through the release of large amounts of synthetic sex pheromone into the cropping system to preemptively delay or prevent successful mating through interference with pheromone-based communication and mate-finding behavior (Byers, 2007). Desensitization or confusion, camouflage, and false-plume following are the mechanisms by which pheromone-based mating disruption is hypothesized to work (Bartell, 1982). Mass trapping is a tactic that removes moths from the population through capture in semiochemical-baited traps positioned in a cropping system (El-Sayed et al., 2006). Attract and kill is a semiochemical-based tool that contains an attractant to lure the target insect and a killing agent or affector to remove individuals from a population (El-Sayed et al., 2009). This approach is similar to mass trapping except that attract and kill removes insects via exposure to a lethal point source, rather than via trap rentention (El-Sayed et al., 2009). Mating disruption (Stüber & Dickler, 1987; Kyparissoudas & Tsourgianni, 1993) and mass trapping (Trematerra, 1993; Aurelian et al., 2012) provide some level of control of the apple clearwing moth, yet these tactics can be labor-and cost-intensive. Attract and kill technology may provide a more effective and economical approach to control this pest in BC.

Many commercially available attract and kill formulations for moth pests incorporate sex pheromone as the attractant and pyrethroid insecticides as the affector (Suckling & Brockerhoff, 1999; Charmillot et al., 2000; Evenden & McLaughlin, 2004; Nansen & Phillips, 2004). The use of species-specific attractants provides an environmentally-sound approach to manage pests with minimal insecticide use (Howse et al., 1998; Gut et al., 2004; El-Sayed et al., 2009). SPLAT® (Specialized Pheromone & Lure Application Technology), is one of many inert media that can be used to release sex pheromone components and insecticide to attract and kill target pests. Though SPLAT was originally developed to release semiochemicals for mating disruption (Jenkins & Isaacs, 2008; Teixeira et al., 2010), the possibility to incorporate insecticide into the matrix provides an opportunity to use this technology as an attract and kill agent (Vargas et al., 2010; ISCA Technologies, 2012; Pastori et al., 2012). SPLAT also provides a less labor-intensive option than mass trapping and mating disruption due to the variety of application methods (i.e., caulking gun, backpack sprayer, tractor, or aircraft) available for this attract and kill formulation (Stelinski et al., 2010; Teixeira et al., 2010; ISCA Technologies, 2012). This variety of delivery methods also provides an easy means to manipulate the size and distribution of SPLAT droplets (Jenkins & Isaacs, 2008).

In order for attract and kill formulations to be effective, the target insect must touch the formulation to be exposed to the killing agent. The attractant must lure the insect directly to the formulation source for exposure to the killing agent. For the insect to experience the adverse effects of the killing agent, the duration of contact must be adequate to ensure proper dosing with the insecticide (El-Sayed et al., 2009). The chemical properties of the lure, such as semiochemical composition, dose and age, and the physical characteristics of the formulation, such as shape, size and color need to be developed for optimal attractiveness and effectiveness of the formulation. Attract and kill techniques tested for the control of the Egyption cotton leafworm, *Spodoptera littoralis* (Boisduval), (Lepidoptera: Noctuidae) produce low levels of mating suppression due to the use of an incomplete pheromone blend in the formulation (Downham et al., 1995). Suboptimal release rate of the pheromone, which depends on the dose and age of dispensers, renders the attract and kill tactic ineffective, as false-plume following to the point sources cannot be achieved (Downham et al., 1995). The shape and size of the attract and kill formulation can influence the release profile of the attractant and affect its attractiveness to the target pest (Vacas et al., 2011). The surface area to volume ratio (SA:V) of the point source affects the semiochemical release. Visual parameters such as shape (Toshova et al., 2007; Gaskett, 2011), size (Charlton & Cardé, 1990) and color (Goyret & Kelber, 2012; KonDo et al., 2012) are utilized by insects that rely on visual cues for close-range host-searching and mate-finding. Visual properties produced by attract and kill formulations may be particularly important for diurnally active pests such as the apple clearwing moth and the peachtree borer, *Synathedon exitiosa* (Say), (Lepidoptera: Sesiidae), where size (Barry, 1978; Judd & Eby, 2013) and color (Barry, 1978; Barry & Nielsen, 1984; Stüber & Dickler, 1987; Chapter 3) of sex pheromone sources mediate close-range orientation.

Characteristics of the affector can also influence the attractiveness of the attract and kill formulation. The pyrethroid, permethrin, inhibits host-searching behavior of *Cotesia vestalis* (Haliday) (Hymenoptera: Braconidae), a parasitoid of the diamond back moth, *Plutella xylostella* (L.), (Lepidoptera: Plutellidae) (Kawazu et al., 2010). No repellency to pyrethroid used as affectors in attract and kill formulations has been documented for moth pests (DeSouza et al., 1992; Nansen & Phillips, 2004; Curkovic & Brunner, 2006). Sublethal effects of insecticidal poisoning on subsequent pheromone-mediated reproductive behavior occur in the Oriental fruit moth, *Grapholita molesta* (Busck), (Lepidoptera: Tortricidae) (Linn & Roelofs, 1984; Evenden et al., 2005) and the pink bollworm, *Pectinophora gossypiella* (Saunders), (Lepidoptera: Gelichiidae) (Floyd & Crowler, 1981; Haynes & Baker, 1985) and may contribute to control obtained by the formulation.

The development of pheromone-based formulations in which more than one species is targeted can be developed for sympatric species that have overlapping flight periods and distinct pheromone communication channels (Evenden, 2005; Jones et al., 2009). A combined pheromone-based lure tested to monitor populations of the forest pests, *Malasoma disstria* (Hübner) (Lepidoptera: Lasiocampidae) and *Choristoneura conflictana* (Walker) (Lepidoptera: Tortricidae) was as attractive to male *M. disstria* and male *C. conflictana* as lures baited with each species pheromone alone (Jones et al., 2009). This approach has also been tested for an attract and kill formulation that targets two tree fruit pests, the Oriental fruit moth and the codling moth, *Cydia pomonella* (L.), (Lepidoptera: Tortricidae) (Evenden & McClaughlin, 2005). The combined formulation was more attractive to Oriental fruit moth males but less attractive to codling moth males as compared to the formulation containing each species' pheromone alone (Evenden & McClaughlin, 2005). This tactic is not limited to moths and has been used to lure multiple forest (Miller et al., 2005) and orchard (James et al., 2000) coleopteran pest species with one formulation. Although attraction to a multi-component aggregation lure was dependent on the region the tests were conducted, *Ips avulsus* (Eichhoff) (Coleoptera: Scolytidae) and *I. grandicollis* (Eichoff), were caught in the highest numbers in traps baited with all three pheromones (Miller et al., 2005). *Carpophilus davidsoni* (Dobson) (Coleoptera: Nitidulidae) and *C. mutilatus* (Erichson), pests of stone fruit in Australia, were equally attracted to the formulation containing both species' pheromones and the pheromone of a conspecific (*C. hemipterus*, L.), and to lures impregnated with each species' pheromone alone (James et al., 2000).

In BC, the codling moth is a serious pest of apples (Dolstad, 1985; Judd et al., 1997) and has a similar distribution and an overlapping flight period (Vakenti & Madsen, 1976) with the apple clearwing moth (Dickler, 1976; Judd, 2008). Since these moths are only distantly related and there is no overlap in the chemical structure of their pheromone signals, the simultaneous release of both species' pheromones from one matrix is feasible (Deland et al., 1994; James et al., 2000; Evenden & McClaughlin, 2005; Miller et al., 2005; Stelinski et al., 2007b; Jones et al. 2009). To determine if the these two species can be managed with the same formulation, the effect of the addition of codlemone (8*E*,10*E*-dodecadienol), the sex pheromone of the codling moth (Roelofs et al., 1971), to the matrix, on the response of male apple clearwing moth is tested.

The objectives of this study were to test the hypotheses that (1) male apple clearwing moths are attracted to their sex pheromone when released from various sizes, shapes and colors of SPLAT droplets, and (2) to determine if the addition of cypermethrin or (3) codlemone affects attraction of male apple clearwing moths to the attract and kill formulation.

2.3 Materials and methods

2.3.1 Preparation of SPLAT and pheromone lures

Droplets of SPLAT® (ISCA Technologies, Riverside, California) used in experiments consisted of 1 g (wet weight) of grey SPLAT, unless stated otherwise. One gram droplets were air dried for 24 h at 23-24°C on polyester strips. Droplets were molded into a hemispherical shape, unless stated otherwise, and transferred to aluminum foil to air for an additional 24-48 h at 35°C. Droplets with a base diameter of 16-18 mm and a final weight of 0.51-0.59 g were stored in glass jars at 2-5°C until deployment into the field. The incorporation of cypermethrin into SPLAT droplets was achieved by mixing SPLAT with 5% (w/w) cypermethrin obtained from the manufacturer with blank splat to obtain the correct cypermethrin dose. Droplets were aired for at least 48 h at 23-24°C in a fume hood and stored at 2-5°C until use in the field. Depending on the experiment, SPLAT droplets and grey rubber septum lures (West Pharmaceutical Services, Lionville, Pennsylvania) were topically loaded with 0.1-, 1- or 10 mg of (3*Z*,13*Z*)-18:OAc (>95% isomeric purity, Pherobank, Wageningen, Netherlands) and/or 0.1- or 1 mg of codlemone (min 97% purity, Bedoukian Research, Danbury, Connecticut) dissolved in 0.2 mL of HPLC-grade hexane (Sigma-Aldrich, St. Louis, Missouri). Lures baited with pheromone were aired for 4 h at 23-24°C in a fume hood before storage in glass jars at -20°C until use in the field.

2.3.2 Experiment 1: effect of pheromone dose, lure type and lure age on the attractiveness of the SPLAT formulation to male apple clearwing moths

Experiment 1 (7 July – 12 August, 2011), and all subsequent trapping experiments (Experiments 2-4, 6) (Table 2.1) were conducted in two commercial apple orchards in Keremeos and Cawston, BC (49.22°N, -119.83°W; 49.15°N, -119.73°W). This experiment was designed to test the effect of pheromone dose, lure type and lure age on male apple clearwing moth trap capture in a no-choice trapping experiment. The attractiveness of one gram droplets of SPLAT and grey rubber septum lures dosed topically with 0-, 0.1-, 1- or 10 mg of (3*Z*,13*Z*)-18:OAc and aged in the field in traps over 5 weeks was compared using a randomized complete block design with 8 replicates of each treatment. Lure age treatments were 1-, 3- and 5 weeks at the time of testing.

Lures were transported to field sites in coolers with ice and individually suspended in traps made with clear sheets of polyester (26.7 X 45.7 cm) rolled into cylinders (Figure 2.1). Traps were held together by wire and lined with polyester sheets (26.7 X 17.1 cm) coated with Tanglefoot (The Tanglefoot Company, Grand Rapids, Michigan, Pennsylvania). Tanglefoot liners were held in place by six large paper clips on the bottom half of the traps. Traps were suspended from trees 1.5 m above the ground and spaced 20 m apart within a linear array (block) containing one trap of each lure type, dose and age combination. Blocks at each site were separated by 20 m. Moth catches were recorded every day and Tanglefoot liners were replaced after the capture of >40 moths. Pheromone dose and lure type treatment positions within blocks were re-randomized every two weeks after the first week for five weeks. SPLAT droplets and rubber septum lures loaded with 0.2mL of hexane were deployed as controls to determine if male moth attraction could be elicited to SPLAT droplets or rubber septum lures in the absence of sex pheromone. No moth capture was observed.

2.3.3 Experiment 2: effect of droplet shape on the attractiveness of the SPLAT formulation to male apple clearwing moths

This experiment (29 July – 10 August, 2011) tested the effect of SPLAT droplet shape on male apple clearwing attraction. The shape of the formulation may provide visual cues to orienting males. The shape of SPLAT droplets may influence pheromone release profiles that could affect the attractiveness of the formulation. SPLAT droplets and traps were prepared as in experiment 1, except that 1 mg of (3*Z*,13*Z*)-18:OAc was mixed into droplets which were molded into hemispheres, cylinders or flattened cylinders. Droplets were 16-, 17- and 19 mm in diameter, respectively. Attraction of male apple clearwing moths to the variously shaped SPLAT droplets in traps was compared using a randomized complete block design with 6 replicates of each treatment (3 linear blocks, replicated in time). Lures and treatment positions within blocks were replaced and re-randomized, respectively, after one week.

2.3.4 Experiment 3: effect of droplet size on the attractiveness of the SPLAT formulation to male apple clearwing moths

Male apple clearwing moth attraction to variously sized SPLAT droplets was tested (16 July – 2 August, 2011). SPLAT droplet size with a constant pheromone load was tested. SPLAT droplets and traps were prepared as in experiments 1 and 2, except that 0.5-, 1- or 2 g (wet weight) of SPLAT was used to generate the differently sized droplets. Droplets had base diameters and final weights of 14 mm and 0.26 g, 17-18 mm and 0.54 g, and 21-22 mm and 1.09 g, respectively. SPLAT droplets were loaded topically with 1 mg of (3*Z*,13*Z*)-18:OAc. Male apple clearwing trap capture was compared using a randomized complete block design with 9 replicates of each treatment (3 blocks, replicated over time). Lures were replaced and treatment positions within blocks were re-randomized every five days for 15 days.

2.3.5 Experiment 4: effect of cypermethrin dose on the attractiveness of the SPLAT formulation to male apple clearwing moths

Experiment 4 (29 June – 19 July, 2012) tested the effect of incorporation of cypermethrin and sex pheromone into SPLAT on catches of male apple clearwing moths. Traps were prepared as in trapping experiments 1-3, but SPLAT droplets contained 0-, 2.5- or 5% cypermethrin in combination with 1- or 10 mg of (3*Z*,13*Z*)-18:OAc added topically for a total of 6 treatments. Male moth capture in traps was compared using a randomized complete block design with 18 replicates of each treatment (6 linear blocks, replicated over time). Lures and treatment positions were replaced and re-randomized every week for three weeks.

2.3.6 Experiment 5: effect of cypermethrin dose on male apple clearwing moth visitation to droplets of SPLAT formulation

Experiment 5 (1 and 8 May, 2012) (Table 2.1) was conducted in two commercial apple orchards in Keremeos, BC (49.19°N, -119.74°W; 49.22°N, -119.83°W). This experiment tested the effect of cypermethrin dose on male moth visitation of SPLAT droplets in the presence of the apple clearwing moth sex pheromone. Droplets of SPLAT containing 0-, 0.625-, 1.25-, 2.5- or 3.75% cypermethrin without pheromone were affixed with clear push pins onto a circular arena (13 cm diam.) made of clear polyester. Orange-striped black SPLAT droplets were used in this experiment as they provide an attractive visual cue to male apple clearwing moths in the presence of their sex pheromone, which elicits high levels of male moth contact with these droplets (Chapter 3). Individual droplets were placed 3 cm apart along the edge of the arena. A grey rubber septum loaded topically with 10 mg of (3*Z*,13*Z*)-18:OAc was positioned in a hole in the center of the arena equidistant from the six droplets (similar to Figure 2.1b). Four arenas were hung 1.5 m above the ground in trees separated by 20 m. Panasonic HDC-SD90 Full HD Camcorders with a high capacity VW-VBK360 3580 mAh Li-ion battery and a 32 GB class 10 SD card with automatic white balance were positioned on platforms placed near the canopy level by each arena. Video recordings were conducted between 1100-1300 and 1300-1500 h, Pacific Daylight Time (PDT), when male flight activity to pheromone traps is highest (Judd & Eby, 2013). Visitation was quantified as the number of males that made contact with each SPLAT droplet with their claspers or tarsi per one hour replicate. A total of twelve replicates were performed (two, one hour replicates at each of the four sites for one and a half days). SPLAT droplets and rubber septum lures were replaced and treatment positions were re-randomized after each replicate.

2.3.7 Experiment 6: effect of codlemone on the attractiveness of the

SPLAT formulation to male apple clearwing moths

This experiment (29 June – 13 July, 2012) tested the effect of codlemone on male apple clearwing moth attraction to SPLAT droplets baited with apple clearwing sex pheromone. SPLAT droplets and traps were prepared as in experiments 1-4 with the exception that 0-, 0.1- or 1 mg of codlemone was added topically to the droplets in addition to 0- or 1 mg of (3*Z*,13*Z*)-18:OAc. A grey rubber septum loaded with 1 mg of both sex pheromones was used as a positive control. Male moth trap capture was compared using a randomized complete block design with 12 replicates of each treatment (6 blocks, replicated in time). Lures were replaced and treatment positions were re-randomized after one week.

2.3.8 Statistical analyses

Analyses were conducted using the R statistical package (R Core Team, 2013) with a significance level of 5%. All data derived from trap capture and behavioral observations were analyzed by generalized linear mixed effects models (GLMM) with error terms fitted to a negative binomial distribution (glmmADMB package) (Table 2.2). Data with no significant random effects were analyzed by generalized linear models (GLM) also with error terms fitted to a negative binomial distribution. Significant results were followed by a *post-hoc* comparison of means using the Tukey's HSD multiple comparison test.

2.4 Results

2.4.1 Experiment 1: effect of pheromone dose, lure type and lure age on

the attractiveness of the SPLAT formulation to male apple clearwing moths

Pheromone dose and lure type (i.e., SPLAT or septum) had an effect on male apple clearwing moth trap capture regardless of the age of the lure. Trap catches increased with pheromone dose across all doses tested in both lure types after one week (χ^2=93.317, df=2, P <0.0001, Figure 2.2a), three weeks (χ^2=74.72, df=2, P <0.0001, Figure 2.2b) and five weeks (χ^2=46.408, df=2, P <0.0001, Figure 2.2c). The effect of lure type on male apple clearwing moth trap capture increased with subsequent weeks. After one week in the field, SPLAT attracts more male moths than similarly baited rubber septum lures (χ^2=18.260, df=1, P <0.0001, Figure 2.2a). However, traps baited with SPLAT are less attractive than septa-baited traps after three weeks (χ^2=20.75, df=1, P <0.0001, Figure 2.2b) and five weeks (χ^2=59.285, df=1, P <0.0001, Figure 2.2c). No significant interaction between pheromone dose and lure type was observed (Table 2.2). Site significantly affected trap capture for both lure types in the first three weeks of the study (Table 2.2).

2.4.2 Experiment 2: effect of droplet shape on the attractiveness of the SPLAT formulation to male apple clearwing moths

The shape of SPLAT droplets affected the number of male apple clearwing moths caught (χ^2=20.51, df=2, P <0.0001, Figure 2.3) in traps. SPLAT lures molded into hemispheres were significantly more attractive than the similarly baited cylinderical shapes tested.

2.4.3 Experiment 3: effect of droplet size on the attractiveness of the SPLAT formulation to male apple clearwing moths

Male trap capture was similar regardless of the size of the SPLAT droplet used to release pheromone (χ^2=1.5482, df=2, P =0.46, Figure 2.4).

2.4.4 Experiment 4: effect of cypermethrin dose on the attractiveness of the SPLAT formulation to male apple clearwing moths

Apple clearwing moth sex pheromone dose (χ^2=70.502, df=1, P <0.0001, Figure 2.5) significantly affected the number of male apple clearwing moths caught in baited traps. Trap capture increased as sex pheromone dose increased. There was no significant effect of cypermethrin in the SPLAT formulation on the number of males captured in traps (Figure 2.5). No significant interaction between cypermethrin and sex pheromone dose was observed.

2.4.5 Experiment 5: effect of cypermethrin dose on male apple clearwing moth visitation to droplets of SPLAT formulation

Cypermethrin dose in this choice-experiment did not affect male moth close-range visitation of SPLAT droplets (Figure 2.6). A similar number of males contacted all droplets (χ^2=8.528, df= 4, P =0.07). Typical contact behaviors of males involve stabbing their claspers against droplets (Figure 2.1b).

2.4.6 Experiment 6: effect of codlemone on the attractiveness of the SPLAT formulation to male apple clearwing moths

The incorporation of the sex pheromone of the codling moth into the SPLAT formulation targeting apple clearwing moth did not affect the attractiveness of the formulation to male apple clearwing moth. Trap capture was similar in traps baited with SPLAT containing apple clearwing moth sex pheromone (χ^2=254.94, df= 1, P <0.0001, Figure 2.7) regardless of codlemone dose. There was no significant interaction between both sex pheromones that influenced orientation of male apple clearwing moths.

2.5 Discussion

Male apple clearwing moths clearly demonstrate long-distance attraction to sex pheromone released from the SPLAT formulation. Male apple clearwing moths are differentially attracted to variously shaped droplets of the SPLAT matrix. Greater moth capture in traps baited with hemispherical droplets could indicate that moths use lure shape as a visual cue or that the physical release profiles of the sex pheromone from the droplets are altered with droplet shape. Pheromone dispenser type (Kehat et al., 1994; Vacas et al., 2009), shape (Tomaszewska et al., 2005) and size (Jenkins & Isaacs, 2008; Vacas et al., 2011), and dispenser composition (Shailaja et al., 1997) affect release rates of pheromone and the attractiveness of a lure (Kehat et al., 1994; Vacas et al., 2009; Vacas et al., 2011). The release of the pheromone through the matrix or membrane is dependent on the shape and size of the dispenser since this alters the SA:V and the amount of the semiochemical released. The interaction of the pheromone with the matrix medium and the physical interaction between multiple pheromone compounds in a matrix can also affect the rate of semiochemical release. These parameters differ between dispenser types. Differences in the release kinetics of pheromone affect the plume structure and the mechanisms by which the control tactic acts on target insects, which affect the attractiveness of the insect to a lure (Suckling, 2000).

Apple clearwing moth sex pheromone applied at doses between 0.1 and 10 mg and released from SPLAT or rubber septum lures is attractive to male moths (Figure 2.2a-c). Male moth capture increased with pheromone dose in both lures regardless of lure age. Male moth trap capture was highest in traps baited with 10 mg of sex pheromone applied topically to the SPLAT matrix and aged for one week; however, moth trap capture did not differ significantly from traps baited with 10 mg of sex pheromone in rubber septum lures and 1 mg of sex pheromone in SPLAT (Figure 2.2a). The attractiveness of rubber septum lures was highest after being aged for three weeks (Figure 2.2b) and remained more attractive than the SPLAT formulation in older lure age treatments (Figure 2.2c). This discrepancy in lure attractiveness is most likely a result of different pheromone release profiles from the different lure types, which enhances with lure age. More grape berry moths, *Paralobesia viteana* (Clemens), (Lepidoptera: Tortricidae) were caught in pheromone traps baited with rubber septum lures containing 0.1 mg of sex pheromone than in traps baited with 0.2- or 0.5 ml of SPLAT-GBM™ (a commercial formulation used for grape berry moth mating disruption) containing 3% of sex pheromone (Jenkins & Isaacs, 2008). Although the SPLAT droplets contained 6 mg or more of the sex pheromone, moth capture decreased with increasing droplet size (Jenkins & Isaacs, 2008). Release rates measured from 1 ml SPLAT-GBM™ droplets indicate a high initial release of pheromone in the first two weeks followed by a period of steady, slow release for the remaining ten weeks (Jenkins & Isaacs, 2008). Similarly, release profiles of 0.38 g droplets of SPLAT-OFM™, a commercially available product for mating suppression of the Oriental fruit moth, indicate a high release rate of pheromone from droplets in the first two weeks, followed by a reduction in the release rate by half during the subsequent seven weeks (Stelinski et al., 2007a).

When pheromone release rates were compared between an attracticide paste and rubber septum lures containing similar amounts of the main sex pheromone component of the oblique banded leafroller, *Choristoneura rosaceana* (Harris), (Lepidoptera: Tortricidae) and the Pandemis leafroller, *Pandemis pyrusana* (Kearfott), (Lepidoptera: Tortricidae), the release rate of the paste was much lower than what was reported for equally baited rubber septum lures (Curkovic et al., 2009). This was consistent with wind tunnel and field experiments in which source contact and moth capture to the paste was only comparable to rubber septum lures when baited with high concentrations of the sex pheromone (Curkovic et al., 2009). Codling moth males were caught in higher numbers in traps baited with rubber septum lures than in traps baited with CM Pherocon caps loaded with the same pheromone dose (Kehat et al., 1994). Rubber septum lures emit pheromone at a slower rate than Pherocon caps (Kehat et al., 1994). On the contrary, pheromone released from rubber septum lures is as attractive as that released from LB Pherocon caps to male European grapevine moths (*Lobesia botrana*, Lepidoptera: Tortricidae) (Anshelevich et al., 1994). The efficacy of lure types appears to be affected by an interaction between the sex pheromone and the lure substrate and differs by species of moth pest.

The rapid decline in male moth trap capture in SPLAT-baited traps over time in the current study, most likely indicates that apple clearwing moth sex pheromone is released at a higher rate from SPLAT than rubber septum lures (Figure 2.2). Although, rubber septum lures may be more effective for long-term attraction of male moths, SPLAT formulations can potentially attract many moths and be effective if deployed immediately before peak adult male emergence. This is especially important because males eclose a couple weeks before females (Aurelian et al., 2012) and could be targeted by pheromone-based control before males have mating opportunities. The rapid release of pheromone from the SPLAT matrix could mean that a number of applications will be required for season-long control. Since there are mechanized application methods for SPLAT deployment into the field (ISCA Technologies, 2012; Stelinski et al., 2010; Teixeira et al., 2010), the application of this attract and kill formulation should remain less labor-intensive than other pheromone-based management tactics.

Male apple clearwing moths were more attracted to hemispherical-shaped SPLAT droplets than to droplets molded into cylinders or flattened cylinders (Figure 2.3). Pheromone dispenser shape affects the release profile of the attractive pheromone compound(s). Codlemone release from two types of polyethylene tube twist tie dispensers, two types of membrane dispensers, and a spiral polymer dispenser, dispensers commercially available for mating disruption control of codling moth, varies regardless of pheromone dose (Tomaszewska et al., 2005). The rates of release of pheromone were consistently dissimilar during the first two weeks of the trial and the remainder of the season for all dispensers and dispenser types (Tomaszewska et al., 2005). Dispenser shape and membrane properties affected pheromone release. The high SA:V of the hemispherical-droplet in the current study most likely allows for a faster pheromone release rate than cylindrical SPLAT droplets, which subsequently induces strong male moth attraction. The higher number of moths caught in traps baited with hemisphere-shaped SPLAT droplets compared to traps baited with droplets with the highest SA:V (flattened cylinders) may be due to visual cues provided by the hemisphere in conjunction with the olfactory cues (Kriston, 1973; Charlton & Cardé 1990; Balkenius et al., 2006). The use of multiple sensory modalities, such as vision and olfaction, may provide a more reliable process to confer the position of potential mates (Hidaka, 1972; Toshova et al., 2007). Once males are in the vicinity of a calling female, visual cues such as shape and size (Mazokhin-Porschnyakov, 1969) can increase attraction to the pheromone or are necessary to initiate copulatory behaviors (Barry & Nielsen, 1984; Koshio & Hidaka, 1995; KonDo et al., 2012). All droplets in the current study were similar in diameter, but varied in height. The flattened cylinder could have been unattractive to male moths because it lacked any resemblance to an apple clearwing female. The size of potential mates in diurnal moths, such as the gypsy moth, *Lymantria dispar* (L.), (Lepidoptera: Erebidae), affects male mating behavior (Charlton & Cardé, 1990). Models that were smaller than average female moths evoked fewer copulatory attempts and altered mating behaviors in male gypsy moths (Charlton & Cardé, 1990).

Male moths were similarly attracted to differently sized droplets of the SPLAT matrix (Figure 2.4). SPLAT droplets of various sizes loaded topically with the same pheromone dose does not influence moth trap capture. In this experiment, all droplets were similar in height but varied in diameter. These results suggest that male apple clearwing moths may rely more on object height than object diameter for visual attraction. This may also suggest that the range of droplet sizes tested was inadequate to observe an effect of droplet size on male moth visual attraction or a significant change in the pheromone release profiles from the droplets. When presented with female moth mimics, peach tree borers contacted models that were 1-2 cm in length and 1.2 cm in diameter more frequently than models that were 4 cm in length and less than 1 cm in diameter in the presence of sex pheromone (Barry, 1978). During close-range pheromone-mediated orientation by apple clearwing moths, 1 g droplets were just as attractive as 3 g droplets (Chapter 3); however, black pheromone baskets, which are several times larger than female apple clearwing moths, positioned in attractively-colored Unitraps were less attractive than predicted (Judd & Eby, 2013). This suggests a size threshold for male apple clearwings for attractive visual stimuli in the presence of sex pheromone. Variously sized cylindrical tablets made of mesoporous material were tested to determine the effect of sex pheromone emission on European grapevine moth attraction to pheromone sources (Vacas et al., 2011). Two tablets, identical in diameter but varying in height, loaded with different doses of pheromone followed dissimilar trends in pheromone emission. The shorter tablet, loaded with a lower dose of pheromone followed a cubic equation, whereas the taller tablet (~3X taller) loaded with a dose three times that of the shorter tablet, followed an asymptotic trend. This major difference in pheromone release was observed after 40 days (Vacas et al., 2011). A similar trend may have been observed in our experiment, if the duration of lures in the field was extended.

The addition of cypermethrin to the SPLAT formulation was tested to establish if cypermethrin affects attractiveness of the pheromone-baited SPLAT formulation to apple clearwing moths. Behavioral observations show no evidence of repellency of cypermethrin to apple clearwing moths at doses between 0.625- and 3.75% cypermethrin. Male moth capture was numerically highest in traps baited without cypermethrin, but was not significantly different from moth capture in traps baited with 2.5- and 5% cypermethrin in the SPLAT formulation (Figure 2.5). These results suggest that pheromone-treated SPLAT with cypermethrin remain attractive to male apple clearwing moths. Similarly, when male apple clearwing moths are provided the opportunity to discriminate between cypermethrin doses at close range, a similar number of males orient to and contact the droplets with or without insecticide (Figure 2.6). These results confirm that apple clearwing moths will readily contact the attract and kill formulation. Spatial repellency of pyrethroids occurs in disease-vectoring mosquitoes (Kawada et al., 2004; Kawada et al., 2008) but has not been documented in agricultural moth pests (DeSouza et al., 1992; Nansen & Phillips, 2004; Curkovic & Brunner, 2006). Female *Anopheles* sp. mosquitoes (Diptera: Culicidae) avoid areas that contain the pyrethroid, metofluthrin, released from impregnated paper (Kawada et al., 2004) and plastic (Kawada et al., 2008) strips; though, nylon strips impregnanted with permethrin (Dusfour et al., 2009) or cypermethrin (Achee et al., 2009) do not induce spatial repellency in female mosquitoes. Sublethal poisoning of pyrethroids, however, affects pheromone-induced behavior in the Oriental fruit moth and the pink bollworm. Voluntary and involuntary sublethal exposures of the Oriental fruit moth to LastCall™OFM formulations reduce subsequent male response to the attract and kill formulation and calling virgin females, one hour post exposure (Evenden et al., 2005). Oriental fruit moth response to their sex pheromone also declines at five hours after exposure to sublethal concentrations of permethrin (Linn & Roelofs, 1984). Similar effects of sublethal insecticide poisoning on pheromone-mediated reproductive behavior occur in the pink bollworm (Floyd & Crowler, 1981; Haynes & Baker 1985). These effects on pheromone-mediated behavior may provide additional modes of control by the attract and kill formulation, but may not be effective if the target pest regains its response to sex pheromone. Although, most contact insecticides, such as pyrethroids, are delivered to the moth pest through contact with their tarsi, knockdown can still be achieved in apple clearwing moths after <1 sec exposures to cypermethrin with the end of their abdomens (and/or claspers) (JJ Kwon, personal observation).

In an effort to develop an attract and kill formulation capable of multi-species control, the effect of the sex pheromone of the codling moth, codlemone, on attraction of male apple clearwing moth was tested. A similar number of apple clearwing moth males were caught in traps baited with apple clearwing moth pheromone with varying doses of codlemone as in the positive control that contained apple clearwing moth pheromone alone (Figure 2.7). This result and the absence of males in traps baited with codlemone alone suggest that there is no chemical or physical interaction between the two pheromones as perceived by male apple clearwing moths. Semiochemical-based management tactics that target multiple species have been tested for other moth pests. Attract and kill formulations tested to control Oriental fruit moth and codling moth, simultaneously, increased trap capture of Oriental fruit moth in the field, the number of moths that contact the formulation and the time spent on the matrix in wind tunnel experiments compared to response to the Oriental fruit moth formulation alone. Codling moth trap capture, however, was reduced in the field when traps were baited with the multi-species attract and kill matrix (Evenden & McClaughlin, 2005). The effect of the apple clearwing moth pheromone on the attractiveness of the combined formulation to male codling moths, remains to be determined. Aerosol pheromone puffers that release both Oriental fruit moth pheromone and codlemone do not enhance mating disruption compared to application of puffers that release each species pheromone separately (Stelinski et al., 2007b). Multi-pheromone lures tested to monitor populations of forest tent caterpillar and large aspen tortrix caught similar numbers of both species as traps baited with each species' pheromone alone (Jones et al., 2009). The release of multiple pheromones by a single dispenser can result in variable responses in different moth species.

Our results lend support to the hypothesis that male apple clearwing moths are attracted to SPLAT baited with their sex pheromone. Increased pheromone dose consistently increased trap capture of male moths. Droplet size, and the incorporation of cypermethrin and codlemone to the SPLAT matrix did not affect male moth attraction to pheromone. Droplet size can affect the release rate of pheromone given that the SA:V of an object changes with size. A 0.5 g droplet loaded with 1 mg of apple clearwing moth pheromone may potentially release pheromone at a higher rate than a 2.0 g droplet with the same pheromone dose and affect apple clearwing moth male attraction. Perhaps droplet size did not have an effect on male moth attraction in our study because the range of droplet sizes used was not large enough to create a significant difference in release profiles to observe a difference in male apple clearwing attraction to the pheromone lures. SPLAT shape and lure type can, however, have an effect on apple clearwing males, as these parameters alter the release profile of pheromones. Overall, the use of SPLAT as a potential medium for attract and kill has potential for this invasive pest of apple in BC.

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Tables

Table 2.1: Trapping experiments conducted on male apple clearwing moths were performed between May-August in 2011 and 2012. Male moths were presented with SPLAT droplets or grey rubber septum lures topically loaded or mixed in to the substrate with a dose of (3*Z*, 13*Z*)-18:OAc suspended from experimental traps. Traps were placed 1.5 m above the ground in trees and separated by 20 m. SPLAT droplets and rubber septum lures were replaced and treatment positions were re-randomized after each week, unless stated otherwise.

Experiment	**Dates conducted**	**Pheromone dose (mg)**	**N of treatments**	**N of replicates**
1 - trapping; effect of dose, lure type and lure age	2 July-12 August, 2011	0, 0.1, 1, 10	8	8
2 - trapping; effect of shape	29 July -10 August, 2011	1	3	6
3 - trapping; effect of size	16 July-2 August, 2011	1	3	9
4 - trapping; effect of cypermethrin dose	29 June-19 July, 2012	1, 10	6	18
5 - observation; effect of cypermethrin dose	1 & 8 May, 2012	10	5	12
6 - trapping; effect of codlemone	29 June-13 July, 2012	0, 1	6	12

Table 2.2: Final generalized linear mixed effects models (GLMM) of experiments tested in the presence of apple clearwing moth sex pheromone (ACM) with significant and non-significant random effects identified. Data with no significant random effects were analyzed by generalized linear models (GLM). Error terms were fit to a negative binomial distribution. (*) indicates significant term effects (P <0.05).

Experiment	Model	Random effects
1 - trapping; effect of dose, lure type and lure age	Week 1: Moths ~ dose* + lure* + (1|site)	site*
	Week 3: Moths ~ dose* + lure* + (1|site)	site*
	Week 5: Moths ~ dose* + lure*	site
2 - trapping; effect of shape	Moths ~ shape* + (1|week)	site, week*
3 - trapping; effect of size	Moths ~ size	site, week
4 - trapping; effect of cypermethrin dose	Moths ~ acmdose* + (site|week)	site*, week*
5 - observation; effect of cypermethrin dose	Visits ~ cypermethrin + (site|time)	time*, site*, date
6 - trapping; effect of codlemone	Moths ~ acmdose* + (site|week)	site*, week*

Figure legends

Figure 2.1 (a) Experimental traps employed in trapping experiments and (b) observation arenas used in observational experiment 1. The male moth in panel b demonstrates typical contact with the posterior abdominal end striking or clasping onto droplets.

Figure 2.2 Median (± Interquartile Range (IQR)) number of male apple clearwing moths captured in traps baited with 0-, 0.1-, 1- or 10 mg of (3*Z*,13*Z*)-18:OAc loaded topically onto SPLAT droplets and rubber septum lures aged for (a) one week, (b) three weeks and (c) 5 weeks in the field. Whiskers represent data that fall within 1.5 X IQR, while outliers are presented by dots that fall between 1.5-3 X IQR. Different letters indicate a significant difference among treatments (Tukey HSD, $P < 0.05$).

Figure 2.3 Median (± Interquartile Range (IQR)) number of male apple clearwing moths captured in traps baited with 1 mg of (3*Z*,13*Z*)-18:OAc mixed into variously shaped SPLAT droplets. Whiskers represent data that fall within 1.5 X IQR, while outliers are presented by dots that fall between 1.5-3 X IQR. Different letters indicate a significant difference among treatments (Tukey HSD, $P < 0.05$).

Figure 2.4 Median (± IQR) number of apple clearwing moth males captured in traps baited with 1 mg of (3*Z*,13*Z*)-18:OAc loaded topically onto variously sized SPLAT droplets. Whiskers represent data that fall within 1.5 X IQR, while outliers are presented by dots that fall between 1.5-3 X IQR.

Figure 2.5 Median (± IQR) number of male apple clearwings captured in traps baited with 1- or 10 mg of (3*Z*,13*Z*)-18:OAc and 0-, 2.5- or 5 % cypermethrin mixed into SPLAT droplets. Whiskers represent data that fall within 1.5 X IQR, while outliers are presented by dots that fall between 1.5-3 X IQR. Different letters indicate a significant difference among treatments (Tukey HSD, $P < 0.05$).

Figure 2.6 Median (± IQR) number of apple clearwing males that made contact with SPLAT droplets containing 0-, 0.625-, 1.25-, 2.5- or 3.75% cypermethrin. Whiskers represent data that fall within 1.5 X IQR, while outliers are presented by dots that fall between 1.5-3 X IQR.

Figure 2.7 Median (± IQR) number of male apple clearwing moths captured in traps baited with 0- or 1 mg of (3*Z*,13*Z*)-18:OAc and 0-, 0.1- or 1 mg of codlemone loaded topically onto SPLAT droplets. Whiskers represent data that fall within 1.5 X IQR, while outliers are presented by dots that fall between 1.5-3 X IQR. Different letters indicate a significant difference among treatments (Tukey HSD, $P < 0.05$).

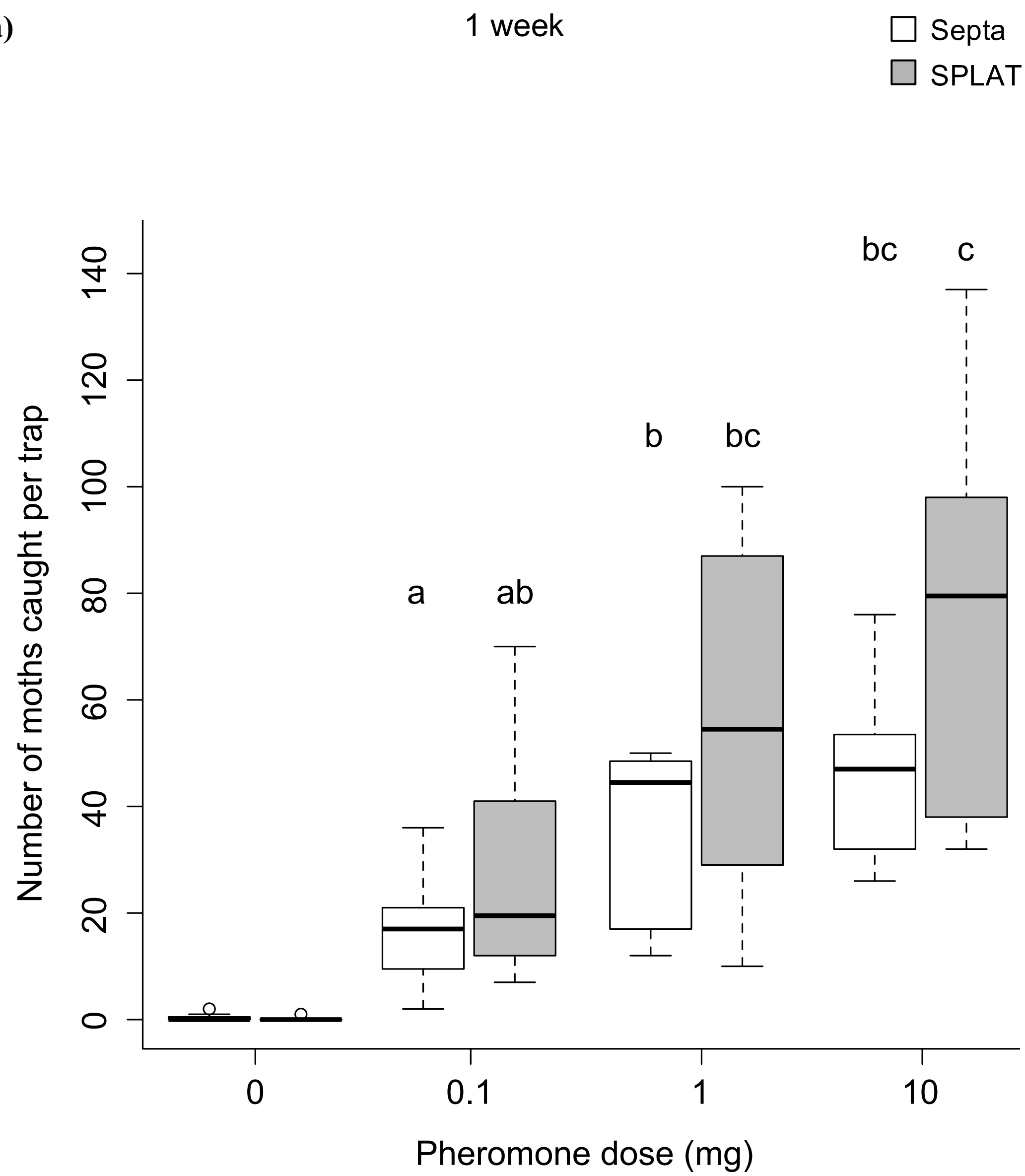
Figures

(a)

(b)

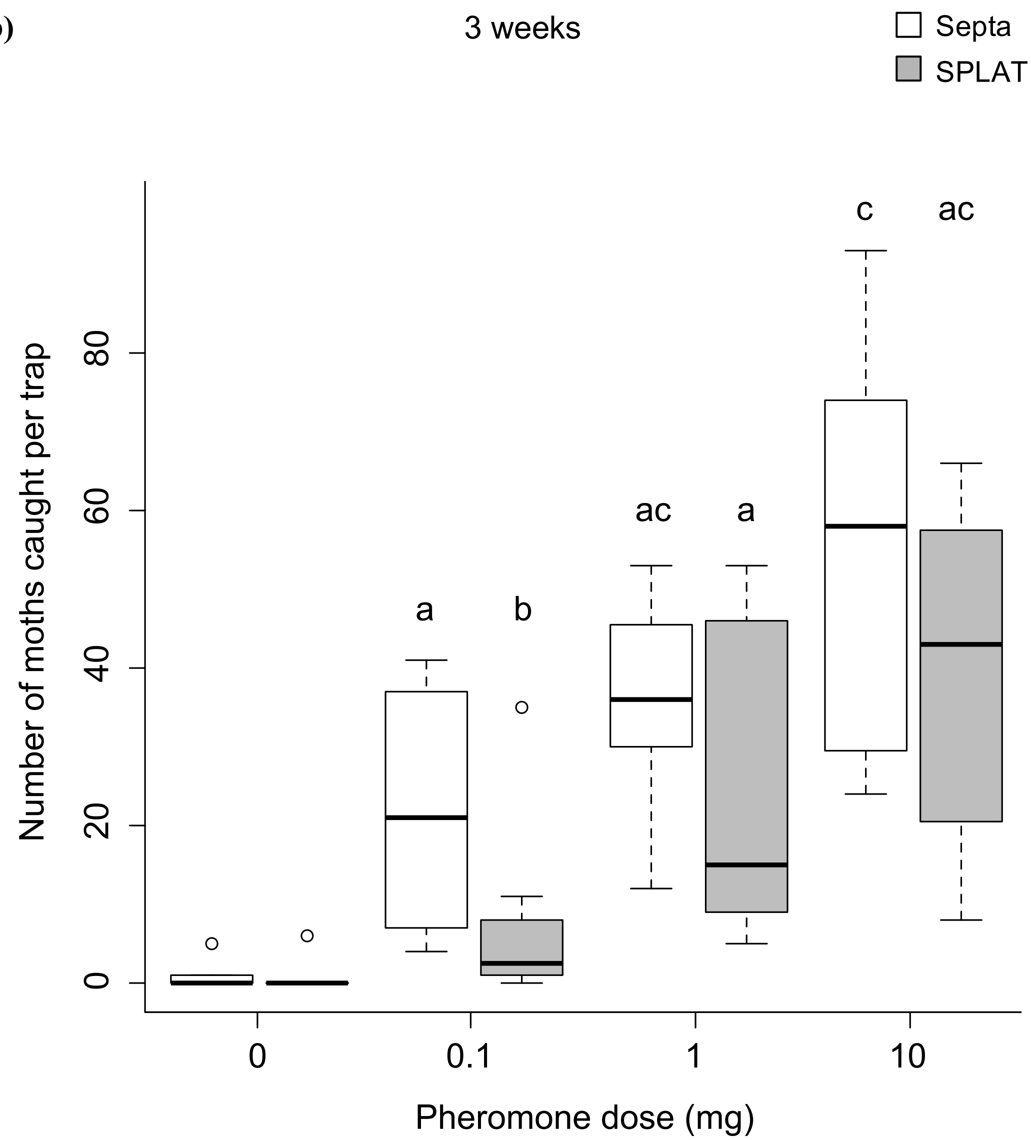
Figure 2.1

(a)



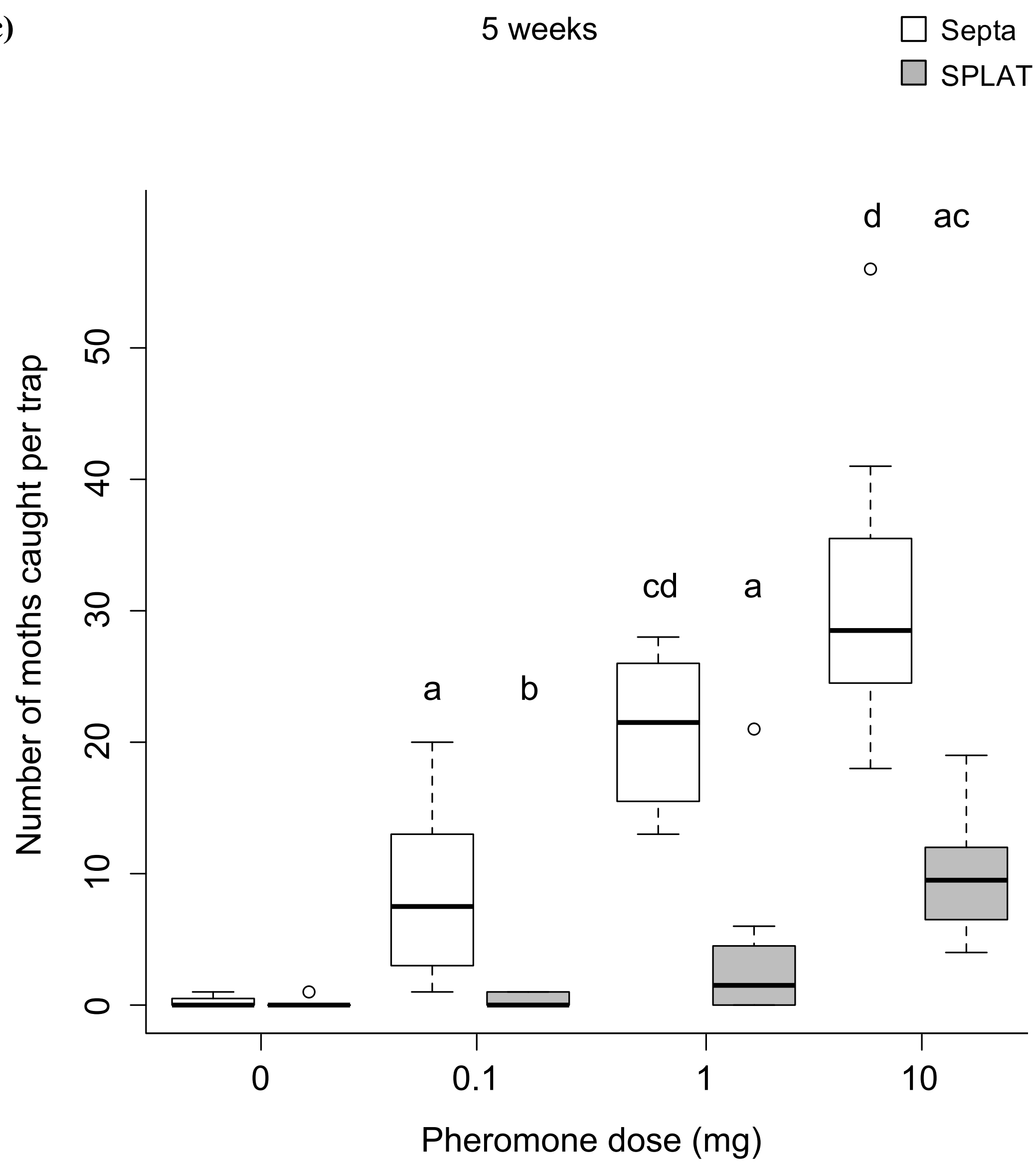
1 week
Septa
SPLAT
Number of moths caught per trap
0
20
40
60
80
100
120
140
a
ab
b
bc
bc
c
0
0.1
1
10
Pheromone dose (mg)

(b)



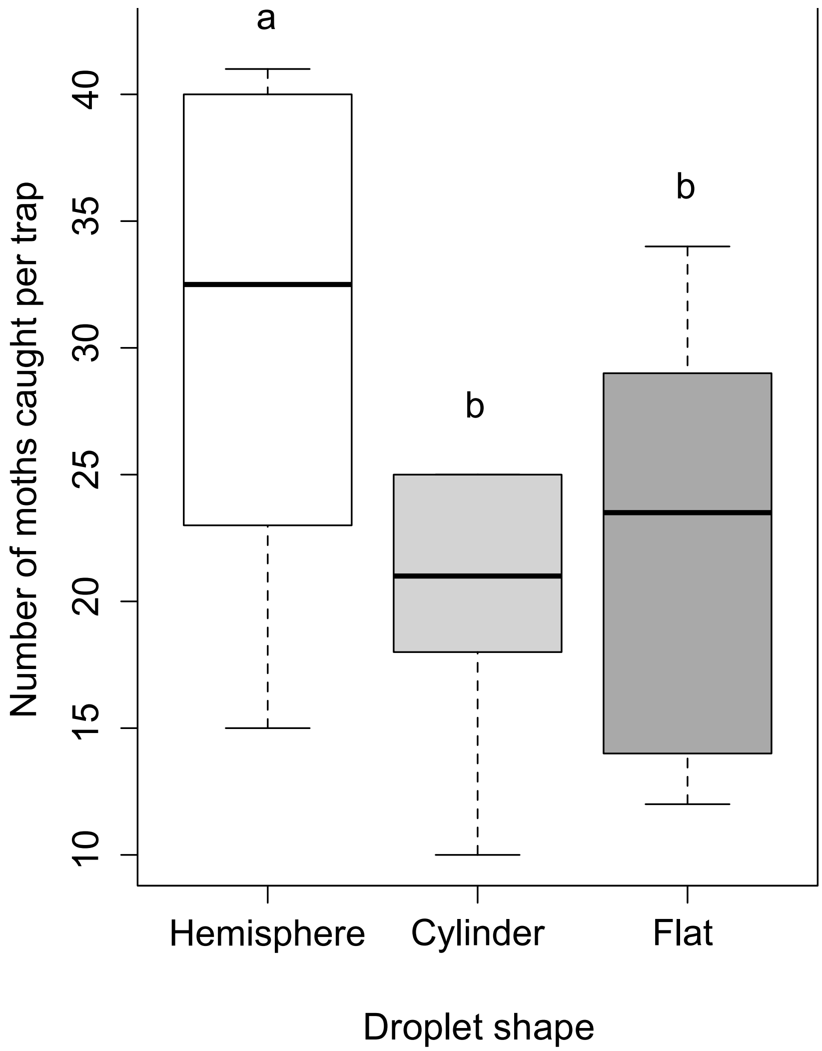
3 weeks
Septa
SPLAT
Number of moths caught per trap
0
20
40
60
80
a
b
ac
a
c
ac
0
0.1
1
10
Pheromone dose (mg)

(c)



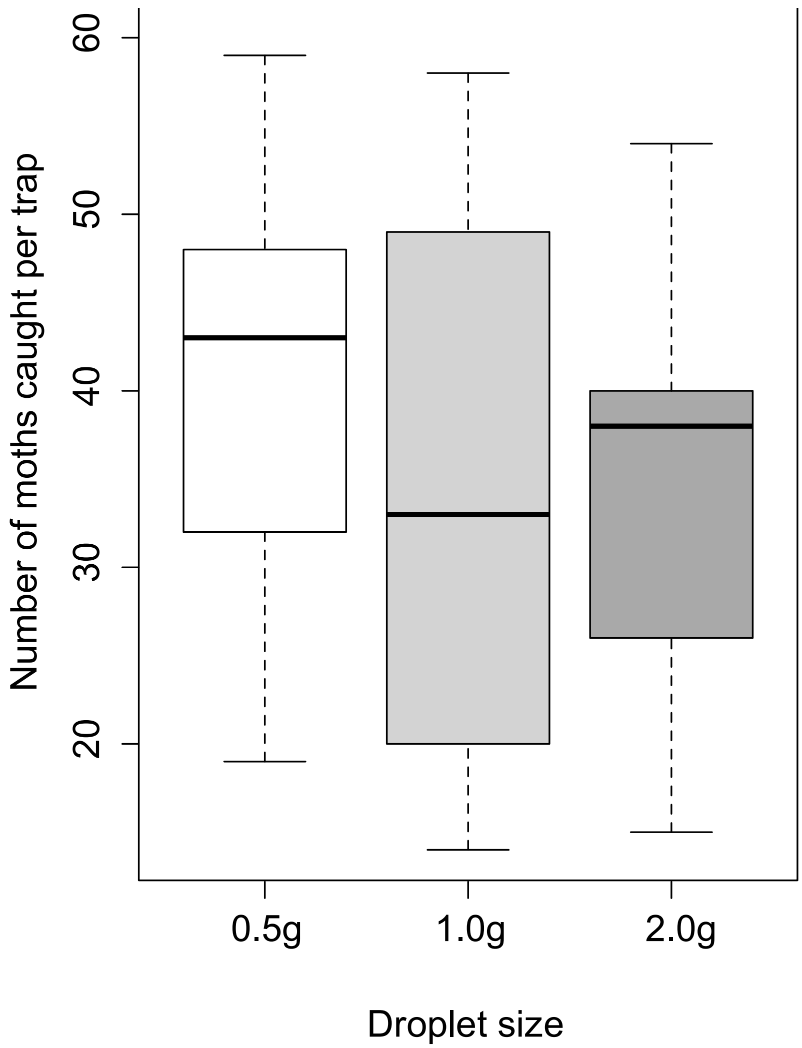
5 weeks
Septa
SPLAT
d
ac
cd
a
a
b
Number of moths caught per trap
0
10
20
30
40
50
0
0.1
1
10
Pheromone dose (mg)

Figure 2.2



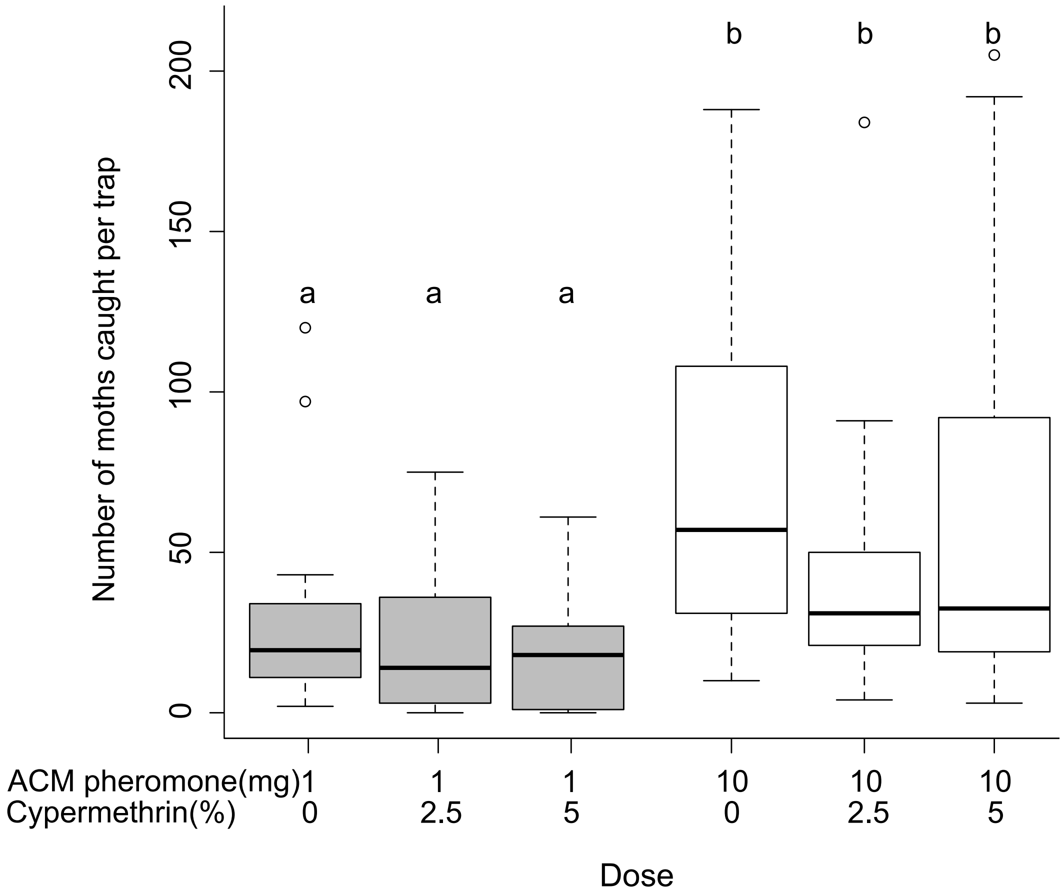
a
b
b
Number of moths caught per trap
10
15
20
25
30
35
40
Hemisphere
Cylinder
Flat
Droplet shape

Figure 2.3



60
50
40
30
20
Number of moths caught per trap
0.5g
1.0g
2.0g
Droplet size

Figure 2.4



b
b
b
a
a
a
200
150
100
50
0
Number of moths caught per trap
ACM pheromone(mg) 1 1 1 10 10 10
Cypermethrin(%) 0 2.5 5 0 2.5 5
Dose

Figure 2.5

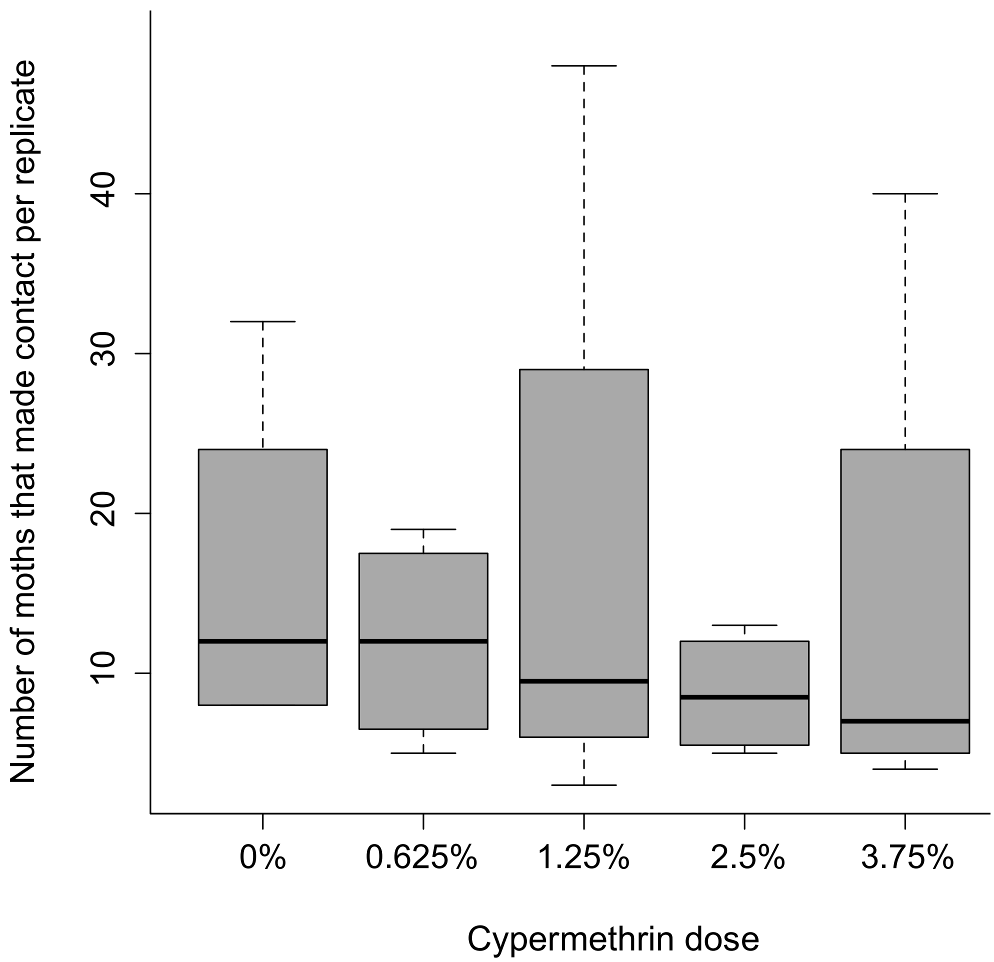

Number of moths that made contact per replicate
10
20
30
40
0%
0.625%
1.25%
2.5%
3.75%
Cypermethrin dose

Figure 2.6

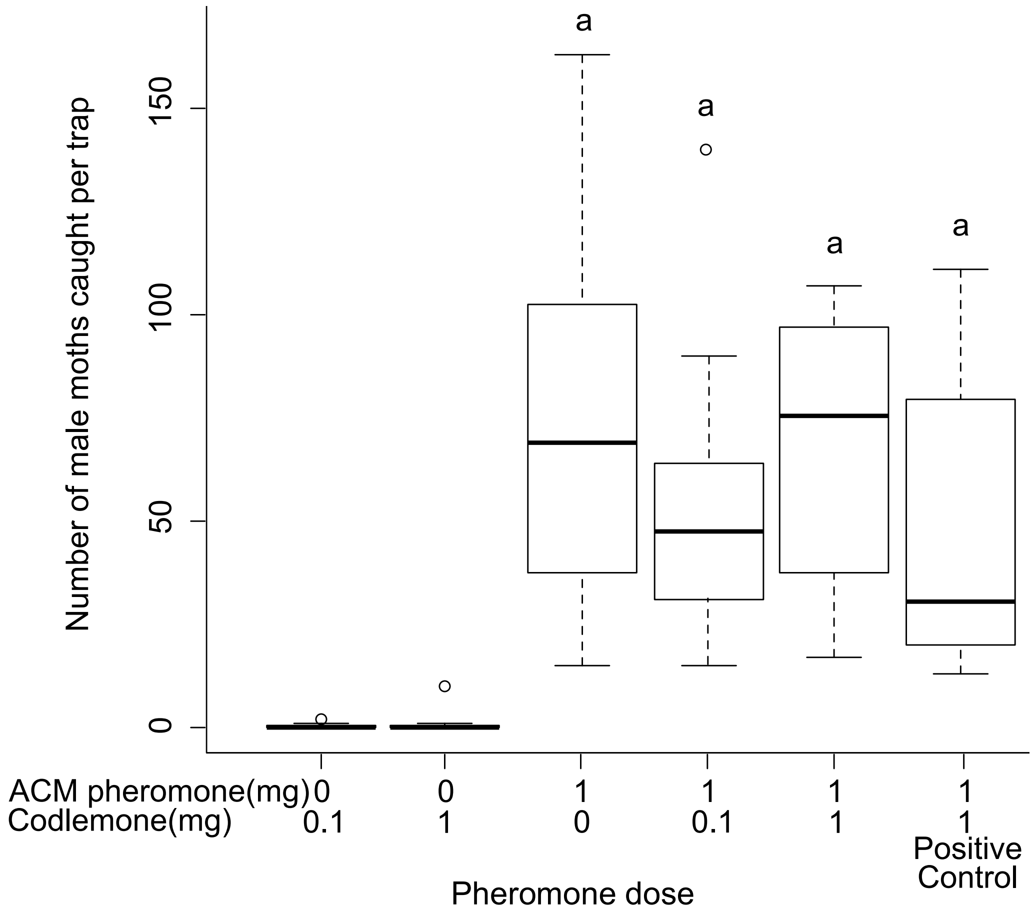

a
a
a
a
150
100
50
0
Number of male moths caught per trap
ACM pheromone(mg) 0 0 1 1 1 1
Codlemone(mg) 0.1 1 0 0.1 1 1
Positive Control
Pheromone dose

Figure 2.7

3. Visual attraction of male apple clearwing moths, *Synanthedon myopaeformis*, to colored SPLAT in the presence of sex pheromone

3.1 Abstract

The diurnally active apple clearwing moth, *Synanthedon myopaeformis* (Borkhausen), (Lepidoptera: Sesiidae) is a serious invasive pest of apple trees in British Columbia (BC), Canada. The effect of visual cues on the close-range orientation of apple clearwing moth males to sources of sex pheromone was tested as part of the development of a pheromone-based attract and kill system for this species. Olfaction elicits long-distance attraction to pheromone-based SPLAT® formulations, while visual cues facilitate short-range discrimination among SPLAT treatments in the presence of pheromone. A one gram orange-striped black SPLAT droplet was a highly attractive SPLAT treatment tested in close proximity to a sex pheromone source. More males touched the orange-striped black SPLAT than freshly killed females positioned near the sex pheromone source. The high level of close-range orientation of male apple clearwing moths to SPLAT may be a result of similar spectral reflectance properties of SPLAT and female apple clearwing moths.

3.2 Introduction

Sex pheromone-mediated communication for mate location is a primitive trait in the Lepidoptera (Löfstedt & Kozlov, 1997). Sex pheromone communication in moths involves detection by males of a female-produced signal, followed by long-distance orientation to the attractive emitters (Schneider, 1992; Hansson, 1995). Adult males may also utilize host-plant volatiles to orient to hosts to facilitate mating (Landolt & Phillips, 1997) or to enhance their attraction to sex pheromone (Yang et al., 2004; von Arx et al., 2011; von Arx et al., 2012). Although both nocturnally and diurnally active moths use sex pheromone communication for mate location (Arn et al., 1986), there is less reliance on olfactory cues in diurnal moths perhaps because these groups utilize visual cues alone or in conjunction with olfactory cues for host recognition (Naumann et al., 1991; Dobson, 1994; Balkenius et al., 2006; Reddy et al., 2009) and mate location (Barry & Nielsen, 1984; Koshio & Hidaka, 1995; Karalius & Būda, 2007; Toshova et al., 2007; KonDo et al., 2012; Monteys et al., 2012). Diurnally active butterflies readily use visual signals for mate detection and recognition (Silberglied & Taylor, 1978; Rutowski et al., 2001; Pinzari & Sbidorni, 2013; Tang et al., 2013).

The use of multiple sensory modalities by moths, such as vision and olfaction, may provide a more reliable process to locate potential mates (Hidaka, 1972; Toshova et al., 2007). The level of reliance on each sensory modality is often dependent on the circadian rhythm of the insect. For example, the nocturnal Elephant hawkmoth, *Deilephila elpenor* (L.), (Lepidoptera: Sphingidae), responds strongly to host-plant odor to elicit foraging behavior, while the diurnally active Hummingbird hawkmoth of the same subfamily, *Macroglossum stellatarum* (L.), preferentially responds to visual stimuli (Balkenius et al., 2006). Similarly, diurnal moths rely more on visual cues than their nocturnal counterparts for mate finding and discrimination (Barry & Nielsen, 1984; Koshio & Hidaka, 1995; Toshova et al., 2007; KonDo et al., 2012). In these systems, mate searching is prompted by the detection of sex pheromone plumes; however, once males are in the vicinity of the calling female, visual cues increase attraction to the pheromone or are necessary to initiate copulatory behaviors. Such cues may include reflectance of ultraviolet (UV) (Silberglied & Taylor, 1978; Robertson & Monteiro, 2005) and visible wavelengths (Wiernasz, 1995; Karalius & Būda, 2007; Otálora-Luna et al., 2013), or physical properties such as shape (Gross et al., 1983; Gaskett, 2011), and size (Pinzari & Sbordoni, 2013). Responses to visual cues are also enhanced by the presence of host-plant volatiles (Raguso & Willis, 2005; Reddy et al., 2009).

The apple clearwing moth, *Synanthedon myopaeformis* (Borkhausen), (Lepidoptera: Sesiidae), is a recently introduced diurnally active pest of apple trees in the Similkameen Valley of BC (Judd et al., 2011). This native of Eurasia (Zhang, 1994) and Africa (Ateyyat & Al-Antary, 2006) was first discovered in Canada in an apple orchard in Cawston, BC in 2005 (Philip, 2006) and identified, soon after, in Ontario (Beaton & Carter, 2006). This moth has since spread south to Washington, USA (LaGasa et al., 2009). Since the larval feeding stage of the apple clearwing moth is protected under the bark, various semiochemical-based control strategies, such as mass trapping (Trematerra, 1993; Aurelian et al., 2012) and mating disruption (Stüber & Dickler, 1987; Kyparissoudas & Tsourgianni, 1993), have been tested to target the adult stage. Though these provide some level of pest control, these tactics can be labor- and cost-intensive. Attract and kill, another semiochemical-based management tactic, may provide a more economical approach to control the apple clearwing moth in BC.

Attract and kill is a technique that involves a lure and an affector. The lure may consist of olfactory cues, such as sex pheromone, visual cues, acoustic cues or a combination of these signals. The affector is a substance that kills the attracted target insect on contact (i.e., via insecticidal poisoning), or indirectly through the activity of sterilants or pathogens (El-Sayed et al., 2009). Most commercially available attract and kill formulations for lepidopteran pests incorporate sex pheromone as the lure and pyrethroid insecticides as the affector (Suckling & Brockerhoff, 1999; Charmillot et al., 2000; Evenden & McLaughlin, 2004). As lepidopteran sex pheromones are usually species-specific signals, attract and kill provides an environmentally-sound approach to manage target insect pests with minimal insecticidal use (Howse et al., 1998; Gut et al., 2004).

SPLAT® (Specialized Pheromone & Lure Application Technology), is a formulation made of an inert wax-based matrix that can be used to release sex pheromone or other semiochemicals and insecticide to attract and kill many moth pests. Although SPLAT was originally developed as a mating disruption tactic to release only semiochemicals (Jenkins & Isaacs, 2008; Stelinski et al., 2010; Teixeira et al., 2010), the successful incorporation of insecticide into the matrix provides the opportunity to use this product for attract and kill (ISCA Technologies, 2012; Vargas et al., 2010; Pastori et al., 2012). SPLAT may provide a less labor-intensive option than other semiochemical-based management tools, such as mass trapping and mating disruption, due to the range of application methods (i.e., caulking gun, backpack sprayer, tractor, or aircraft) available for this formulation (ISCA Technologies, 2012; Stelinski et al., 2010; Teixeira et al., 2010).

The development of a pheromone-based attract and kill formulation to target the apple clearwing moth is possible because its sex pheromone has recently been identified as (3*Z*,13*Z*)-octadecadienyl acetate (3*Z*,13*Z*)-18:OAc) (Voerman et al., 1978; Judd et al., 2011). Since the apple clearwing moth is a diurnally active moth and SPLAT is commercially available in an array of colors, visual cues can also be tested to improve the level of source contact with the formulation. If male apple clearwing moths utilize multi-sensory modalities to locate mates, incorporation of visual cues may increase male attraction to the formulation at close-range. Male apple clearwing moths orient at close-range to the "red" bands on the abdomen of adult females (Stüber & Dickler, 1986). Similarly, male peachtree borers, *S. exitiosa* (Say), (Lepidoptera: Sesiidae), orient to objects with stripes that closely resemble the colors of adult females (Barry, 1978).

The objectives of this study were to test the hypothesis that (1) male apple clearwing moths exhibit visual discrimination among variously colored SPLAT in the presence of their sex pheromone, and (2) to determine which visual cues or spectral qualities facilitate the greatest contact with these lures.

3.3 Materials and methods

3.3.1 Preparation of SPLAT and pheromone lures

All droplets of SPLAT® (ISCA Technologies, Riverside, California) were prepared with 1g (wet weight) of SPLAT, unless stated otherwise. One gram droplets were placed on polyester strips and aired for 24 h at 23-24°C. Droplets were then molded into a hemispherical shape, transferred to aluminum foil, and dried for an additional 24 h at 35°C. Droplets had a base diameter of 16-17 mm and a final weight of 0.60-0.61 g. Droplets were stored in glass jars at 2-5°C until use in the field. Depending on the experiment, SPLAT droplets and grey rubber septum lures (West Pharmaceutical Services, Lionville, Pennsylvania) were topically loaded with 0.1-, 1- or 10 mg of (3*Z*,13*Z*)-18:OAc (Pherobank, Wageningen, Netherlands) dissolved in 0.2 mL of HPLC-grade hexane (Sigma-Aldrich, St. Louis, Missouri). Lures were aired for 4 h at 23-24°C in a fume hood and stored in glass jars at -20°C until deployment in the field.

3.3.2 Experiment 1: effect of SPLAT color on male moth capture in traps

Experiment 1 (16-24 July, 2011) (Table 3.1) was conducted in a commercial apple orchard in Cawston, BC (49.15°N, -119.73°W) and was designed to test the effect of SPLAT color on male apple clearwing moth capture in a no-choice trapping experiment. The attractiveness of variously colored (grey, black, green, orange, white, and yellow) one gram droplets of SPLAT topically treated with 1 mg of (3*Z*,13*Z*)-18:OAc was compared using a randomized complete block design with six replicates of each treatment.

Cylindrical traps were made with clear sheets of polyester (26.7 X 45.7 cm) rolled into cylinders to minimize visual cues associated with the trap itself (Figure 3.1a). Traps were held together by wire and lined with Tanglefoot-coated (The Tanglefoot Company, Grand Rapids, Michigan, Pennsylvania) polyester sheets (26.7 X 17.1 cm), held in place on the bottom half of the traps by six large paper clips. Traps were suspended from trees 1.5 m above the ground and spaced 20 m apart within a linear array (block) with 20 m between blocks of traps. Moth trap capture was recorded every day; Tanglefoot liners were replaced when saturated with >40 moths. Halfway through the experiment, treatment positions within blocks were re-randomized but lures were not replaced.

3.3.3. Experiment 2: effect of color on male moth close-range orientation to SPLAT droplets

Experiment 2 (2-11 August, 2011), and all subsequent observational experiments (Experiments 3-6) (Table 3.1) were conducted in two commercial apple orchards in Keremeos, BC (49.19°N, -119.74°W; 49.22°N, -119.83°W). Experiment 2 tested the effect of SPLAT color on male apple clearwing close-range orientation in a choice experiment in the presence of the sex pheromone. Six droplets of the variously colored SPLAT used in field experiment 1 but without pheromone were affixed with clear push pins 3 cm apart on a circular arena (13 cm diam.) made of clear polyester. A grey rubber septum topically loaded with 10 mg of (3*Z*,13*Z*)-18:OAc was positioned in a small hole in the center of the arena equidistant from the six droplets (Figure 3.1b). Two arenas were placed 1.5 m above the ground in trees and separated by 20 m. One hour observations were performed when temperatures were above 29°C and during 1100-1500h PDT, when male flight activity to pheromone traps is highest (Judd & Eby, 2013). Visitation was quantified as the number of males that made contact with each SPLAT droplet with their claspers or tarsi per one hour replicate in a total of six replicates. SPLAT droplets and rubber septum lures were replaced and SPLAT droplet position on the arenas was re-randomized after each replicate.

To determine if male moth attraction could be elicited to the variously colored SPLAT droplets in the absence of the sex pheromone lure, a similar experiment was performed 30-31 July, 2012 in which the centrally positioned grey rubber septum contained 0.2 mL of hexane as a control. No moth contact or attraction was observed.

3.3.4 Experiment 3: effect of SPLAT color with or without UV reflectance on male moth close-range orientation to droplets

This experiment (19 and 24 July, 2012) tested the effect of UV reflectance on male apple clearwing moth close-range orientation to SPLAT. Droplets and arenas were prepared as in experiment 2 but two arenas of the six colors were prepared for each of the two UV treatments (+UV and -UV). A UV-absorbing agent developed and used by Andersson and Amundsen (1997) to manipulate UV reflectance of bird plumage and flowers (Johnson & Andersson, 2002) was used to remove UV reflectance in SPLAT. The UV-absorbing compounds, Parsol® MCX (ethylhexyl methoxycinnamate, UVB absorber) and Parsol® 1789 (butyl methoxydibenzoylmethane, UVA absorber), (DSM Nutritional Products, Basel, Switzerland), were mixed in equal amounts and dissolved in J. T. Baker® mineral (paraffin) oil (Avantor Performance Materials, Center Valley, Pennsylvania, USA) at 40:60 (w/w), with gentle heating. Six colored droplets painted with the UV-absorbing agent were placed on each of two arenas with a central grey rubber septum topically loaded with 0.1 mg of (3*Z*,13*Z*)-18:OAc. Two other arenas had paraffin oil-painted droplets arranged around the pheromone lure. Four arenas were suspended from trees 1.5 m above the ground and separated by 20 m at each of four sites. Panasonic HDC-SD90 Full HD Camcorders with a high capacity VW-VBK360 3580 mAh Li-ion battery and a 32 GB class 10 SD card, with automatic white balance were positioned on platforms, staked into the ground near the canopy level at each site. Video recordings were performed when temperatures were above 29°C and between 1100 and 1500 h PDT. Visitation was quantified as the number of males that made contact with SPLAT droplets with their claspers or tarsi per one hour replicate. A total of eight replicates were performed for each UV treatment. At each site, SPLAT droplets and rubber septum lures were replaced and treatment positions were re-randomized after each replicate.

3.3.5 Experiment 4: close-range orientation of male moths to orange-

striped black SPLAT

Experiment 4 (25-26 July, 2012) tested the orientation of male apple clearwing moths to orange-striped black SPLAT, in the presence of the sex pheromone. This experiment was designed to determine if an orange stripe, similar to those found on the tergum of female apple clearwing moths, would increase the close-range orientation of males to the black SPLAT droplet. Orange-striped black droplets were prepared in similar fashion as described above, with the addition of a thin orange SPLAT line to the droplet applied with a syringe. One gram SPLAT droplets of black, orange-striped black, orange, and grey were arranged on arenas with a central grey rubber septum topically loaded with 1 mg of (3*Z*,13*Z*)-18:OAc. Video observations were conducted as in experiment 3. Ten replicates were performed.

3.3.6 Experiment 5: close-range orientation of male moths to orange-striped black SPLAT compared to dead females

Experiment 5 (6-7 August, 2012) compared male apple clearwing moth close-range orientation to orange-striped black SPLAT to that of a dead female apple clearwing moth in the presence of the sex pheromone. One gram orange-striped black droplets and freshly killed females (<20 h deceased) were arranged on arenas with a central grey rubber septum topically loaded with 10 mg of (3*Z*,13*Z*)-18:OAc. Video observations were conducted as in experiments 3 and 4. Twelve replicates were performed.

3.3.7 Experiment 6: close-range orientation of male moths to variously sized orange-striped or -swirled black SPLAT compared to dead females

This experiment (10-11 August, 2012) tested the effect of SPLAT droplet size on male apple clearwing close-range orientation to orange-striped or orange-swirled black droplets. Orange-swirled black droplets were prepared as outlined for SPLAT droplets tested above, except that black SPLAT and orange SPLAT (at 75:25 w/w) were mixed together. One- and three- gram orange-striped black and orange-swirled black droplets and freshly killed females were arranged on arenas with a central grey rubber septum topically loaded with 10 mg of (3*Z*,13*Z*)-18:OAc. Video observations were conducted as in experiments 3-5. Twelve replicates were performed.

3.3.8 Spectral reflectance from variously colored SPLAT with or without UV

Six droplets of SPLAT in each color were prepared as described in experiment 1 but without pheromone. Three droplets of each color were lightly painted with the UV-absorbing agent used in experiment 3 using a paint brush (-UV), while the other three were painted with paraffin oil (+UV). Droplets were arranged on a black felt background and subjected to three spectral reflectance measurements (i.e., 9 measurements for each color and +/- UV treatment). Measurements on the dorsal side of the abdomen of seven dead female apple clearwing moths were also performed. Spectral reflectance was measured using a

JAZ-PX spectrophotometer with a pulsed Xenon Arc light source (Ocean Optics, Dunedin, Florida, USA) and a 400 μm diameter silica core fibre optic reflection probe (QR400-7-UV-VIS, Ocean Optics) fitted with a custom built 45° black anodized aluminum attachment. A Spectralon white standard (WS-1-SL, Ocean Optics) and dark reference was used to calibrate the spectrophotometer before each set of sample measurements. The spectrophotometer was set to complete 50 scans to average with 0-box car smoothing (averaging of adjacent pixels) and perform measurements with the lamp flashing at 100 Hz (10 ms). Measurements for each color with or without UV were averaged.

3.3.9 Statistical analyses

Analyses were conducted using the R statistical package (R Core Team, 2013) with a significance level of 5%. Data were analyzed by generalized linear mixed effects models (GLMM) with error terms fitted to a negative binomial distribution (glmmADMB package) (Table 3.2). Data with no significant random effects were analyzed by generalized linear models (GLM) with error terms fitted to a negative binomial distribution. Significant results were followed by a *post-hoc* comparison of means using the Tukey's HSD multiple comparison test.

3.4 Results

3.4.1 Experiment 1: effect of SPLAT color on male moth capture in traps

Capture of males was similar (χ^2=4.1173, df = 5, P = 0.53, Figure 3.2) in traps baited with variously colored SPLAT droplets treated with 1 mg pheromone.

3.4.2 Experiment 2: effect of color on male moth close-range orientation to SPLAT droplets

SPLAT color in this choice-experiment had an effect on male apple clearwing close-range orientation (Figure 3.3). Males contacted the black droplet significantly more times than they contacted the other colors or the centrally located rubber septum lure containing the pheromone source (χ^2=341.91, df = 5, P < 0.0001). Typical contact behaviors of males include hitting their claspers against the droplet (Figure 3.1b).

3.4.3 Experiment 3: effect of SPLAT color with or without UV on male moth close-range orientation to droplets

Male apple clearwing moths only contacted black droplets in the –UV and +UV treatments in this experiment. Significantly more male apple clearwing moths made contact to droplets that reflected UV compared to those that did not (χ^2=17.336, df = 1, P <0.0001, Figure 3.4).

3.4.4 Experiment 4: close-range orientation of male moths to orange-striped black SPLAT

Significantly fewer male moths contacted the orange droplet as compared to the black or orange-striped black SPLAT (χ^2=162.256, df = 3, P <0.0001, Figure 3.5). Close-range orientation was numerically highest to orange-striped black SPLAT, but the number of moths that contacted orange-striped black and black SPLAT did not differ significantly.

3.4.5 Experiment 5: close-range orientation of male moths to orange-striped black SPLAT compared to dead females

More male apple clearwing moths contacted the orange-striped black SPLAT than freshly killed females in the presence of a sex pheromone source (χ^2=30.026, df = 1, P <0.0001, Figure 3.6). Orange-striped black SPLAT appears to provide a strongly attractive visual cue for apple clearwing males.

3.4.6 Experiment 6: close-range orientation of male moths to variously sized orange-striped or -swirled black SPLAT compared to dead females

Males oriented differentially to the variously sized orange-striped and -swirled black SPLAT and freshly killed females (χ^2=22.301, df = 4, P <0.001, Figure 3.7). Moth visitation was significantly higher to one gram orange-striped black SPLAT than to three gram orange-swirled black SPLAT. There is a non-significant trend for greater orientation of male moths to SPLAT compared to dead females.

3.4.7 Spectral reflectance from variously colored SPLAT with or without UV

Ultraviolet reflectance (300-400 nm wavelengths) was successfully removed in each colored droplet treated with the UV-absorbing compounds (Figure 3.8a). Some of the reflectance in the other wavelengths was altered with the addition of the UV-absorbing agent, but the presence or absence of UV remained stable. The abdominal orange stripe on female apple clearwing moths exhibit similar percent reflectance of UV as observed in black SPLAT (Figure 3.8b, c). Percent reflectance gradually increased in the yellow-orange-red wavelengths (500-700 nm).

3.5 Discussion

Male apple clearwing moths demonstrate spectral discrimination during close-range orientation to pheromone sources. Sex pheromone is required to initiate and facilitate long-distance orientation to the variously colored SPLAT, but once in the vicinity of the pheromone source, visual orientation dictates contact with SPLAT droplets. This is similar to the visually oriented close-range courtship behavior of male apple clearwing moths (Stüber & Dickler, 1987), and other diurnal moth species (Barry & Nielsen, 1984; Koshio & Hidaka, 1995; Toshova et al., 2007; KonDo et al., 2012). Male apple clearwing moths demonstrate spectral sensitivity to UV and behavioral preference to black SPLAT or black with orange SPLAT presented as a stripe or swirl in the droplet matrix. Male moth contact of SPLAT droplets in the observation experiments (Figure 3.1b) is reminiscent of the copulatory behavior of this species described by Stüber and Dickler (1987). This behavior involves orientation to the pheromone source followed by stabs to the female's abdomen aimed at the "red" stripe on the dorsal side (Stüber & Dickler, 1987). This type of mating behavior also occurs in other diurnal Sesiids with striped abdomens including the peachtree borer, *S. exitiosa*

(Barry, 1978; JJ Kwon, personal observation), and the lilac borer, *Podosesia syringae* (Harris) (Nielsen et al., 1975).

Response to SPLAT by male apple clearwing moths requires an initial upwind oriented response to sex pheromone. Males were not captured in traps without a pheromone source (Kwon et al., unpublished) or observed near platforms with colored SPLAT droplets without pheromone in the current study. In the no-choice trapping experiment, a similar number of males was captured in traps baited with grey, black, green, orange, white, or yellow SPLAT with topical pheromone treatment (Figure 3.2). This suggests that male moths orient to the source of the sex pheromone and become trapped before close-range copulatory behaviors occur. Long-distance mate location behavior using sex pheromones also occurs in other diurnally active moths such as *S. exitiosa* (Barry, 1978), *P. syringae* (Nielsen et al., 1975), the Japanese nine-spotted moth, *Amata fortunei* (Orza), (Lepidoptera: Arctiidae) (KonDo et al., 2012) and the white-tailed zygaenid moth, *Elcysma westwoodii* (Vollenhoven), (Lepidoptera: Zygaenidae) (Koshio & Hidaka, 1995). Mate finding via female-produced sex pheromones is a primitive trait in the Lepidoptera (Löfstedt & Kozlov, 1997) that is retained in day flying moths. Although females typically release small quantities of the signal (Haynes et al., 1983; Bäckman et al., 1997), apple clearwing moth males are attracted to sex pheromone over a wide range of doses between 0.1 and 10 mg (Judd, 2008; Judd et al., 2011). Pheromone dose of (3*Z*,13*Z*)-18:OAc varied in the observational studies in the current work (Table 3.1) to ensure sufficient visitation numbers of males throughout the flight period (Judd, 2008).

Male apple clearwing moths preferentially orient to and contact black SPLAT when given the opportunity to visually discriminate between variously colored SPLAT in choice arenas in the presence of pheromone (Figure 3.3). Similarly, African vine borers, *Melittia oedipus* (Oberthür), (Lepidoptera: Sesiidae), are strongly attracted to yellow visual cues in the presence of food sources (Reddy et al., 2009). The addition of an orange stripe to mimic the stripe on the abdomen of female apple clearwing moths (Figure 3.8b, c) did not significantly increase visitation rates compared to black SPLAT alone (Figure 3.5). Reflectance of UV from black SPLAT significantly affected the attraction of apple clearwing moths (Figure 3.4). This is consistent with spectral sensitivity measurements of male and female apple clearwing moth eyes (Eby et al., 2013) that indicate two strong peaks of sensitivity in the green (500-560 nm) and UV (330-370 nm) regions of the spectrum. Spectral sensitivity of apple clearwing moth eyes to red light (660-700 nm) gradually increases with longer wavelengths but does not display a strong peak. Apple clearwing moth eyes are sensitive to green light (Eby et al., 2013) and all SPLAT colors reflected green wavelengths in the current study (Figure 3.8a), however, green SPLAT was not attractive to male apple clearwing moths (Figure 3.3). The intensity of reflectance may have an effect on male attraction.

Male apple clearwing moths were attracted to black and orange SPLAT at close range, which displayed the lowest intensity of reflectance in the green region (~5% reflectance). The intensity of visual light reflected from an object can provide achromatic information that may be relevant in visual discrimination

(Goyret & Kelber, 2012). Honey bees rely on long-wave receptors (green-sensitive photoreceptors) to detect variation in quantities of light for motion detection and spatial recognition (achromatic) (Lehrer et al., 1988); whereas, color detection (chromatic) depends on the comparison of signals in the green-, blue-, and UV-sensitive photoreceptors (Briscoe & Chittka, 2001). Diurnally active hummingbird hawkmoths use both achromatic and chromatic cues to control proboscis movement during hovering flight, but rely on achromatic cues in the absence of chromatic differentiation in flowers (Goyret & Kelber, 2012). A reliance on achromatic information for flower foraging occurs in the nocturnal hawk moth, *Manduca sexta* (L.) (Lepidoptera: Sphingidae) (Goyret, 2010). The ability to detect achromatic differences in light intensity may be more prevalent in nocturnal insects, but this innate ability may be useful to diurnal insects when fine visual discrimination is required quickly. Male apple clearwing moths have the ability to utilize chromatic cues but also appear to gather achromatic information, such as reflectance intensity which they may use to identify potential mates at close-range. Since apple clearwing moths are very dark (Figure 3.8c) in comparison to the objects in their natural environment, males may be more attracted to objects with low levels of reflectance in the green wavelengths because this contrast could indicate the presence of an apple clearwing moth female. Low reflectance in this region also occurs in the orange-striped black SPLAT that attracts male apple clearwing moths.

A low level of ultraviolet reflectance (~5% reflectance) is important for male apple clearwing moth close-range attraction. This low level of UV reflectance was only observed in black SPLAT, which was consistently attractive to males at close-range in all observational experiments. The specificity of response of apple clearwing moth males to low levels of UV reflectance could be due to a low level of UV reflectance from the orange stripe on females in comparison to the level of UV reflected by the rest of their body, although further quantification of the UV reflectance from female bodies is necessary to support this hypothesis. Honey bees, butterflies, and other diurnally active insects are sensitive to patterns expressed in UV light for host-plant discrimination and/or mate recognition (Briscoe & Chittka, 2001). Butterflies often rely on sexually dimorphic wing patterns that reflect UV light more readily than patterns expressed in visible light (Silberglied & Taylor, 1978; Silberglied, 1979; Robertson & Monteiro, 2005) during close-range female and male interactions. UV expression also varies in diurnally active Lepidoptera. Female white cabbage butterfly (*Pieris rapae crucivora,* Boisduval) (Lepidoptera: Pieridae) wings strongly reflect UV while male wings strongly absorb UV (Obara & Hidaka, 1968); whereas, female large grass yellow butterflies (Lepidoptera: Pieridae) (*Eurema hecabe*, L) (Kemp, 2007) and orange sulphur butterflies (*Colias eurytheme*, Boisduval) (Lepidoptera: Pieridae) (Papke et al., 2007) rely on the UV reflective scales of males to evaluate their mates. Female *C. eurytheme* will reject males that do not reflect UV (Silberglied & Taylor, 1978). Reliance on UV expression also promotes the avoidance of interspecific copulation among closely related Lepidoptera species, such as the sulphur butterflies, *C. eurytheme* and *C.*

philodice, especially among species that share pheromone components (Silberglied & Taylor, 1973).

The ability to detect some levels of red light would also explain male apple clearwing moth attraction to the bands on conspecific females (Stüber & Dickler, 1987) and to the orange-striped black SPLAT in the current study. The three properties identified here: low levels of reflectance in the visible green region, low levels of UV reflectance, and reflectance in the orange region constitute a visual cue that elicits the greatest attraction of male apple clearwing moths and are also present in the spectral reflectance of the female abdomen (Figure 3.8b). This may imply that orange-striped black SPLAT provides an adequate visual mate-finding cue for male apple clearwing moths. The addition of female mimics in traps acts as a mate-finding cue for male *Heliothis zea* (Boddie) (Lepidoptera: Noctuidae) and increases moth capture (Gross et al., 1983) in pheromone traps. Male apple clearwing moths oriented to and contacted orange-striped black SPLAT droplets more frequently than freshly killed females (Figure 3.6). This suggests that orange-striped black SPLAT can provide a good visual courtship stimulus that is more attractive than real females; however, it is also possible that males are deterred by olfactory cues from dead females. A more refined mimic would consist of orange-striped black SPLAT droplets with UV eliminated from the orange stripe.

It is likely that male apple clearwing moths have two photoreceptors and the potential for dichromatic vision (Mazokhin-Porshnyakov, 1969) active in the green and UV wavelengths. Other insects that display this type of color vision system include *S. tipuliformis* (Clerck) (currant clearwing moth, Lepidoptera: Sesiidae) (Karalius & Būda, 2007), *Libelulla quadrimaculata* (L.) (four-spotted chaser, Odonata: Libellulidae) (Mazokhin-Porshnyakov, 1959), *Periplanata americana* (L.) (American cockroach, Blattodea: Blattidae) (Walther, 1958), and *Musca domestica* (L.) (housefly, Diptera: Muscidae) (Mazokhin-Porshnyakov, 1960). Trichromatic vision with spectral sensitivity towards UV, blue, and green light, is prevalent in butterflies, honeybees and bumblebees, (Briscoe & Chittka, 2001; Mazokhin-Porshnyakov, 1969), as well as some diurnal moths such as the hummingbird hawkmoth, *M. stellatarum* (Goyret & Kelber, 2012).

Orange-swirled droplets were tested to determine if a more commercially feasible product would elicit short-range orientation behavior from male apple clearwing moths as contact is crucial for optimal effectiveness of attract and kill formulations. Differently sized droplets were tested to establish if size is a potential visual cue utilized by apple clearwing moths in conjunction with spectral reflectance. One gram striped-droplets were more attractive than the three gram droplets but all other size and pattern configurations elicited intermediary attraction (Figure 3.7). This may imply that there is an upper size threshold for the visual stimulus that elicits courtship behavior in this species. Judd and Eby (2013) found that placement of pheromone lures in black pheromone baskets inserted in non-saturating yellow bucket Unitraps lowered trap capture of male apple clearwing moths compared to similarly baited traps with the pheromone positioned in green or white baskets. The explanation for deterrence from the black baskets could be due to the large size of the baskets compared to female apple clearwing moths (Judd & Eby, 2013). Similarly, black pheromone-baited delta traps with yellow stripes, designed to mimic female currant clearwing moths, are less attractive to male currant clearwings than yellow delta traps (Suckling et al., 2005). When male peach tree borers were presented with female moth mimics, models that were 1-2 cm in length and 1.2 cm in diameter were more attractive than models 4 cm long and less than 1 cm wide, in the presence of sex pheromone (Barry, 1978).

Our results lend support to the hypothesis that male apple clearwing moths utilize multi-modal sensory systems to locate pheromone sources. The incorporation of visual cues, such as spectral reflectance and size, to olfactory cues consistently increased contact of male apple clearwing moths with pheromone sources. SPLAT is a promising medium for use in attract and kill to target apple clearwing moths, as orange-striped black SPLAT displays similar spectral reflectance to the abdominal stripe of female apple clearwing moths. In addition, these moths have the ability to discriminate orange-striped black SPLAT from the background (host plant) and other lures.

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Tables

Table 3.1 Trapping experiments conducted on male apple clearwing moths were performed in July in 2011. Male moths were presented with SPLAT droplets topically loaded with a dose of (3*Z*, 13*Z*)-18:OAc suspended from experimental traps. Traps were placed 1.5 m above the ground in trees and separated by 20 m. Observational experiments conducted on male apple clearwing moths were performed when temperatures were above 29°C and between 1100-1500 h PDT. Male moths were presented with unbaited SPLAT droplets and/or other treatments affixed onto circular arenas with one central grey rubber septum topically loaded with a dose of (3*Z*, 13*Z*)-18:OAc. Arenas were placed 1.5 m above the ground in trees and separated by 20 m. SPLAT droplets and rubber septum lures were replaced and SPLAT droplet positions on the arenas were re-randomized after each one hour replicate.

Experiment	Dates conducted	Pheromone dose (mg)	N of treatments	N of replicates
1 - trapping; effect of color	16-24 July, 2011	1	6	6
2 - observation; effect of color	2-11 August, 2011	10	6	6
3 - observation; effect of +UV	19 & 24 July, 2012	0.1	6	8
4 - observation; orange-striped	25-26 July, 2012	1	4	10
5 - observation; orange-striped + dead female	6-7 August, 2012	10	2	12
6 - observation; variously sized orange-striped and -swirled	10-11 August, 2012	10	5	12

Table 3.2 Final generalized linear mixed effects models (GLMM) of experiments with significant and non-significant random effects identified. Data with no significant random effects were analyzed by generalized linear models (GLM). Error terms were fit to a negative binomial distribution. (*) indicates significant term effects ($P < 0.05$).

Experiment	Model	Random effects
1 - trapping; effect of color	Visits ~ color + (1|site)	site*, week
2 - observation; color	Visits ~ color* + (1|date)	time, date*
3 - observation; effect of +UV	Visits ~ color*	time, site, date
4 - observation; orange-striped	Visits ~ color* + (time|site)	time*, site*, date
5 - observation; orange-striped + dead female	Visits ~ treatment* + (time|site)	time*, site*, date
6 - observation; variously sized orange-striped and -swirled	Visits ~ treatment* + (time|site)	time*, site*, date

Figure legends

Figure 3.1 (a) Experimental traps employed in Experiment 1 and (b) observation arenas used in Experiments 2 through 6. A male moth in panel b demonstrates typical display of contact with the end of the abdomen striking or clasping onto droplets.

Figure 3.2 Median (± Interquartile Range (IQR)) number of male apple clearwing moths captured in traps baited with 1 mg of (3*Z*,13*Z*)-18:OAc loaded topically onto colored SPLAT droplets. Whiskers represent data that fall within 1.5 X IQR, while outliers are presented by dots that fall between 1.5-3 X IQR.

Figure 3.3 Median (± IQR) number of male apple clearwing moths that made contact with colored SPLAT droplets. The rubber septum was included as a treatment in the analyses as some moths contacted the source of the pheromone. Whiskers represent data that fall within 1.5 X IQR, while outliers are presented by dots that fall between 1.5-3 X IQR. Different letters indicate a significant difference among treatments (Tukey HSD, $P < 0.05$).

Figure 3.4 Median (± IQR) number of male apple clearwing moths that made contact with SPLAT droplets with and without UV. Only black treatments are shown as no contact occurred to the other colors.

Whiskers represent data that fall within 1.5 X IQR, while outliers are presented by dots that fall between 1.5-3 X IQR. Different letters indicate a significant difference between treatments (Tukey HSD, $P < 0.05$).

Figure 3.5 Median (± IQR) number of male apple clearwing moths that made contact with black, orange-striped black, orange and grey SPLAT droplets. Whiskers represent data that fall within 1.5 X IQR, while outliers are presented by dots that fall between 1.5-3 X IQR. Different letters indicate a significant difference among treatments (Tukey HSD, $P < 0.05$).

Figure 3.6 Median (± IQR) number of male apple clearwing moths that made contact with orange-striped black SPLAT and freshly killed females. Whiskers represent data that fall within 1.5 X IQR, while outliers are presented by dots that fall between 1.5-3 X IQR. Different letters indicate a significant difference between treatments (Tukey HSD, $P < 0.05$).

Figure 3.7 Median (± IQR) number of male apple clearwing moths that made contact with orange-striped and -swirled black SPLAT of varying size and freshly killed females. Whiskers represent data that fall within 1.5 X IQR, while outliers are presented by dots that fall between 1.5-3 X IQR. Different letters indicate a significant difference among treatments (Tukey HSD, $P < 0.05$).

Figure 3.8 Relative spectral reflectance curves for (a) six colors of SPLAT and (b) the dorsal region of the abdomen of female apple clearwing moths (with black +UV and orange –UV SPLAT for comparison). Closed circles indicate droplets +UV, while open circles indicate droplets –UV. (c) Female apple clearwing moth with tergum visible.

Figures

(a)

(b)

Figure 3.1

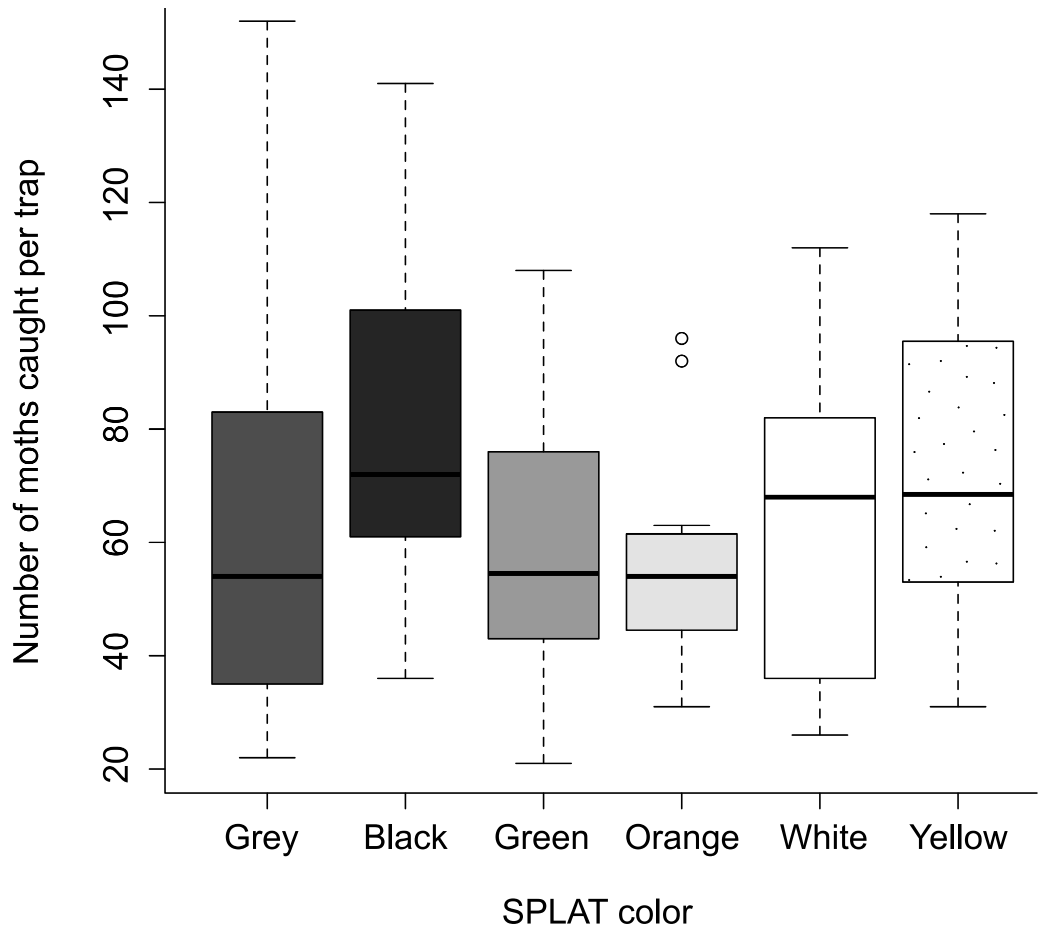

Number of moths caught per trap
20
40
60
80
100
120
140
Grey
Black
Green
Orange
White
Yellow
SPLAT color

Figure 3.2

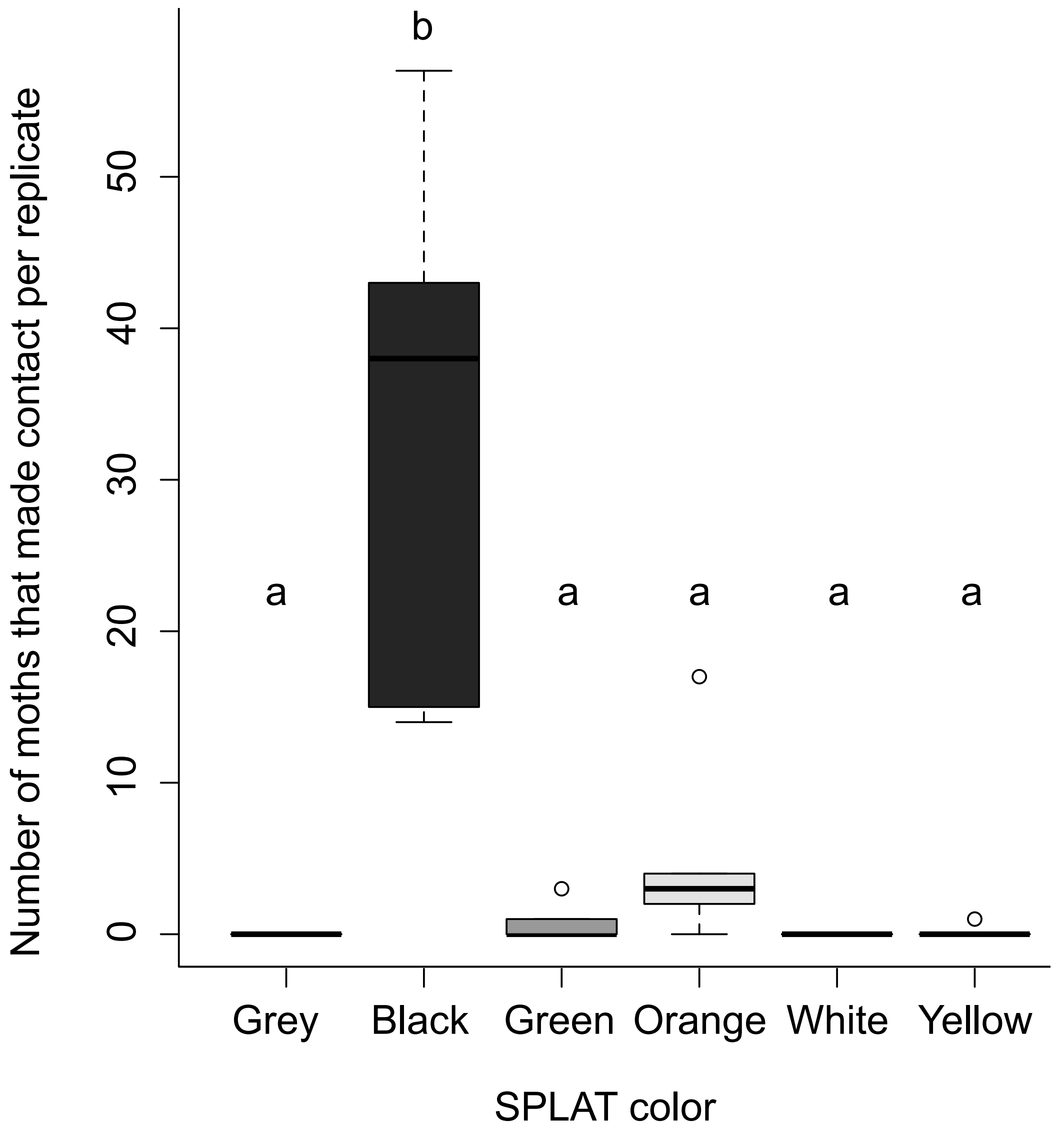

b
a
a
a
a
a
0
10
20
30
40
50
Number of moths that made contact per replicate
Grey
Black
Green
Orange
White
Yellow
SPLAT color

Figure 3.3

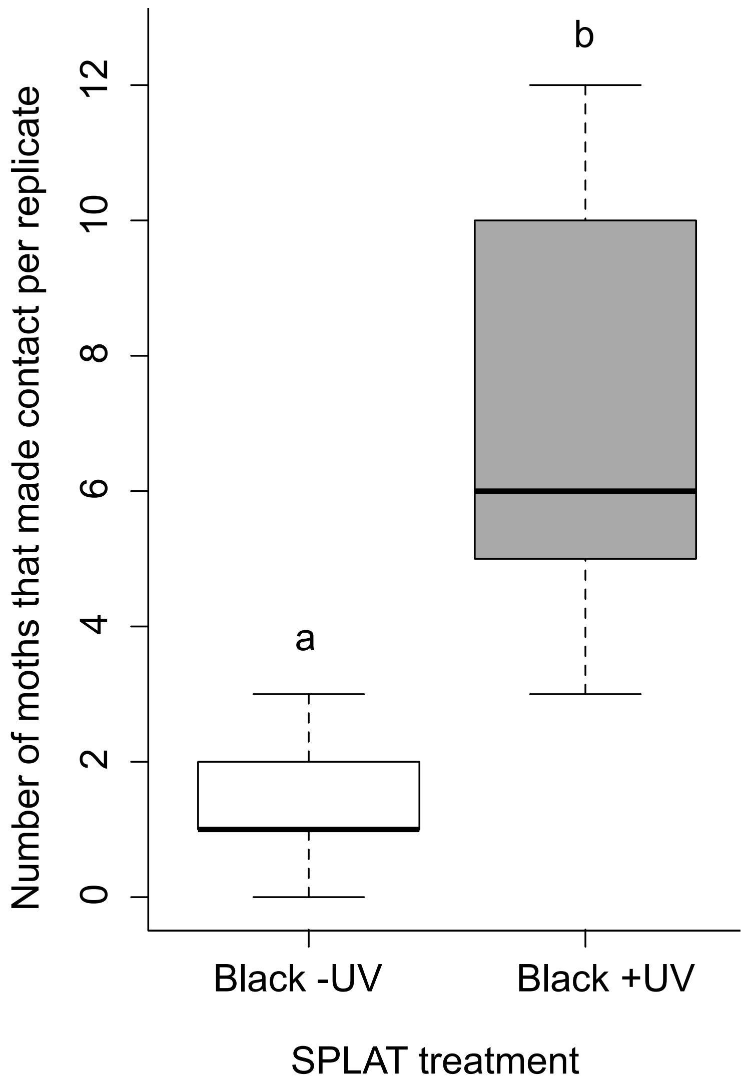

b
a
Number of moths that made contact per replicate
0
2
4
6
8
10
12
Black -UV
Black +UV
SPLAT treatment

Figure 3.4

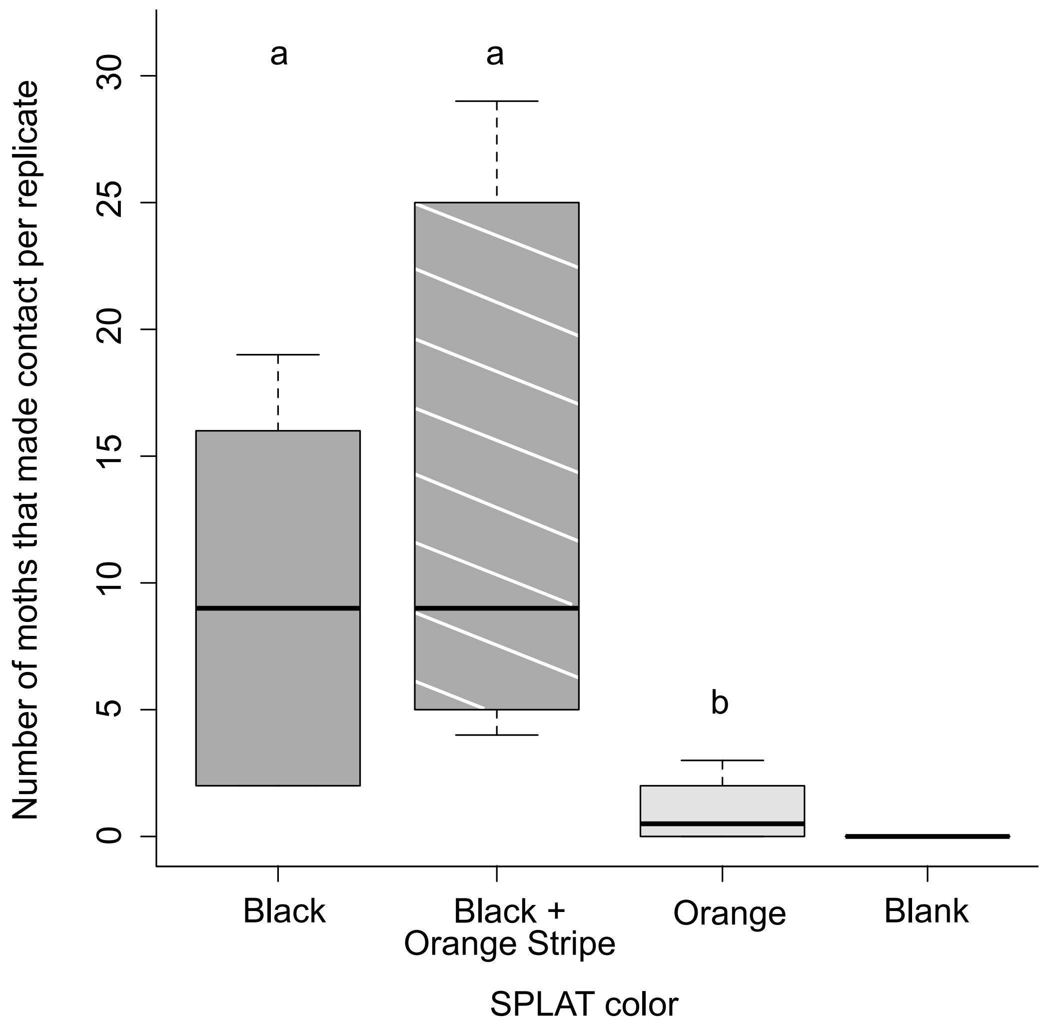

a
a
b
30
25
20
15
10
5
0
Number of moths that made contact per replicate
Black
Black + Orange Stripe
Orange
Blank
SPLAT color

Figure 3.5

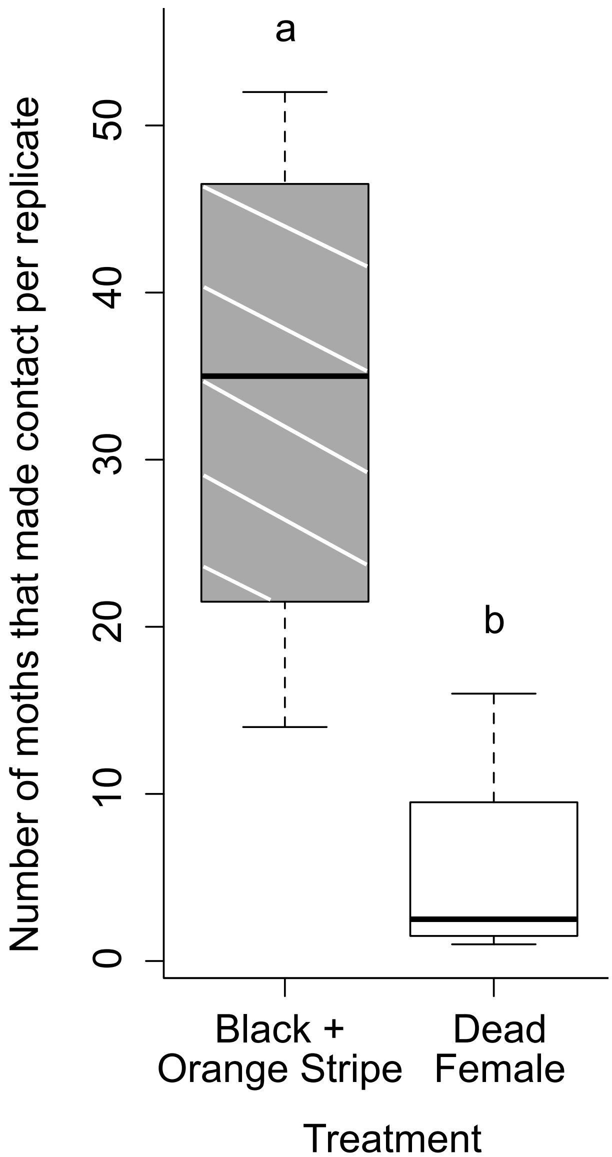

a
b
Number of moths that made contact per replicate
0
10
20
30
40
50
Black +
Orange Stripe
Dead
Female
Treatment

Figure 3.6

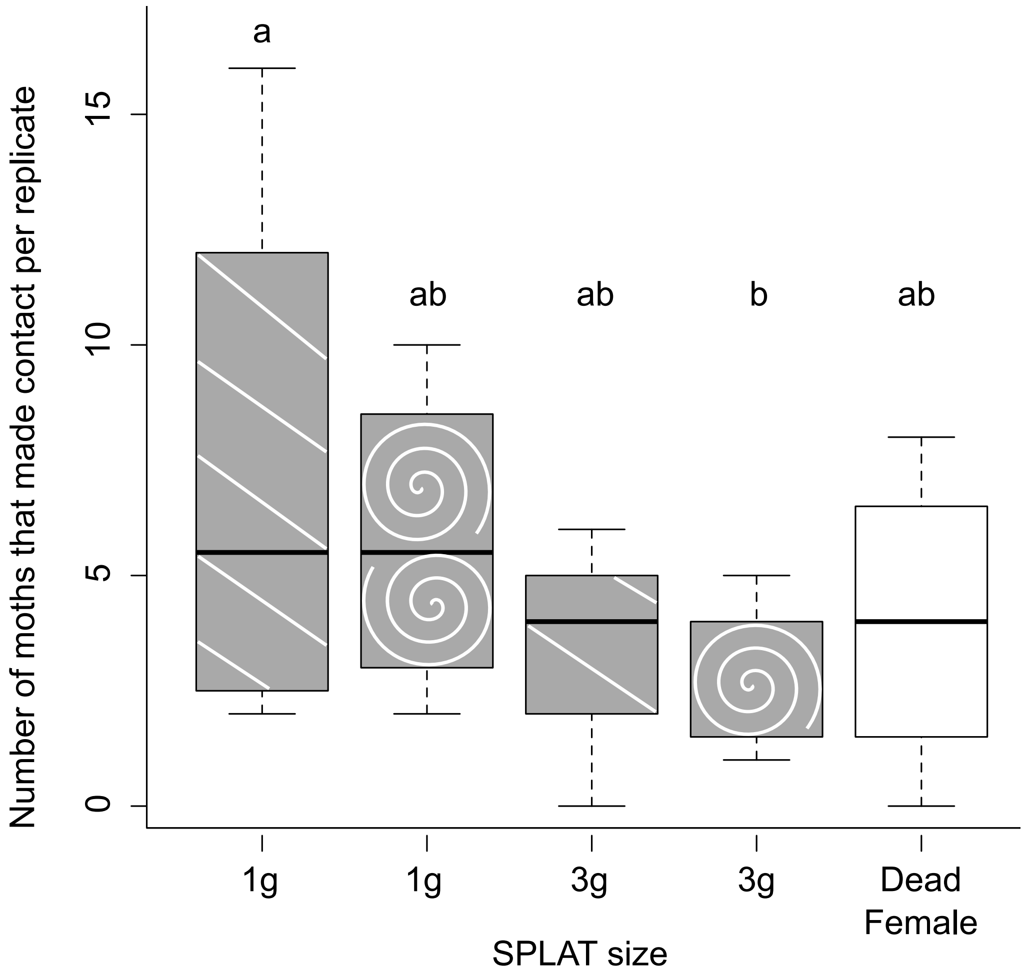
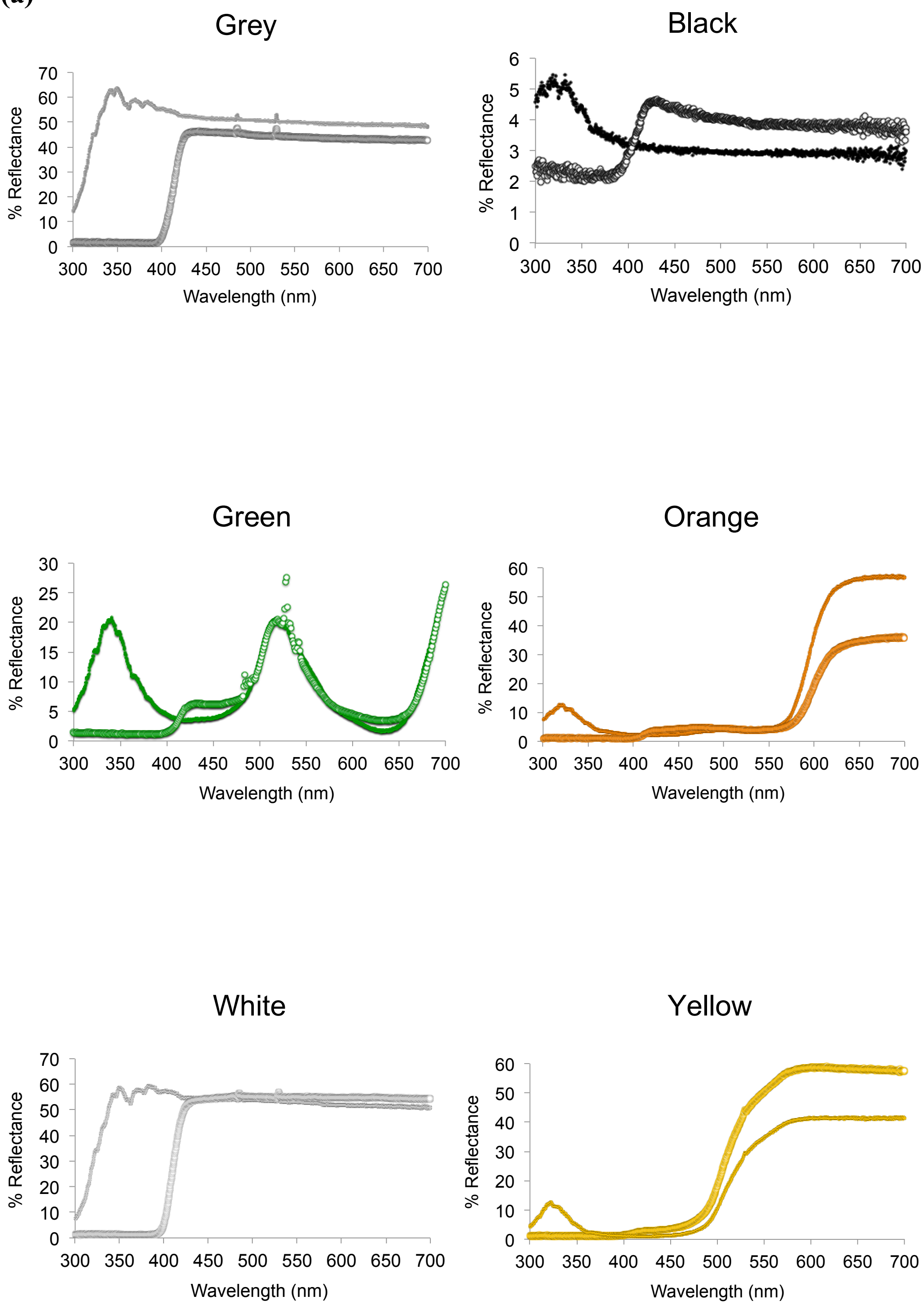
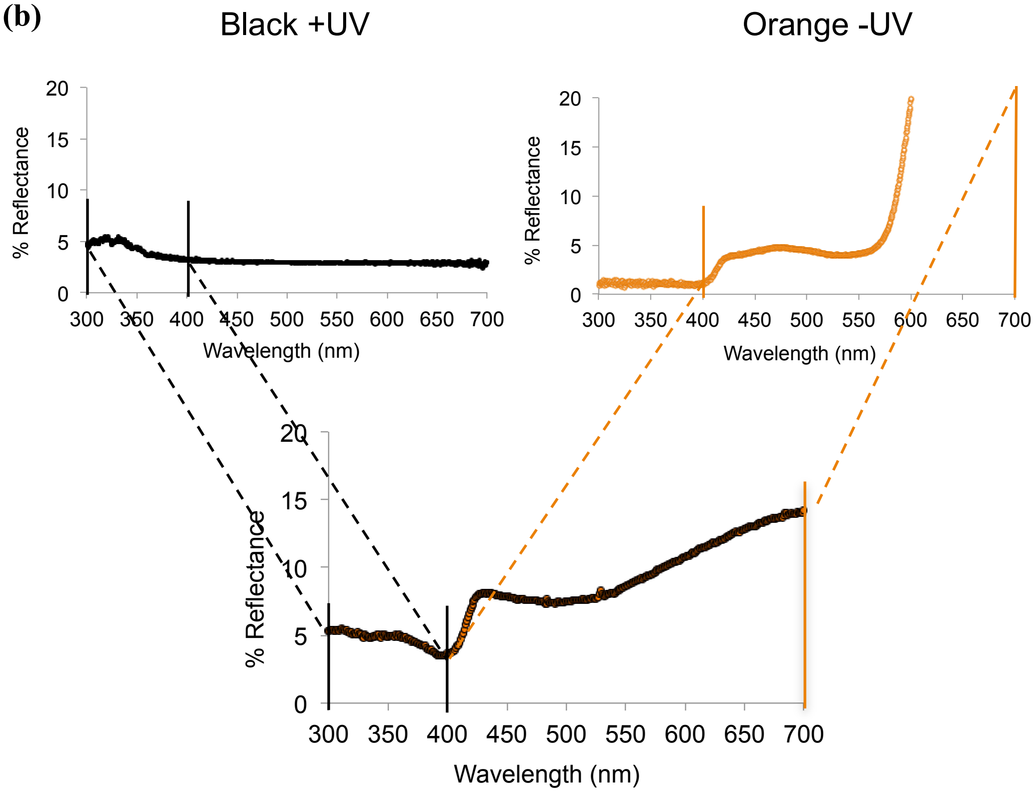

a
ab
ab
b
ab
15
10
5
0
Number of moths that made contact per replicate
1g
1g
3g
3g
Dead
Female
SPLAT size

Figure 3.7

(a)


Grey
Black
Green
Orange
White
Yellow
% Reflectance
Wavelength (nm)
300 350 400 450 500 550 600 650 700

(b)


Black +UV
Orange -UV
% Reflectance
Wavelength (nm)
20
15
10
5
0
300 350 400 450 500 550 600 650 700

(c)

Figure 3.8

4. Conclusions

4.1 Research summary

The apple clearwing moth, *Synanthedon myopaeformis* (Borkhausen), (Lepidoptera: Sesiidae) is an invasive pest of apple trees in BC and due to its recent introduction, there are no registered semiochemical-based control tactics for this target insect in Canada (Judd, 2008). Since the Similkameen Valley is under area-wide management for codling moth (Judd & Gardiner, 2005), control tactics that target the apple clearwing moth must comply with the program. This program and the growth of organic apple production in the region make it difficult to control the apple clearwing moth with insecticide treatments. Since the larvae are cryptic feeders (Dickler, 1976; Alford, 2007), the development of a pheromone-based attract and kill formulation with SPLAT®, an inert matrix, and multiple attractants that target adult moths was initiated.

In order to maximize the effectiveness of the attract and kill formulation, the parameters of SPLAT were manipulated to ensure the highest level of attraction of male apple clearwing moths to the lure released from the matrix; these were droplet shape, droplet size and droplet color. A pheromone dose response was performed in SPLAT and compared to similarly baited rubber septum lures to determine an attractive dose of apple clearwing moth sex pheromone for use in SPLAT and the duration of lure activity. Male moth trap capture increased with pheromone dose in both lure types regardless of age. One-week old SPLAT baited with 10 mg of sex pheromone caught the highest number of moths, but after the fifth week, rubber septum lures became more attractive than SPLAT lures. The release rate measured from similar SPLAT products indicates a high initial release rate that would explain the high level of attraction to SPLAT lures after the first week (Jenkins & Isaacs, 2008). Attract and kill formulations made with SPLAT may require frequent applications throughout the adult flight period, but the use of mechanized application methods for SPLAT makes this strategy feasible. Although droplet size did not affect male apple clearwing moth trap capture, hemisphere-droplets caught significantly more moths than other tested shapes of SPLAT droplets. Since diurnally active moths rely on visual cues in close-range orientation to objects (Barry & Nielsen, 1984; Koshio & Hidaka, 1995; KonDo et al., 2012), the shape and height of the hemisphere-droplet may have resembled the body shape of an apple clearwing moth female more closely than the other tested shapes. Though the range of droplet sizes tested in this study was equally attractive to apple clearwing moth males, peachtree borers (*Synanthedon myopaeformis*, Say) (Lepidoptera: Sesiidae), discriminate between variously sized female mimics (Barry, 1978). This suggests that apple clearwing moths may also respond to visual cues of objects to secure potential mates. The presence of variously colored droplets in traps, however, did not affect long-range orientation to sex pheromone sources.

For attract and kill to provide sufficient control of target pests, the insect must contact the formulation to ensure the delivery of the toxicant (El-Sayed et al., 2009). The addition of cypermethrin, a pyrethroid insecticide, to the SPLAT formulation did not affect male apple clearwing moth attraction or close range orientation to the sex pheromone. Similarly, incorporation of the sex pheromone of the sympatric codling moth into the SPLAT formulation did not affect the attractiveness of the formulation to male apple clearwing moths. This suggests that there is room to develop a SPLAT-based attract and kill formulation that targets multiple species with overlapping flight periods in apple orchards in BC.

Increased exposure to the insecticide in an attract and kill formulation ensures that the target insect receives an adequate dose of the toxicant (El-Sayed et al., 2009). The addition of multiple cues can enhance the efficacy of the attract and kill formulation by evoking an increased number of contacts of the formulation and longer durations of contact by the target pest. After the mediation of long-range orientation by sex pheromone, the color of droplets had a significant effect on close-range orientation to the sex pheromone source. Black SPLAT was highly attractive and induced the highest number of contacts by male apple clearwing moths. Spectral reflectance measurements of black SPLAT and the orange stripe on female apple clearwing moth abdomens were coincidentally similar. Both reflect low levels of UV which may indicate a visual cue that male apple clearwing moths use to determine the presence of a conspecific female. Male apple clearwing moth (Stüber & Dickler, 1987) and peachtree borer (Barry, 1978) courtship behavior involve orientation to the orange stripes on female moths. Orange SPLAT was the next most attractive color, which reflected the second lowest level of UV compared to the other SPLAT colors. Spectral measurements of apple clearwing moth compound eyes suggest that apple clearwing moths are capable of detecting UV wavelengths (Eby et al., 2013). The spectral reflectance profile of the orange stripe on the fourth female tergite in the yellow-red wavelengths was most similar to the reflectance profile of orange SPLAT. An orange stripe was added to black SPLAT droplets in an attempt to mimic the visual cues provided by female apple clearwing moths. Orange-striped black droplets were similarly attractive to male moths as black droplets alone but were more attractive than dead females. To test a more commercially feasible product, droplets with orange SPLAT swirled into black SPLAT were tested. Since mechanized application of SPLAT results in variously sized droplets, different sizes of swirled orange and black SPLAT were also tested. All droplets produced intermediary attraction by male apple clearwing moths but one gram orange-striped black droplets were more attractive than three gram orange and black-swirled droplets.

4.2 Future directions

The work conducted in my thesis provides the baseline data necessary for further refinement of an attract and kill formulation for control of the apple clearwing moth in BC. Further research involving large plot experiments needs to be conducted to develop the application protocol and to determine if control of the apple clearwing moth can be achieved. Parameters that can affect the efficacy of the product include deployment methods, the density of droplets deployed per hectare, the placement of droplets within apple orchards, and the age of droplets. Although droplet placement within the canopy does not affect citrus leafminer (*Phyllocnistic citrella*, Stainton) (Lepidoptera: Gracillariidae) suppression in citrus orchards, moth suppression increases with droplet density, while fewer moths contact and are attracted to droplets aged 21 and 35 days (Stelinski & Czokajlo, 2010). The effect of droplet size, which is affected by the application method, should be revisited in large plot experiments to determine the efficacy of control when semiochemical and insecticide release is kept constant among plots and when the release of these active ingredients varies among plots. These parameters should also be tested on codling moth to determine if one formulation can effectively achieve multi-species control.

Further refinements of the visual components of the attract and kill formulation should be tested. Although black SPLAT closely mimics the level of UV reflected off of the orange stripe on female apple clearwing moth abdomens, it lacked the appropriate reflectance in the yellow-red wavelength region. Orange SPLAT resembles the reflectance profile of the orange stripe on female apple clearwing moths but the increased intensity of reflectance in the yellow-red region and the intensity of UV reflectance may not induce optimal visual attraction of male moths. The elimination of UV from the orange stripe on black-striped droplets should be tested to determine if this improves the visual cue in the attract and kill formulation, and if it attracts and promotes increased contact to droplets by male apple clearwing moths. The spectral reflectance measurement of the rest of the female body could help determine if further visual components in SPLAT can be refined.

The attraction of apple clearwing moths to droplets that radiate different temperatures could be investigated to determine if apple clearwing moths are attracted to black SPLAT droplets due to thermal cues in addition to UV reflectance. The black fire beetle, *Melanophila acuminate* (DeGeer), (Coleoptera: Buprestidae) uses thoracic infrared (IR) pit organs to detect and locate forest fires in order to lay their eggs on freshly killed coniferous trees (Schmitz & Bleckmann, 1998). The sun-basking butterfly, *Troides rhadamanthus plateni* (Staudinger) (Lepidoptera: Papilionidae) have thermoreceptors located on the antennal club which most likely measures ambient temperature (Schmitz & Wasserthal, 1993). Since the arenas and droplets were presented to male apple clearwing moths in direct sunlight, if they are capable of detecting IR radiation, the darkest droplet may emit higher levels of IR radiation than the rest of the droplets and more closely resemble IR radiation emitted from a dark apple clearwing moth female.

A variety of experiments on the toxicant in the attract and kill formulation need to be performed on the apple clearwing moth. Lethal and sublethal effects after exposure to the toxicant for various lengths of time need to be investigated to ensure that knockdown, and possible behavior-modifying effects are achieved in the field. The effects of insecticide exposure on subsequent long-distance pheromone-mediated behavior and close-range vision-mediated behavior can also be examined. Sublethal insecticidal poisoning affects pheromone-mediated reproductive behavior in the Oriental fruit moth, *Grapholita molesta* (Busck), (Lepidoptera; Tortricidae) (Linn & Roelofs, 1984; Evendent et al., 2005) and the pink bollworm, *Pectinophora gossypiella* (Saunders), (Lepidoptera: Gelichiidae) (Floyd & Crowler, 1981; Haynes & Baker, 1985).

In conclusion, my research has documented the sex pheromone dose, the droplet shape, and the droplet color required to develop an attract and kill formulation made with SPLAT and cypermethrin to target the apple clearwing moth in BC. In addition, codlemone did not have an effect on male apple clearwing moth attraction to the formulation, which suggests the possibility of simultaneous control of the apple clearwing moth and the codling moth with one product. This research provides a basis for future research in the development of a commercially available attract and kill formulation that could play an imperative role in an Integrated Pest Management program to control this serious pest of apple trees in BC.

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