Orientation and Mating Disruption of the Navel Orangeworm

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Interpretive Summary:

The pheromone of the navel orangeworm (NOW) is now known to be a multi-component system (Coffelt et al. 1979; Leal et al. 2005; Millar et al. 2005), but the precise ratio of constituents and the release rate needed to create a lure that merely equals the level of attraction of a single caged female in the field has so far remained unclear. Such new information would most obviously be useful to the development of pheromone-baited monitoring traps and, very likely, also would be highly useful to development of the mating disruption technique. Mating disruption seems to work best when the disruptant is formulated as the complete natural blend. This is probably true for any type of formulation (e.g., from "puffers" to microcapsules), but it most certainly is applicable to formulations that rely in part on males being attracted to point sources (Cardé and Minks 1995; Cardé 2007).

The recent discovery of new NOW pheromone components and of previously unknown features of their mating behavior (Girling and Cardé 2006), combined with two new experimental approaches, provide fresh opportunities to investigate ways to improve lure attractivity and efficacy of mating disruption of NOW. The first new device is a piezo-electric sprayer, which allows emission of a precise and known quantity of each pheromone component (Girling and Cardé, in press). This enables us to determine with behavioral assays how faithfully any synthetic blend matches the attractiveness of natural pheromone from a calling female. The second new system permits recording and analysis of ultrasound signals. Our recent acoustic recordings demonstrate that NOW produce ultrasound while courting, but so far we have not proven that these sounds facilitate mating. A third approach will examine mating disruption using our wind tunnel assays. Our current wind tunnel studies have clearly established that the pheromone consists of four components, and the complete blend achieves the highest proportion of source location with the greatest rapidity. This information is crucial to the design of highly efficacious lures and very likely an optimal mixture for mating disruption.

Objectives:

1. Using the new piezo-electric sprayer system and synthetic pheromones newly available from our colleagues, determine the rate of emission and the precise ratio of major to minor synthetic pheromone components in the blend to maximize attraction of male NOW and compare the in-flight and close-range male orientation and courtship behaviors evoked by females, female extracts, and synthetic blends.
2. Determine the extent to which prior exposure to synthetic pheromone impairs subsequent responsiveness to females and pheromone lures. These wind tunnel studies will help forecast successful strategies for field deployment of formulated pheromone for mating disruption.
3. Investigate the use of sound in the close-range orientation and courtship behaviors of NOW.

Materials and Methods:

Objective 1: Determine the rate of emission and the precise ratio of major to minor synthetic pheromone components in the blend to maximize attraction of male NOW and compare the in-flight and close-range male orientation and courtship behaviors evoked by females, female extracts, and synthetic blends.

We have established in wind tunnel observations that the mating sequence of the NOW involves a series of interactive male and female behaviors (Girling and Cardé 2006) that had been overlooked in previous work (Phelan and Baker 1990). The earlier work had stated that there was no discernible courtship sequence and that the male simply mated with the calling female. We have found that the failure of the male to accomplish a series of tactile and positional maneuvers in proper sequence results either in the female flying away or a female "escape run," which subsequently can result in mating, if the male recontacts her and he executes the proper behaviors. The failure of the previous study to observe these reactions likely stems from the confined, artifical bioassay setup then used. Knowing this behavioral sequence permits us to see how it might be modified when air is permeated with pheromone. This may offer a simple laboratory method for testing various blend combinations for mating disruption.

A piezo-electric sprayer system, described by El-Sayed et al. (1999), has been modified and tested. With this device we produce an aerosol from a solution of ethanol to which synthetic pheromone is added (Fig.1). The system consists of drawn glass capillary tip, with an opening of 10-50 µm i.d., which is vibrated at ultrasonic frequency (~120 kHz) by a piezo-ceramic disc. This disperses the solution in micro-droplets which results in an aerosol of the solution that evaporates completely within 5 cm of the tip. A motor driven micro-injection syringe pump (CMA/Microdialysis, Carnegie Medicine AB, Stockholm, Sweden) is used to deliver the odorant solution, via micro tubing (0.12mm i.d.), to the capillary tip at a rate of 10 µl min. This system allows precise and known amounts and ratios of pheromone to be released, which previously had not been feasible. Due to large differences in volatility of the identified components of the NOW blend, rubber septa and other dispensers would release these compounds in very different ratios from those applied to or contained in such dispensers. The sprayer system allows us to determine the exact ratio of compounds needed to elicit optimal male response, so that this can be specified for use in field puffers for mating disruption. We have described the physical features of this system and the kinds of plumes that it produces (Girling and Cardé, in press).

In combination with this new sprayer, we are using video recording of the male's flight track in a 3-meter-long wind tunnel. We will determine how the calling female, female pheromone extract, and blends of synthetics modulate male orientation. Previous studies have shown that such detailed monitoring of flight behavior can contribute to understanding blend completeness. For example, Witzgall and Arn (1990), also using video analysis of flights in a wind tunnel, showed that males flying along a plume of the complete pheromone blend have a more direct upwind flight trajectory than when the pheromone blend is incomplete. Witzgall and Arn proposed this criterion (essentially track linearity) as a guide to verifying blend completeness. We will use a similar approach to understanding blend completeness, but with the additional feature of analyzing flight characteristics immediately before and during landing. Furthermore there is some possibility that the "secondary" or minor components of the blend mediate "late-stage" behaviors such as persistence at the source and landing, but not the ones involved in long-distance (flying) orientation.

With respect to our supply of insects, we rear a large, continuous colony of NOW, originally obtained from Dr. Bas Kuenen. There is a daily availability of sufficient adult males and females for all of the tests proposed.

We are collaborating with Drs. Jocelyn Millar and Bas Kuenen to design and plan experiments to utilize these new technologies to investigate the most effective ratio of synthetic compounds to best mimic males' responses to virgin females. We are relying on their collaboration in supplying lures and solutions (blends and ratios) suitable for our tests. Because we will use different methods of analysis, our work will complement rather than duplicate their wind tunnel studies. Our proposed work could help in design of "lure-and-kill" formulations, which rely on male contact with the lure; of importance in males receiving a lethal or debilitating dose of insecticide in the lure and kill method will be the time of the male's contact with a lure, a parameter that we will measure. We will collaborate on the design and evaluation of these experiments with Drs. Kuenen and Millar to minimize duplication of effort.

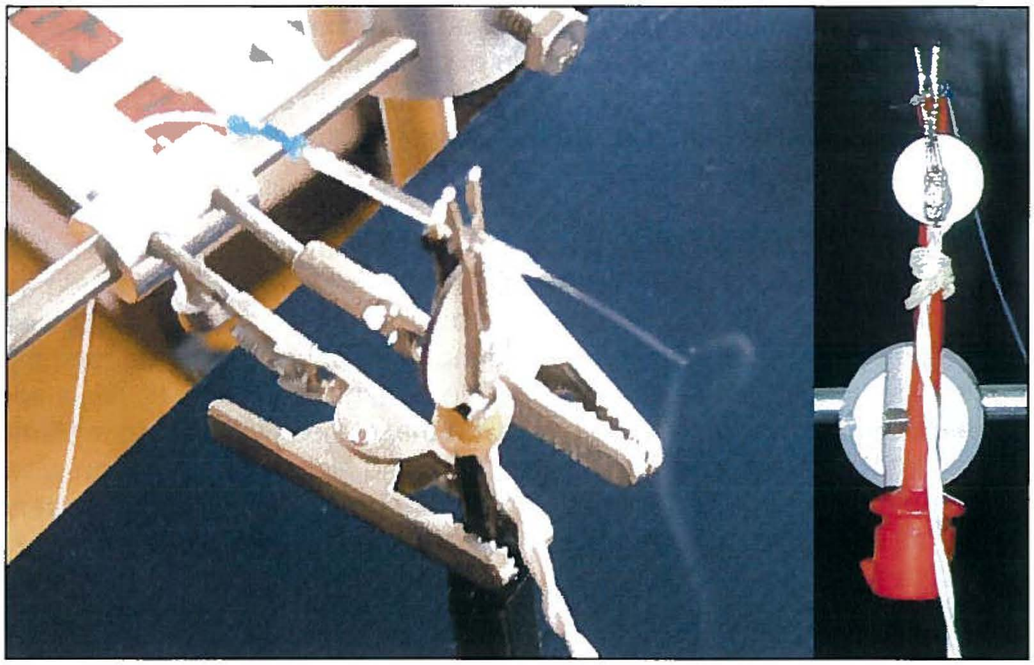

Figure 1. Piezo-electric sprayer system producing an aerosol in still air conditions. The round piezo disc in the center of the apparatus vibrates the drawn glass capillary at ~120 kHz, resulting in the production of the aerosol strands at the capillary tip.

Objective 2: Determine whether exposure to synthetic pheromone impairs subsequent responsiveness to females and pheromone lures.

Prior exposure of males to pheromone (as occurs in orchards treated with mating disruptant) can either raise the males' response threshold (increase the quantity of pheromone required to evoke response) or entirely eliminate responsiveness (Cardé and Minks 1995; Cardé 2007). Such habituation is considered to be a major mechanism in achieving mating disruption. The effects of full or partial blends in causing habituation can be tested in the wind tunnel (e.g., Sanders 1985). We have a wind tunnel system that allows the odors to be presented as a homogeneous (Justus and Cardé 2002) or heterogeneous (Schofield et al. 2003) cloud and to use two- or three-dimensional video recording to determine how well male moths can orient to point sources of pheromone (either an individual female or a synthetic lure).

We have several wind tunnel setups for such experiments. Because the male NOW in its initial flight path after take off seems to fly upward before returning to the height of the plume, we need to use our largest tunnel (3 m long and about 1 by 1 m in cross-section) for these trials. At the tunnel's upwind end we will simulate the placement of multiple point sources of disruptant or host plant leaves treated with other formulation types such as microcapsules in the tunnel. We will release males individually at the tunnel's downwind end and determine how well males locate calling females (or point sources of synthetic pheromone designed to mimic females). Some males will serve as experimental controls and will not have had prior exposure to disruptant or pheromone. Treatment males will have been subjected to varying doses and times of exposure to pheromone. Such pre-flight exposure can be undertaken by either holding moths individually in screened cages in the wind tunnel prior to testing, or males can be held separately in closed cages with pheromone before introduction into the wind tunnel. The main questions to be explored are whether prior exposure induces a substantial habituation and whether the strength of the habituation is related to how well the disruptant blend matches the natural pheromone and, to some extent, dose and time of pre-exposure. Habituation is readily induced in some moth species and in those species it likely contributes greatly to the efficacy of mating disruption (Cardé and Minks 1995; Cardé 2007). However, in some moths such as the pink bollworm, habituation seems to be of little importance (Cardé et al. 1998).

Objective 3: Investigate the use of ultrasound in the mating sequence of NOW.

Ultrasonic signals have been identified as important in the courtship of a number of arctiid moths (e.g., Conner, 1987; Portilla *et al*, 1987; Krasnoff and Yager, 1988; Sanderford and Conner, 1995). In addition, many pyralid moths (including phycitines), closely related to NOW, have been proposed to use ultrasound in their copulation sequences; these males produce ultrasonic pulses with their tegulae while wing fanning (Trematerra and Pavan, 1995; Trematerra, 1997). Trials with recently acquired ultrasound recording and analysis equipment suggest it will allow us to investigate whether NOW also uses ultrasound in its mating behavior and therefore whether the inability to fully mimic a male's response to a calling by using synthetic pheromones is due to a lack of some ultrasound signal. We will pursue this area of research only if ultrasound is discovered to be a key component of NOW mating behavior.

Results and Discussion:

Objective 1: Determine the rate of emission and the precise ratio of major to minor synthetic pheromone components in the blend to maximize attraction of male NOW and compare the in-flight and close-range male orientation and courtship behaviors evoked by females, female extracts, and synthetic blends.

Using our just-finished study on the mating sequence of the navel orange worm (Girling and Cardé 2006) as a starting point, we are examining the features of in-flight and post-flight orientation to various combinations of the pheromone blend. As four components are involved, we are comparing the "complete blend" vs. the single major component vs. three treatments, each with the complete blend minus one component. This subtractive approach is the most efficient way to verify the contribution of individual components to blend efficacy as it allows us to use five treatments to establish the contributory role of each of the four components. The in-flight maneuvers of males are being analyzed in two-dimensional following video recording and the persistence of males at the source is also recorded.

We had anticipated having a definitive (that is the natural) ratio of the four- or three-component blend to test in mid 2006. Drs. Millar and Kuenen had unanticipated difficulties with the purity (and therefore the behavioral activity) of the major pheromone component in a newly synthesized batch, which was only revealed in field tests conducted in the summer of 2006. This in turn has set back completion of our tests to the summer of 2007. We are now evaluating ratios in our wind tunnel tests. Our strategy is to use the best estimate of the most active blend in the subtraction assay mentioned above, and then refine the ratio by varying the proportion of one active component.

Our work with the piezo-disk delivery system has turned out to be quite challenging. Because the NOW is quite sensitive to ultrasound, we needed to use the 120 kHz system (the frequency is well above bat cries) as moths are insensitive to these ultrahigh frequencies. This system in practice is, to put it simply, finicky to adjust and therefore we ran our pilots with another moth, *Cadra cautella* (the almond moth), which we routinely use for wind tunnel studies and has a completely defined pheromone blend (attraction levels of 90-95% routinely in our wind tunnel). We have completed these studies (Girling and Cardé, in press), but currently plan to use filter paper dispensers because we can test more moths on a given day with this much simpler bioassay system.

Objective 2: Determine whether exposure to synthetic pheromone impairs subsequent responsiveness to females and pheromone lures.

These tests are contingent on definition of the complete blend (Objective 1), work that is progressing very well and rapidly, but is not yet complete. Such tests comprise a crucial perspective on which formulation types (e.g., numerous point sources vs. high dose, widely spaced dispensers or complete vs. partial blends) are apt to be efficacious for mating disruption (Cardé and Minks 1995).

Objective 3: Investigate the use of ultrasound in the mating sequence of NOW.

Our findings so far indicate that courting males produce a distinct ultrasonic pulse several times a second (Fig.2). Such sound pulses accompany wing-fanning behavior of males. This acoustic cue could facilitate a female's identification of a courting male as a conspecific and influence her propensity to allow mating to proceed (Girling and Cardé 2006). We are now performing experiments to establish whether such male-produced sounds influence female acceptance behavior. These tests include ablation of the female's tympana (ears) and interfering with the male's ability to produce ultrasound pulses while wing fanning. Our understanding of the normal courtship sequence (Girling and Cardé 2006) in NOW is crucial to the interpretation of these manipulations.

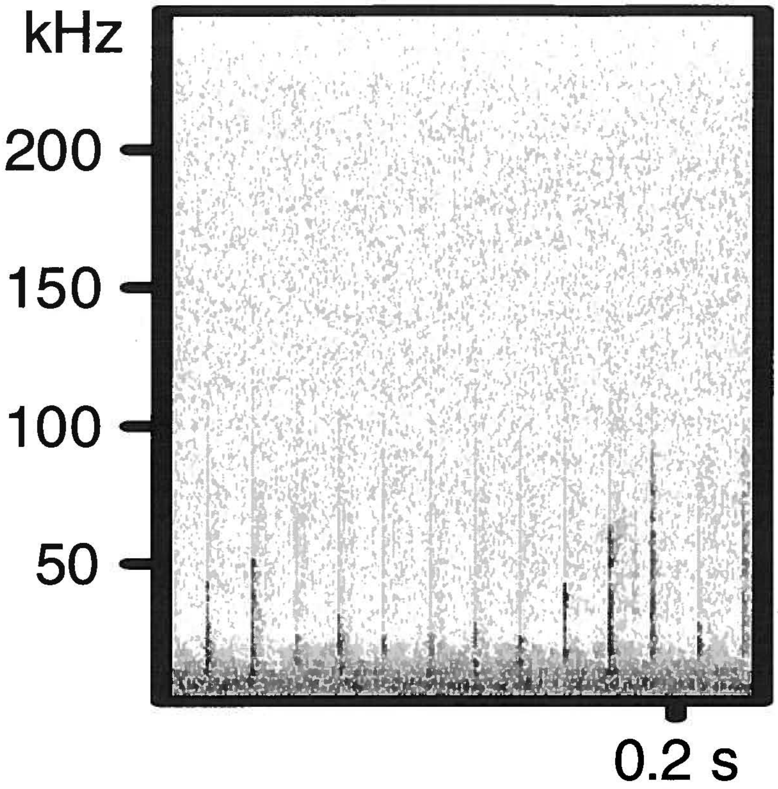

Figure 2. Sound spectrogram produced by a wing-fanning NOW male stimulated by pheromone.

Conclusions and Practical Applications:

We have in hand a reliable wind tunnel assay which will enable us to characterize the which components of the NOW blend contribute to orientation at a distance and in close vicinity of the synthetic source and to conditions that simulate mating disruption. Progress in these two endeavors has been hampered by inconsistencies in the purity of the synthetic compounds available to us, but these difficulties have been resolved (Millar, personal communication). Because of this issue, our work in mid-2006 instead emphasized development of the piezo release system and exploration of the possibility the acoustic signals facilitate mate location/ or recognition at relatively close range. Recent studies with other moth pests such as the peach twig borer (Jumean et al. 2005) have shown that acoustical signals enhance a male's ability to locate a pheromone source. If this proves to be true in the NOW, it might explain some anomalies in the comparative efficacy of live NOW females versus synthetic blends in traps.

The completion of these studies also has two practical applications. There remains a need to have a reliable pheromone-baited trap and our wind tunnel and acoustical studies we expect to help in devising such a monitoring device by refinement of the most attractive blend and determination if acoustical signals augment attractiveness. Our experiments on mating disruption, also to be carried out in a wind tunnel, will provide some insight into how mating disruption is best effected—and this in turn will suggest what combination of pheromone components and formulation types (beyond the puffer system) ought to be field tested.

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