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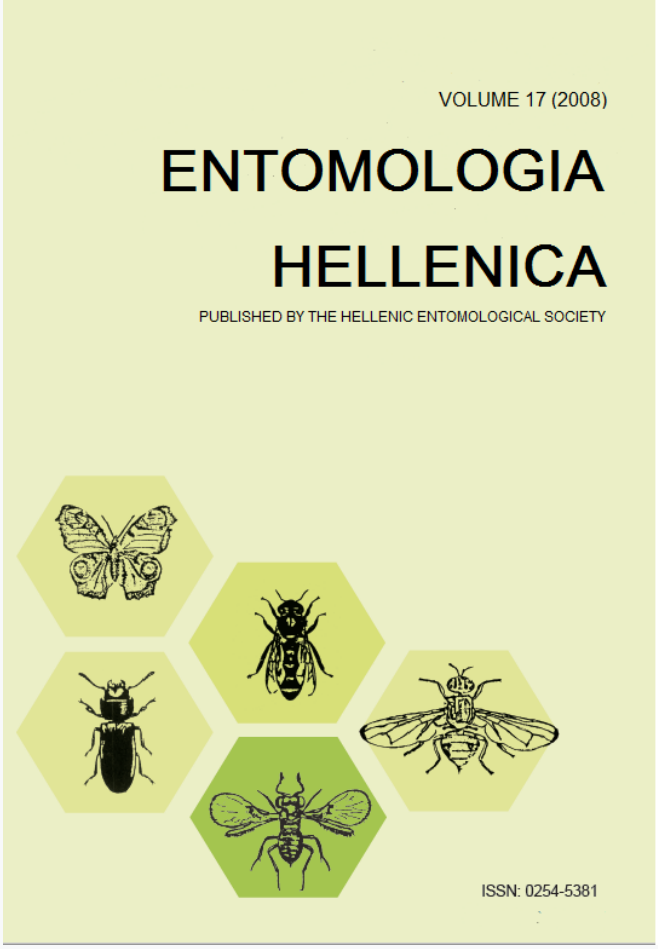

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Preliminary studies for the attract-and-kill strategy against *Culex pipiens*

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ABSTRACT

The attract-and-kill strategy requires an intelligent and an efficient combination of an attractant and a killing agent such as a pheromone and an insecticide respectively. The production of the synthetic oviposition pheromone of the mosquito species *Culex quinquefasciatus* (Diptera: Culicidae) was already achieved and its combination with three different insecticides were tested. Furthermore three larvicides, an insect growth regulator (pyriproxyfen), an organophosphate (temephos) and a microbial (*Bacillus thuringiensis* subsp. *israelensis*) were tested in the laboratory against *Culex pipiens* biotype *molestus* (Diptera: Culicidae) as agents that can keep water free from mosquito larvae. Larvicidal activity, over a 50-day period, revealed good results primary for temephos and secondary for pyriproxyfen. Temephos killed all the hatched larvae (100%) while pyriproxyfen was effective the first five days (>90%) and for the following days mortality was in a rate between 60 and 80%. The results from oviposition bioassays revealed that except temephos all the tested larvicidals repel gravid females of laying eggs for the first two days. However, when synthetic pheromone is combined with the three larvicidals, temephos and microbial agent followed the same attractant pattern as synthetic pheromone independently.

Introduction

Culex pipiens represents a mosquito species complex whose taxonomical status is still a matter of argument (Bourguet et al. 1998, Olejnicek and Gelbic 2000). The principal members of the complex are: *Cx.* biotype *pipiens*, *Cx. pipiens* biotype *molestus*, and *Cx. quinquefasciatus*. Biotype *molestus* prefer hypogeous habitats, it does not require blood meal to produce its first batch of eggs (autogeny), it is able to mate in confined spaces (stenogamy), it does not hibernate (homodynamy) and it is mammophilous, which means it prefers to feed mainly on mammals (Bourguet et al. 1998). The latter one explains why this biotype occurs more frequently in human environments and females have been reported to bite human indoors and outdoors in many urban areas in Europe (in Latin *molestus* means nuisance). Except nuisance, their medical importance is another matter of discussion and Lundström (1999) suggests that *Cx. p.* biotype *molestus* should be collected and processed for isolation of West Nile virus in order to evaluate the occurrence of the virus.

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The main tendency for the control of vectors without the presence of disease is to perform integrated programs minimizing chemicals by using environmentally friendly substances (Mulrennan 1995). Furthermore other agent such as oviposition pheromones (Laurence and Pickett 1985), plant extracts (Isoe et al. 1995) and skatole waters (Olagbemiro et al. 2004), which serve as oviposition attractants, could be valuable tools in mosquito control programs. Semiochemicals are biomolecules that spread information between individuals and their finding has introduced the attract-and-kill strategy with many advantages (Stetter and Folker 2000). The oviposition pheromone of *Culex quinquefasciatus* was recently synthesized and tested for its bioactivity on *Cx. pipiens* with very promising results (Michaelakis et al. 2005).

In the present study the main objective was to evaluate the attractiveness of a synthetic pheromone (SP) separately or when it is combined with a killing agent. Additionally, the effect of three commercially available larvicides was tested over a 50-day period.

The used larvicides were: an insect growth regulator IGR (pyriproxyfen), an organophosphate (temephos) and a microbial (*Bacillus thuringiensis* subsp. *israelensis*-Bti). These larvicides were chosen because of their frequent use in mosquito control programs worldwide and currently registered in Greece. The mode of action of pyriproxyfen has already been studied in detail and as a member of the IGRs group it has a remarkable larvicidal activity and a good efficacy against many mosquito species in a variety of mosquito breeding sites (El-Shazly et al. 2002, Lee 2001, Rettich 1977). Additionally, pyriproxyfen is effective against mosquito larvae and causes minimum undesirable effects on the environment and public health (Mulla et al. 1989). Similarly, temephos was used as a larvicide in many mosquito control programs and its efficacy has been also well documented (Novak et al. 1990, Cilek et al. 1991). Finally, the environmental safety of microbial agents has been confirmed in numerous laboratory and field tests. *Bacillus thuringiensis* occurs in the soil micro-ecosystem and subspecies *israelensis* is a commonly used mosquito larvicide (Aronson et al. 1986, Barjac and Sutherland 1990, Becker et al. 2003).

Materials and Methods

Mosquito rearing

A *Cx. pipiens* biotype *molestus* colony was used maintained in the Benaki Phytopathological Institute, (Kifissia, Greece) for more than two decades. Adults were kept in wooden framed cages (33×33×33cm) with 32x32 mesh per square inch wire screening. The conditions were set at 20±2°C, 80±2% R.H. and under a photoperiod of 14:10 (L:D). Cotton wicks saturated with 10% sucrose solution were provided to the mosquitoes as food source. Females laid their eggs in round, plastic containers (10cm in diameter, 5cm in depth) filled with 150mL of tap water. Egg rafts were removed daily and placed in cylindrical enamel pans (35cm in diameter, 10cm in depth) in order to hatch. Larvae were reared under the same temperature and photoperiod and were fed daily with baby fish food (TetraMin, Baby Fish Food) at a concentration of 0.25g/L of water until pupation. Pupae were then collected and introduced into the adult rearing cages.

Synthetic pheromone

The oviposition pheromone (6-Acetoxy-5-hexadecanolide) was obtained

according to a method recently described (Michaelakis et al. 2005). SP is a synthetic mixture of four diastereomers synthesized with a simple (five steps), efficient, high yielding (45% total yield) and low cost procedure (use of low cost reagents).

Control agents

Commercial products (commonly used in Greece) of 0.5% pyriproxyfen and 50% temephos were tested at the doses of 2mg/L and 0.15mL/L, respectively. The initiation of this study is dated back to the period 2004-2005, when temephos was the commonly used larvicide in Greece. Since 2006, temephos has been banned from Europe. In Greece it remained during 2007 only for essential use. Despite this fact, temephos is one of the active substances suggested by WHO for mosquito larval control programs.

The dosages were equivalent to the lowest recommended label rates for each active substance. For Bti the highest recommended label dose was chosen: 2μL/L [Bt (serotype H-14), 13.2g/L (or 1200 International Toxic Units/mg)].

Larvicidal bioassays

The bioassay method followed was based on the standard test for determining the susceptibility or resistance of mosquito larvae to insecticides (WHO 1981). However, in the present study, instead of 3rd and early 4th instars larvae, one-day egg rafts were used. Aqueous biocide stock solutions were prepared in conical flasks as follows: Four to six consecutive dilutions were prepared as working solutions in a 3-litre glass jar, depending on the active ingredient, to obtain the desirable concentration. Before their use, glass jars were stored uncovered under similar conditions with mosquito rearing. Glass jars filled with tap water where used as control. Bioassays were performed on day 5, 20, 35 and 50 after preparation of dilutions (day 0). Every 3 days the jars were weighted and tap water was added up to initial volume to supplement water loss due to evaporation.

100ml of each stock solution were added in 250ml glass beaker and one newly laid egg rafts (less than 20h old) were transferred by means of a wooden stick on the water surface (70±5 eggs per egg raft). In addition, 1mL of baby fish food solution (TetraMin, Baby Fish Food) was added to each beaker every 2 days to provide larvae with food.

Oviposition bioassays

Two-choice oviposition experiments were set in sieve covered wooden framed cages (33x60x33cm). Two to three days old male and female adult mosquitoes were removed daily from the maintenance cages (not containing oviposition beakers) and introduced into the bioassay cages. The bioassay cages were kept under the above-mentioned rearing conditions. Two glass beakers (10cm in diameter, 5cm in depth), one containing 100mL distilled water and the other 100mL distilled water plus SP, were placed into the cages in approximately 40cm distance between each other as more centrally as possible in order to provide oviposition sites. The dilution of 1μg SP was followed, according to the method described by Michaelakis et al. (2005) with the following deviation: instead of glass cover slips filtered paper was used (Bruno and Laurence 1979).

A similar procedure was followed in order to assess the effect of the presence of a larvicide in the oviposition site. In this set of experiment the beaker with the water and SP had been replaced by a larvicide in the recommended by the label effective dose. Each oviposition bioassay lasted six days.

Data recording and analysis

For larvicidal bioassays: adult emergence for control and each treatment was recorded for a period of 50 days (post treatment days: 5, 20, 35 and 50). The efficacy of each tested larvicide was assessed as the total number of emerged adults from each treatment compared to the number of adults emerged from controls. Five replicates were used for each larvicide and storage day (Fig. 1).

For oviposition bioassays: For the evaluation of all oviposition mediums all data were log (x+1) transformed and means of treatments and controls were compared by using Students *t* test. Any *F* test significant at $P<0.05$ was followed by a least significant difference test to compare treatment means.

The number of egg rafts was counted and removed every 24 hours after the introduction of the oviposition beakers into the bioassay cages. The number of egg rafts in the treated beaker was converted to percentages of the total number of egg rafts in both beakers. These results refer to three experiments for each case.

Results and Discussion

All insecticides were evaluated against newly hatched larvae of *Cx. p. molestus* at a specific concentration. The tested bioassay solutions were stored for 50 days under constant conditions before use (post treatment days). The efficacy of all larvicides is shown in Fig. 1 where adult emergence is presented for the control and each insecticide and for every post treatment day.

The results indicated that temephos killed all the hatched larvae (100% mortality). Although pyriproxyfen significantly differed from the control, the evaluation showed excellent mortality level only for the first five days. Moreover, pyriproxyfen was also found to be highly effective in the pupal stage with mortality ranging from 80 to 95 % (data not shown). These results agree with the already known mode of action of pyriproxyfen (Schaefer et al. 1988).

As far as Bti is concerned, it had no bioactivity against newly hatched larvae of *Cx. pipiens* (Fig. 1). Efficacy of Bti was not significantly different from the control. The bioactivity of the Bti derives from a variety of factors (Becker et al. 2003). The most important of these factors are: a) Species and instar sensitivity (Larvae lose their sensitivity to bacterial toxins as they develop), b) Temperature, size of the water body, state of nutrition and sunlight also play a significant role in Bti bioactivity and c) The protein crystal (inactive protoxin) must be ingested by the target insect, and this depends on its feeding habits.

As concerning the oviposition bioassays, the results of the SP with the use of filtered paper, during a period of 6 days, are shown in Fig. 2. During the first three days the attraction was significantly different from the control (~60-72%) and after the fourth day the attraction was almost the same as the control.

In Fig. 2 except the SP attraction, the oviposition effect by temephos, Bti and pyriproxyfen are also shown. For the first two days Bti and pyriproxyfen repelled gravid females from laying eggs. For the rest four days the attraction level reached almost control levels. These findings are in accordance with the negative effect on oviposition activity by larvicides (Maw 1970, Ritchie and Long 2003, Beehler and Mulla 1993). On the contrary, temephos did not differ significantly from control, during the 6-day period.

Even though Bti showed repellent activity, its combination with SP rendered water attractive (from 16% attraction to

57% for the first day, Fig. 2 and 3). The attraction reached higher levels during second and third day where attraction was 68% and 69% respectively (Fig. 3). After the third day the attraction of SP with Bti followed the same pattern with SP alone. Although the combination of SP with pyriproxyfen on the first day resulted to higher attractant levels (from 18.6% attraction to 61%), during the following 5 days it was proved to be deterrent (Fig. 2 and 3), indicating that the presence of SP did not improve oviposition in water with pyriproxyfen for a long period. As previously mentioned, temephos had no negative oviposition effect and water with both SP and temephos followed the same attractant course as SP independently (Fig. 2 and 3).

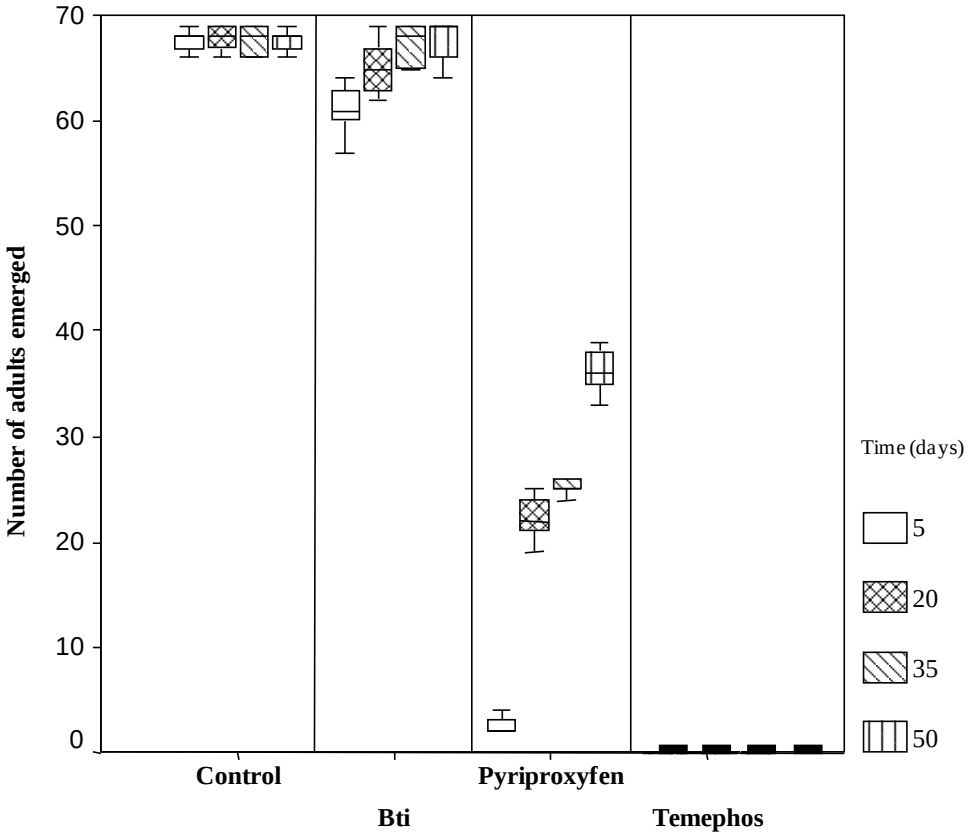

FIG. 1. Results for adult stage emergence (number) for each treatment (control, pyriproxyfen, temephos and Bti) against newly hatched larvae. Mean number of 5 replicates (±SE) for every post treatment day.

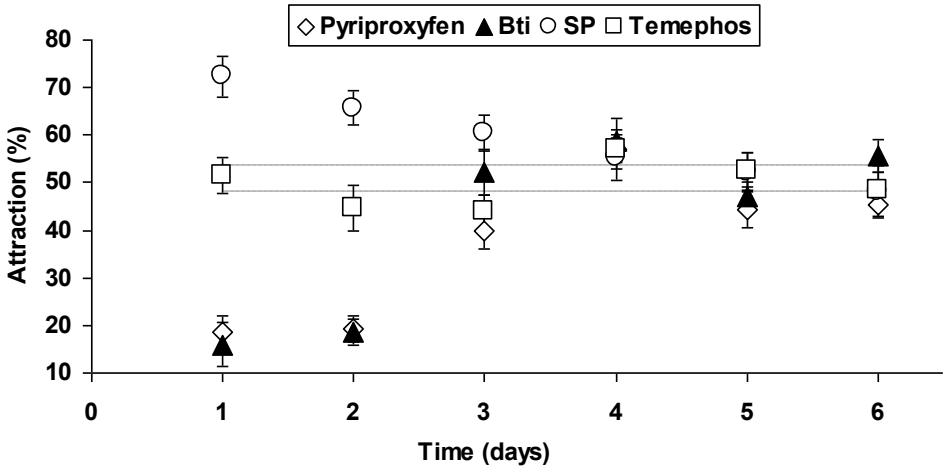

FIG. 2. Response to dose of 1μg of SP (○) and how Bti (▲), temephos (□) and pyriproxyfen (◊) affect oviposition. Broken lines represent the upper and lower values of the control mean ± SE (50.1±2.1%, n=10).

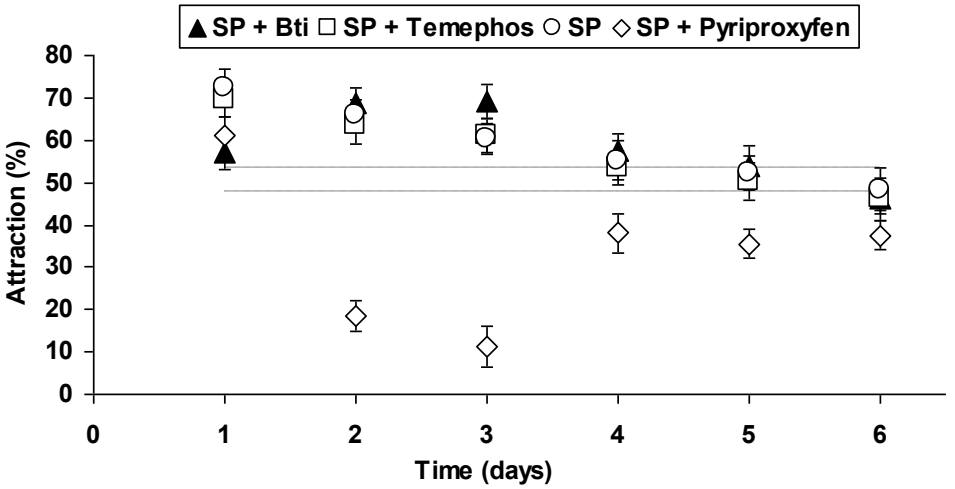

FIG. 3. Response to dose of 1μg of SP combined in water with Bti (▲), temephos (□) and pyriproxyfen (◊). Broken lines represent the upper and lower values of the control mean ± SE (50.1±2.1%, n=10).

Overall, SP after the third day stopped attracting gravid females independently of the larvicidal agent. This happens due to the fact that pheromones are volatile and thus they do not remain stable for a long period. These preliminary studies are the basic step for the implementation of attract-and-kill strategy. The following step will be the use of microencapsulated oviposition pheromone, a method used before for *Sesamia* males, (Mihou et al. 2007) which will prolong the slow release capability of SP.

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KEYWORDS: *Culex pipiens* biotype *molestus*, synthetic oviposition pheromone, pyriproxyfen, temephos, *Bacillus thuringiensis* subsp. *israelensis.*

Προκαταρκτικές μελέτες της στρατηγικής προσέλκυσης-και-θανάτωσης ενάντια στο *Culex pipiens*

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ΠΕΡΙΛΗΨΗ

Η στρατηγική της προσέλκυσης-και-θανάτωσης απαιτεί αποτελεσματικούς τρόπους συνδυασμού μεταξύ του παράγοντα προσέλκυσης (φερομόνη) και του παράγοντα θανάτωσης (εντομοκτόνο). Μετά την ολοκλήρωση της σύνθεσης της φερομόνης ωοθεσίας του κουνουπιού *Culex quinquefasciatus* (Diptera: Culicidae) κρίνεται ο συνδυασμός της με εντομοκτόνο. Επιπλέον, τρία προνυμφοκτόνα δοκιμάστηκαν εργαστηριακά ενάντια σε κουνούπια *Culex pipiens* biotype *molestus* (Diptera: Culicidae): ένας ρυθμιστής ανάπτυξης (pyriproxyfen), ένα οργανοφωσφορικό (temephos) και τέλος ένας βάκιλλος (*Bacillus thuringiensis* subsp. *israelensis*). Μελέτη προνυμφοκτόνου δράσης, για περίοδο 50 ημερών, έδωσε ικανοποιητικά αποτελέσματα για το temephos (100% θνησιμότητα) ενώ για το pyriproxyfen η θνησιμότητα ήταν >90% για τις πρώτες 5 ημέρες και 60-80% για τις υπόλοιπες ημέρες. Τα πειράματα ωοθεσίας έδειξαν ότι εκτός από το temephos όλα τα άλλα προνυμφοκτόνα αποτρέπουν την εναπόθεση ωών για τις πρώτες δύο ημέρες. Τέλος, όταν η συνθετική φερομόνη συνδυάστηκε με ένα από τα προνυμφοκτόνα διαπιστώθηκε ότι η παρουσία του βακίλου ή του temephos στο νερό δεν επηρεάζει την προσελκυστική ικανότητα της φερομόνης ωοθεσίας.