1997-98 CALIFORNIA ALMOND BOARD FINAL REPORT

Project Title: **Pheromone-based Monitoring and Mating Disruption of Navel Orangeworm.**

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Summary

The navel orangeworm (NOW), Amyelois transitella, is a key pest of nut crops in California. Despite intensive research on methods of managing NOW and minimizing NOW infestations, this insect continues to be a significant problem for many growers. The major component of the female-produced sex pheromone for this insect was identified as Z11,Z13-hexadecadienal in 1979 (Coffelt et al. 1979b), but this compound alone is not as attractive to male moths as virgin females, for reasons which are not yet clear. Because of inconsistencies in its efficacy, the pheromone has not yet been used to any extent either as a trap bait or for mating disruption. The goal of this project is to determine the cause of and resolve the inconsistencies in efficacy, so that the NOW pheromone can be developed into a useful and efficacious tool for both monitoring and control of NOW.

In 1997, two significant milestones were reached. First, we were finally able to locate a reliable commercial supplier of pheromone on a kilogram scale, allowing mating disruption trials to proceed. These large-scale NOW mating disruption trials using timed release puffer devices resulted in virtual shut-down of trap catches in sticky traps and egg traps. Furthermore, NOW damage levels at harvest were lower than in control blocks in all three commodities in which the pheromone was tested, demonstrating the potential for insect control. These positive results suggesting that pheromone-based mating disruptionhas good potential for NOW control, even with only the single component pheromone, has catalyzed the redesign, reengineering, and large-scale production of the "puffer" pheromone release devices for use in control of NOW and other moth pests.

The second part of the project, the identification of a highly attractive NOW pheromone blend for use in monitoring traps, as distinct from mating disruption, has not yet been successful, but progress has been made. The establishment of a reliable wind tunnel bioassay method clearly demonstrated that the NOW pheromone consists of more than one component; the major component alone is not as attractive as live females or extracts of females. Testing of fractions of extracts from NOW females indicated that most or all of the active material was associated with the fractions containing the major, konwn component of the pheromone, plus related compounds such as isomers. In parallel with the laboratory bioassays, we have attempted to carry out field trials with different attractant formulations. These trials have been inconclusive to date due to low moth populations at field test sites; even traps baited with virgin female moths caught few male NOW.

We have not requested a renewal of funding for continued work on developing a better attractant because of our slow progress to date. However, this portion of the project will be continued at UC Riverside, with J. G. Millar being primarily responsible for chemistry work (i.e., analyses, and synthesis of test compounds), and Dr. Ring Cardé taking charge of lab and field trials of attractant formulations.

Procedures

1. **Insects**: Navel orangeworm cultures were maintained on a bran and honey diet in the laboratory at UC Riverside as previously described (Coffelt et al. 1979a). Male and female pupae were separated, and the emerging adults were maintained in 30 cm square screen cages until needed. Virgin females were used either as trap baits in field tests, or for preparation of pheromone gland extracts. Male insects were used for conducting coupled gas chromatography-electroantennogram detection (GC-EAD) and wind tunnel studies at UCR. All insect cultures were maintained at 20-25° C.

2. **Preparation, analysis and fractionation of pheromone extracts**: Virgin female insects were put on a reverse light cycle, as they normally produce pheromone just before and around dawn. Pheromone glands were dissected out of 1-3 day old virgin females at the end of the dark cycle. Briefly, the abdomen of the female was gently squeezed to extrude the gland on the end of the ovipositor, and the gland was clipped off with iris scissors. The gland was soaked in pentane (25 microliters) for 10 min, and the pentane was then transferred to a clean vial. Extracts from several thousand females have been prepared to provide raw materials for analyses, fractionations, and bioassays.

Pheromone gland extracts were analyzed by gas chromatography on several capillary columns of differing polarity (DB-5, DB-WAX, DB-17), by coupled GC-electroantennogram detection (GC-EAD), using DB-5 and DB-WAX columns, and by GC-mass spectrometry. Compounds were identified by comparison of retention times and mass spectra with those of synthetic standards, prepared as described in our 1995 report.

Pheromone gland extracts were fractionated by high pressure liquid chromatography on silica gel, eluting with THF/hexane mixtures. Extracts were fractionated in several different ways, to try and determine exactly which parts of the extract are necessary for full biological activity. Individual fractions and recombinations of fractions were then tested in wind tunnel bioassays as described below.

3. **Wind tunnel tests**. Dr. Ring Cardé, an international authority on lepidopteran behavior and pheromones, recently joined the faculty of UCR, and has entered into an active collaboration with us on this project. In particular, a large plexiglass wind tunnel has been built and tested by Dr. Ring Carde's research group for conducting tests with

pheromone extracts and fractions. Briefly, air is pushed with a fan through a baffle to get laminar airflow, and into the tunnel. Test compounds are loaded on filter paper wicks, male moths are released at the downwind end, and their behaviors are observed. Extensive optimization of the experimental conditions (e.g., light levels, humidity, wind velocity, age of male moths, time of day, etc.) was required to get high and reproducible numbers of male moths to fly to live females or pheromone sources.

4. **Testing of attractant blends.** The syntheses of most of the analogs and the known major component of the pheromone in high purity, for use in wind tunnel and field tests of attractant baits, were described in last year's report.

To test the effects of the various isomers and analogs as synergists or inhibitors of the major pheromone component, grey rubber septum lures were loaded with mixtures of the major component with 10% of each analog, with a total load of 110 micrograms per septum. Septa were then placed in Pherocon 1C sticky traps, with 5 replicates per treatment, and traps were deployed in almond orchards for periods of 4 weeks. The results of the first test indicated that one of the additives increased the attractiveness of the standard component, so a second trial tested baits loaded with a fixed amount of the major component (100 μg) and variable amounts of the additive (0-100μg). Traps were counted twice weekly, and baits were replaced after 2 weeks in both trials.

5. **Mating disruption trials**. The technical grade pheromone loaded into puffers for mating disruption trials was custom synthesized by a Bedoukian Research. Puffer cans were loaded at Dr. Shorey's loading facility at Kearney Agricultural Center, using a mixture of Dymel 134a propellant, and the stabilized pheromone in a solvent carrier. Puffer machines releasing the navel orangeworm (NOW) pheromone component Z11,Z13-hexadecadienal at 15-min intervals during the nighttime hours of moth activity, were suspended at 2/3 tree height in a 40-acre pistachio orchard, a 40-acre walnut orchard, and an 80-acre almond orchard. The machines were attached to branches at 2/3 tree height. For the 40-acre block, they were positioned in perimeter trees only, while for the larger block, five machines were also placed in trees, equally spaced along the midline of the orchard. Numbers of puffers per acre varied, being 1/acre in pistachios, 2/acre in walnuts, and less than 1 per acre (65 per 80 acres) in almonds. In each case, NOW pheromone was

released in an amount calculated to give approximately 2 g per acre per 100 days. This was accomplished through having the concentration of NOW pheromone in each puffer aerosol can at the level to provide 0.4 mg NOW pheromone per puff in the case of pistachios and almonds and 0.2 mg/puff in the case of walnuts. In walnuts, a blend of NOW and codling moth pheromones was released from March 15 through harvest on September 10. In pistachios, NOW pheromone alone was released from June 1 through harvest in late August. In almonds, due to a lack of supply of pheromone, NOW pheromone (in combination with peach twigborer pheromone) was not placed in the orchards until late June (when a midsummer flight of NOW moths was already underway, and the test lasted through harvest in late August. All three puffer-protected blocks were monitored for NOW male moth activity with sticky traps baited with living female moths. The blocks were also monitored for NOW egg laying activity with egg traps.

Results and Discussion

1. **Laboratory testing of attractant formulations**. To carry out detailed studies of the NOW attractant blend, we have enlisted the aid of Dr. Ring Cardé, who recently joined the UCR Entomology department. Dr. Cardé is an expert on the use of wind tunnels in studying the responses of moths to pheromones, and the participation of his research group, as described below, was crucial to the progress of the project.

The known synthetic pheromone of the navel orangeworm moth is comprised of a single chemical (Coffelt et al. 1979b). Previous efforts to verify whether additional pheromone components exist have been hampered by the lack of a reliable bioassay. Past assays using wind tunnels failed to obtain consistent orientation of males to calling virgin females, extracts of female pheromone glands, or the single component synthetic pheromone. Reliable bioassays were vital to being able to determine whether there are indeed further components to the pheromone, and in the identification of any new components.

We have tried to approach the bioassay question from two directions. First, for laboratory bioassays, a protocol was developed to assay NOW males in the wind tunnel.

We started with what should be the best pheromone attractant: pheromone emanating from calling virgin females. Our initial effort yielded upwind orientation in only 15.8 % of the males, with only 5.2% of the males landing on the cage holding the females. Because many environmental factors affect the response of male moths to pheromone, the first step was to determine the optimal environmental conditions for male attraction. Among the many factors tested, several substantially influenced the success of the bioassay. We determined that males have a window of response encompassing the 2 hours preceding sunrise, and that light levels during the bioassay have to be low enough not to disturb the males, while being high enough to allow observation of the males' behavior. We also found high humidity, a wind velocity of 0.5 cm s^{-1}, and 26° C to be optimal. Male moth age was also important, with the best levels of response being obtained from 1-2 day old moths.

Studies with other moths (Mafra-Neto & Cardé 1994) indicate that males have a higher level of attraction to pheromone when the pheromone is presented as a diffuse (turbulent) pheromone plume rather than a narrow, ribbon-like plume, as is commonly used in wind tunnel assays. We found that a higher proportion of NOW males progressed upwind when the odor was presented as a wide plume rather than when the plume was narrow.

In our wind tunnel bioassays, a cage containing a male is placed downwind in the path of the pheromone plume. Unlike many other moths, following contact with the pheromone plume NOW males usually take off in a looping flight downwind and then re-contact the plume 30 to 100 cm downwind of the platform where they initiated flight. Before progressing upwind to the source of odor, males spend a variable amount of time trying to pass the 'barrier' formed by the cage intercepting the plume, and many males cease plume following before they circumvent this obstacle. These observations allowed us to design a collapsible release system that removed the cage from the moth's upwind path immediately following his take off. This new procedure eliminated a major source of variability and inconsistency in attraction.

With the improved bioassay protocol, ~80% of the males flew upwind and 50% landed on cages containing calling females. We then tested serial dilutions of extractions

of the pheromone gland, ranging from 10 female gland equivalents to 0.036 gland equivalents, to determine the dose of pheromone that elicits the highest proportion of upwind flight and landing on the source by males. The highest levels of attraction upwind and landing were found at dosages near 0.36 female gland equivalents (Fig. 1); this is now our standard 'control' stimulus.

Several other important pieces of information were also obtained. First, the response of males to extracts of female pheromone glands is the same as the response to live females, indicating that all the components of the pheromone are indeed present in extracts, and that there are no inhibitory compounds present.

Second, in electroantennogram studies, which measure the response of the insects' antennae to chemical stimuli, additional components elicited small but significant responses, although an antennal responses alone does not establish that these components mediate the attractive behavioral response. The compounds which give the most consistent and significant responses are the EZ and ZE isomers of the major pheromone component. However, because of their structural similarity to the main component of the pheromone, these compounds would be expected to give at least some response. Thus, their possible role as pheromone components needs further checking with behavioral bioassays (see below).

Third, the synthetic major component of the pheromone was less attractive to males than an equivalent dose of the female extract, indicating either that the female extract contains additional components which enhance attraction, or that the synthetic material contains some inhibitory impurities.

Fourth, wind tunnel bioassays of liquid chromatography fractions of a combined sample of hundreds of female gland extracts indicated that attractant activity was associated only with the area around the main pheromone component. Viewing the extract as 3 groups of compounds (compounds eluting before the main pheromone component [before], compounds eluting with the main pheromone component [main], and compounds eluting after the main pheromone component [after]), fractions were cut in four different ways, as follows:

FRACTION

RUN #	BEFORE	MAIN COMPOUND	AFTER
Run 1	Fraction 1	Fraction 2	Fraction 3
Run 2	Fraction 4		Fraction 5
Run 3	Fraction 6	Fraction 7	
Run 4	Fraction 8		

Bioassays of the resulting fractions indicated that activity appeared to be associated only with the cluster of compounds around the main component of the pheromone (Fraction 2; Fig. 2). Addition of the compounds eluting before (Frac. 4) or after (Frac. 7) did not appear to enhance the activity of the main pheromone fraction (Fig. 2). Furthermore, the complete recombination (Frac, 8) appeared to be no better than the main pheromone fraction. In combination, these results suggest that any biologically active trace components are similar in structure to the main pheromone component (i.e., isomers or analogs), and that the active components are aldehydes (i.e., they cannot contain other functional groups such as alcohols or acetates because these types of compounds elute in the other fractions).

In a parallel effort to determine which minor components might possibly be enhancing the attractiveness of the female extracts, we have screened a number of binary blends composed of the main pheromone component in combination with small quantities of several isomers and compounds related to the pheromone (determined from a consideration of biosynthetic pathways used to make pheromone components, and consideration of the pheromone components for related moth species). None of the binary blends were as attractive as an equivalent amount of the female gland extract (Fig. 3).

Taken together, the results discussed above suggest several possibilities, as follows:

1. The "true" NOW pheromone blend is composed of more than two components, all of which must be present to get optimal attraction of male moths.

2. One or more key trace components may still remain to be identified, due to instability (i.e., we are not seeing the compounds in extracts with our instruments because the compounds are degrading during analysis), or due to being present in very small quantities.

To investigate these possibilities, several further experiments are planned, as follows:

1. Fractionate an extract by preparative gas chromatography. If the recombined fractions are less attractive than the crude extract, this will provide compelling evidence that there are one or more thermally unstable components to the pheromone.
2. Comparison of female extract with a blend of female extract and the synthesized main component of the pheromone. If the synthetic material contains inhibitory impurities, it should decrease the attractiveness of the female extract. Conversely, no decrease in attraction would suggest that the decreased activity of the synthetic material is because of missing trace components.

The results of one or both of these two experiments will determine how to proceed further towards determination and reconstruction of the "true" pheromone.

2. **Field trials**. In a parallel effort, we carried out field trials in 1997 with binary blends of the main pheromone component with possible minor components. The results of these experiments should be treated with caution because of the low numbers of moths trapped overall, even in traps baited with virgin female moths.

In the first field trial, one of the added components, an isomer of the main component, appeared to increase the attractiveness of the major pheromone component (~3 fold; Fig. 3). A second test suggested that the optimum ratio of the major to the minor component was about 100 : 3.3 (Fig. 4). Furthermore, careful analysis of a combined extract prepared from many female moths revealed the presence of trace quantities of this isomer.

Because of the low numbers of moths caught in these two trials, they will be repeated during the coming field season. At the time of writing, we have our first set of

traps in the field, targetting the first flight of NOW this year, which is late due to the cool spring.

3. **Mating disruption trials**. The scheduling for mating disruption trials in 1997 was thrown off by the failure of two companies to provide the synthetic pheromone on time. However, in mid-season, a reliable supplier was finally found, and several hundred grams of pheromone were obtained, allowing large scale field trials to proceed, albeit with later start dates than originally planned.

The sticky traps consistently showed a reduction in male moth attractancy in the pheromone-treated areas ranging from 95 to 100%, relative to untreated control blocks. Furthermore, in no instance was a NOW egg found on an egg trap in the pheromone-treated areas during the time that the puffers were operating, although more than 100 eggs total were found on egg traps in the control orchards. At harvest, in pistachios, 0.4% of nuts sampled from near the center of the puffer-protected orchard were infested with NOW larvae, compared to 3.3% of nuts in an untreated, control orchard (Table 1). In walnuts, 0.0% of all nuts sampled from the puffer-protected orchard were infested with NOW larvae, compared to 1.6% of nuts in a control orchard (which had received 3 applications of Confirm for worm control) (Table 2). In almonds, 1.2% of nuts sampled from near the center of the puffer-protected orchard were infested with NOW larvae, compared to 11.3% in a control orchard (Table 3). These results are promising with regard to the potential for NOW pheromone released nightly from puffers to provide control of larvae of this pest. Further work is needed before a commercially viable system for mating disruption of NOW can be recommended with confidence. The strategy for pheromone release which now appears to have the most potential would have puffers releasing NOW pheromone at 0.2 mg per puff, placed in trees along the perimeters of 40, 160, or 640-acre blocks, at 66-foot separations, with alternate machines being at 40% and 80% of tree height. Puffers releasing NOW pheromone at 0.4 mg per puff would be placed also at 132-foot separations along transects through those orchards that are larger than 40 acres, with the transects separated from each other by 0.25 mile.

Nevertheless, the results to date from the NOW mating disruption trials have been promising enough that a major grower, Paramount Farms, is providing technical and financial backing for the pheromone puffer technology for use in control of both NOW and other insect pests. This, in combination with locating a reliable manufacturer capable of producing synthetic pheromone in multi-kilogram quantities, has eliminated two key impediments to the further development and commercialization of the NOW pheromone.

Conclusions

1. In 1997, large-scale NOW mating disruption trials using timed release puffer devices resulted in virtual shut-down of trap catches in sticky traps and egg traps. Furthermore, NOW damage levels at harvest were lower than in control blocks in all three commodities in which the pheromone was tested, demonstrating the potential for insect control.
2. The establishment of a reliable wind tunnel bioassay method by Dr. Cardé's group has clearly demonstrated that the NOW pheromone consists of more than one component. Testing of various fractions of female pheromone extracts indicated that the additional components must be similar in structure to the main component, but the additional component(s) have not yet been conclusively determined. Two further experiments will provide more information about the missing component(s).
3. Field tests of trial bait formulations provided indications that one of the isomers of the main pheromone component may increase the attractiveness. These results will be rechecked in 1998, if possible in orchards with a known history of NOW infestation to ensure relatively large populations of NOW.

Acknowledgments

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Literature cited

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Coffelt, J.A., K.W. Vick, P.E. Sonnet, and R.E. Doolittle. 1979b. Isolation, identification and synthesis of a female sex pheromone of the navel orangeworm, Amyelois transitella. J. Chem. Ecology 6:955-966.

Mafra-Neto, A. & Cardé, R.T. 1994 Fine-scale structure of pheromone plumes modulates upwind orientation of flying moths. *Nature* **369**:142-144.

Table 1. Results of whole-orchard experimentation with puffers for control of navel orangeworm infestations in pistachios, Madera County - 40 acres, protected by 40 puffers continuously releasing NOW pheromone from June 1- September 1, 1997.

	Puffer block	Control block
Pheromone monitoring traps		
Mean NOW males/trap/week (4 traps)	0.3	
4 control traps		4.4
Percentage infested nuts at harvest		
8 sampling stations in perimeter trees	0.6%	
5 stations at least 100 m into the block	0.4%	
4 control stations		3.3%
Mean NOW eggs per egg trap/week	0.0	0.3

Note: Neither puffer-treated nor control pistachios received any insecticide treatments .

Table 2. Results of whole-orchard experimentation with puffers for control of navel orangeworm infestations in walnuts, Tulare County - 40 acres, protected by 80 puffers continuously releasing codling moth (CM) plus navel orangeworm (NOW) pheromones from Mar. 15 - Sept. 10, 1997.

	Puffer block	Control block
Pheromone monitoring traps		
Mean NOW males/trap/week (4 traps)	0.0	
4 control traps		0.5
Percentage NOW-infested nuts at harvest		
4 stations at least 100 m into the block	0.0	
4 control stations		1.6
Mean NOW eggs/egg trap/week	0.0	1.0

Note: The walnut control block was treated 2-4 times with Confirm for CM/NOW control. The puffer block received no applications for worm control.

Table 3. Results of whole-orchard experimentation with puffers for control of navel orangeworm infestations in almonds, Kern County, CA - 80 acres, protected by 65 puffers continuously releasing peach twigborer plus NOW pheromones from June 20 through September 1, 1997.

	Percentage infested nuts at harvest	
	Puffer block	Control block
8 sampling stations in perimeter trees	2.3	
5 stations at least 100 m into the block	1.2	
4 control stations		11.3

Note: Both puffer and control almonds received one hull-split Guthion treatment

Figure captions

Fig. 1. Results of wind tunnel bioassays with different doses of NOW female pheromone gland extract.

Fig. 2. Results of wind tunnel bioassays with liquid chromatography fractions of NOW female pheromone gland extract. Fraction numbers correspond to fractions described in the text. Fraction 9 is a blank control.

Fig. 3. Results of wind tunnel bioassays with binary blends of the main component of the pheromone with possible minor components.

Fig. 4. Attractiveness of test attractant blends to NOW male moths in field trials.

Fig. 5. Effect of addition of different doses of an isomer of the main pheromone component on the attraction of NOW male moths in field trials.

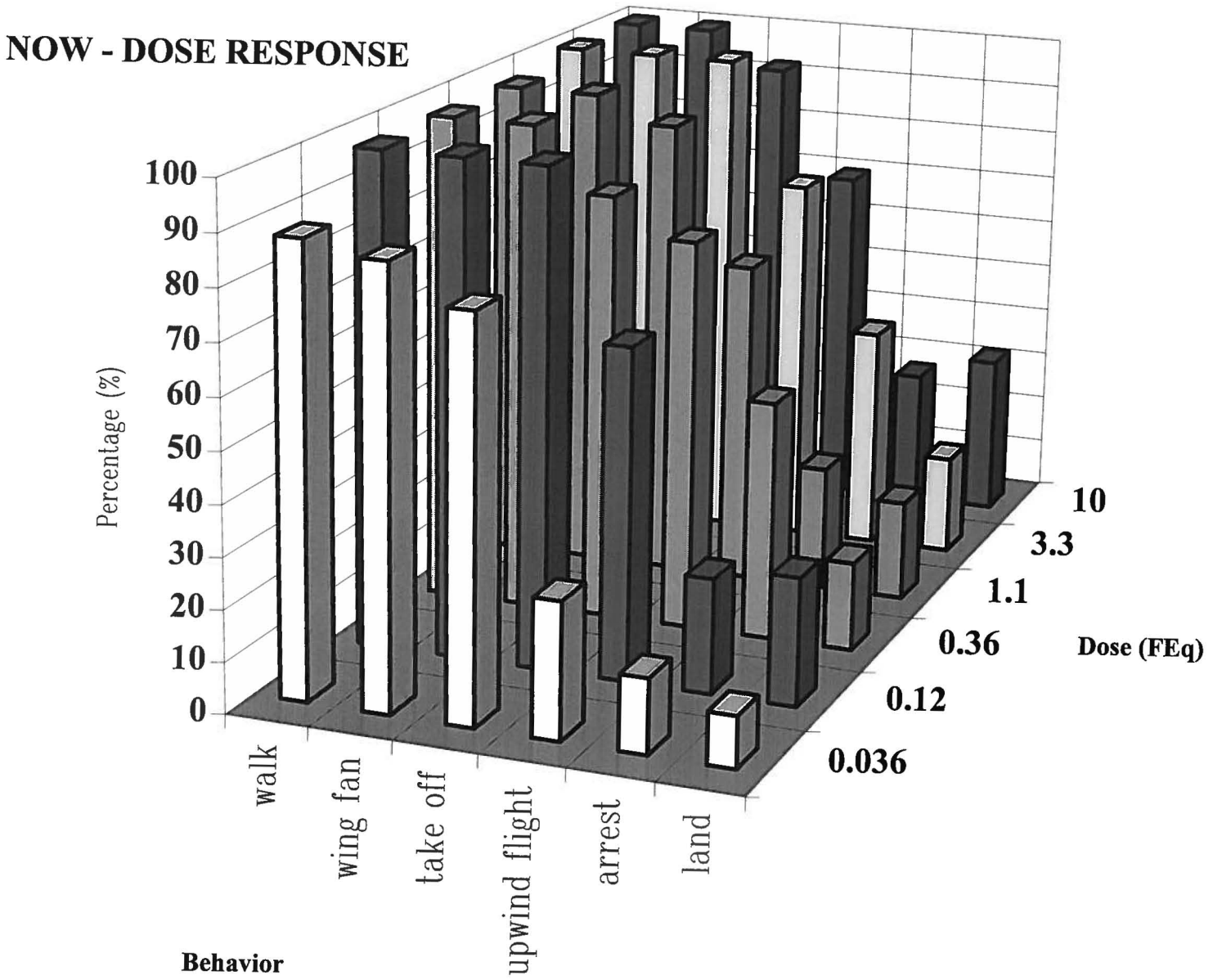
NOW - DOSE RESPONSE
Percentage (%)
100
90
80
70
60
50
40
30
20
10
0
walk
wing fan
take off
upwind flight
arrest
land
Behavior
10
3.3
1.1
0.36
0.12
0.036
Dose (FEq)

Fig. 2

NOW - HPLC

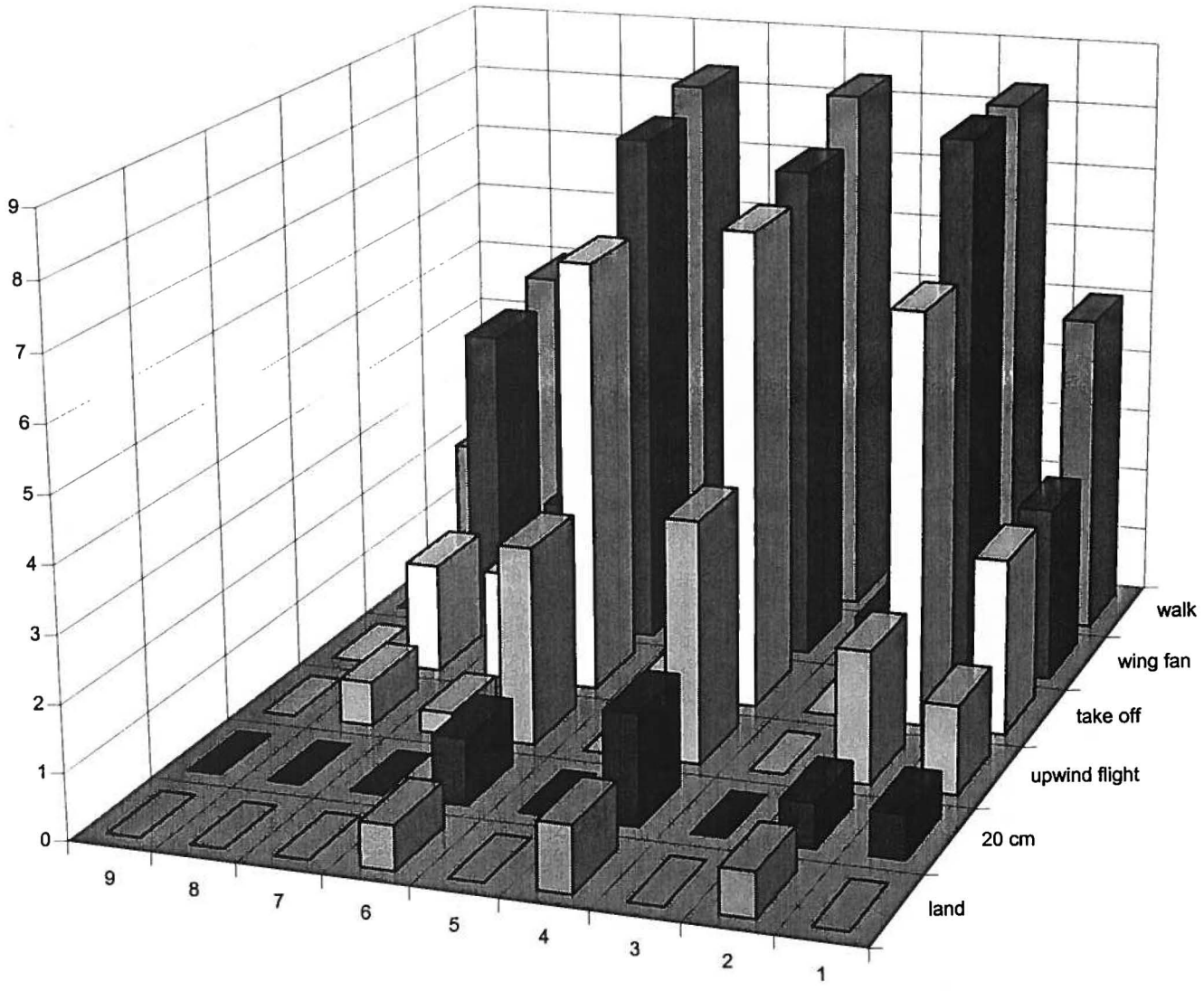

Fig. 3

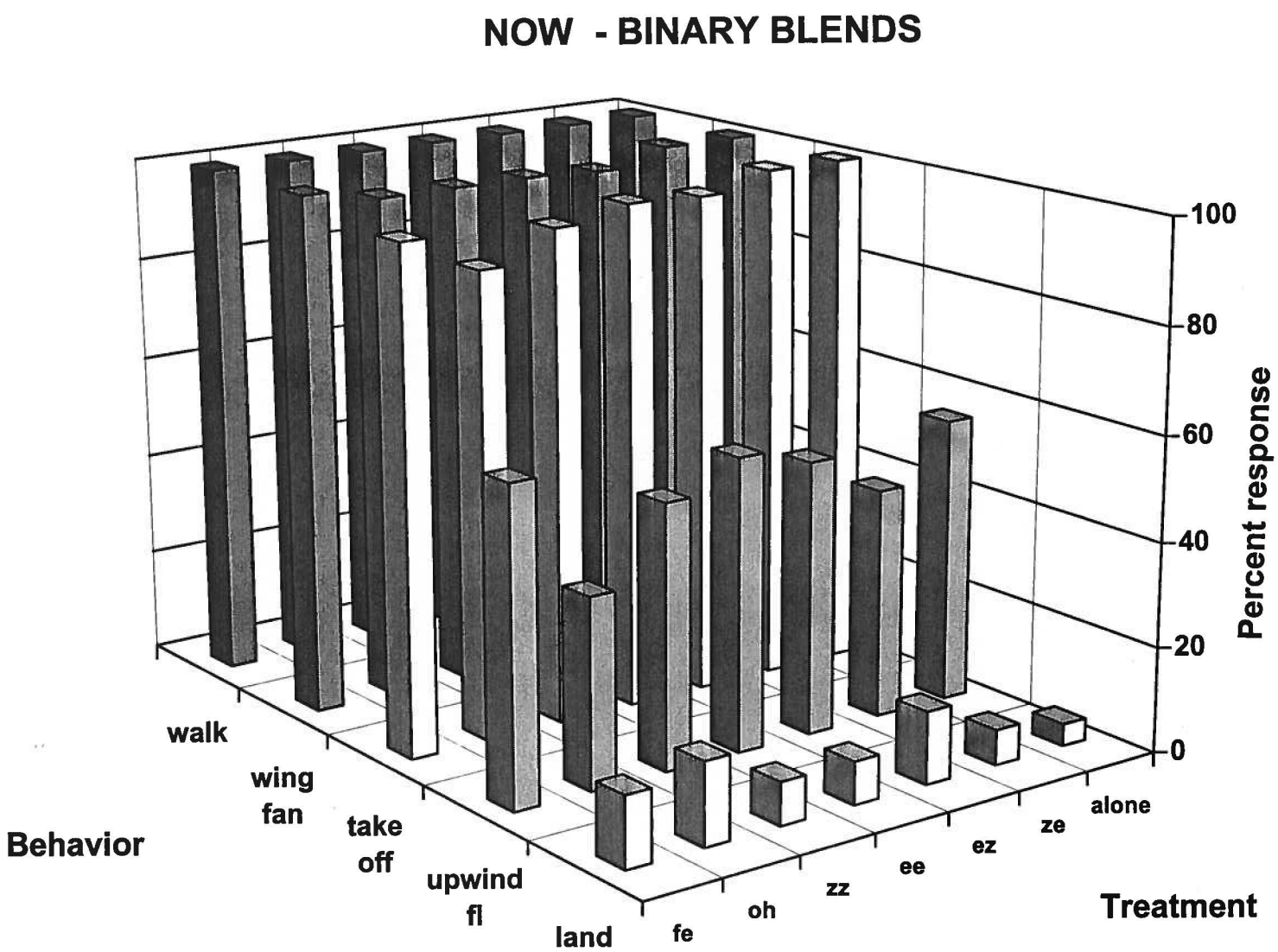
NOW - BINARY BLENDS
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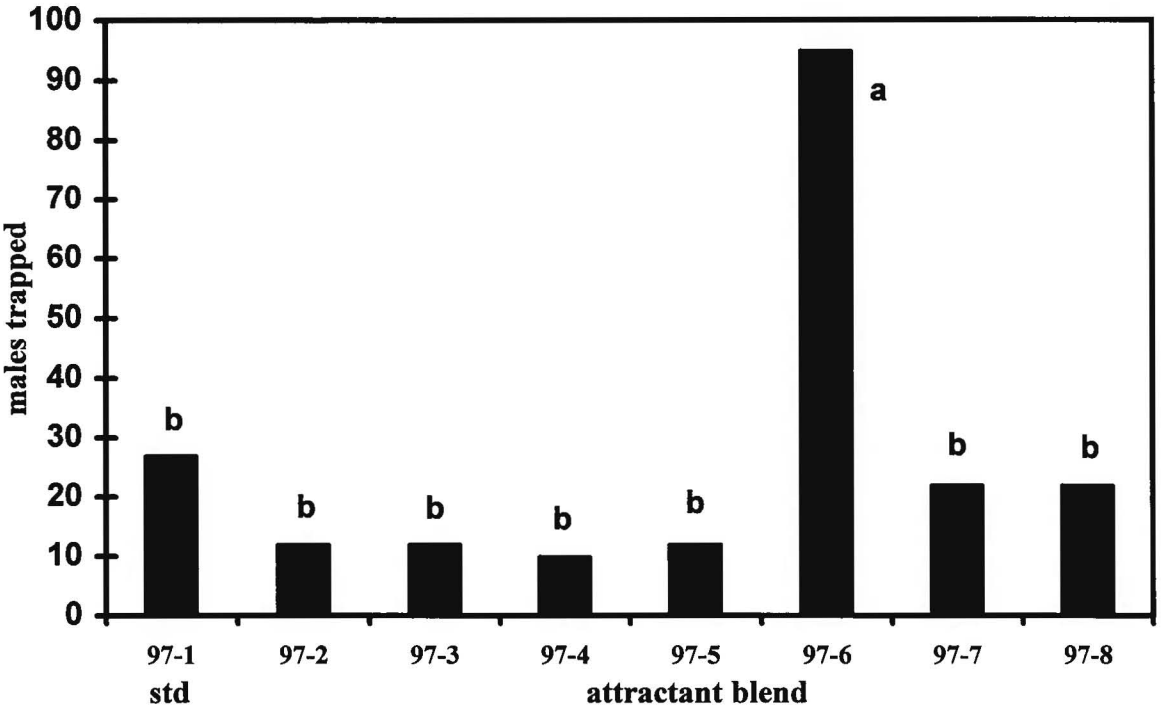
Fig. 4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97-3
97-4
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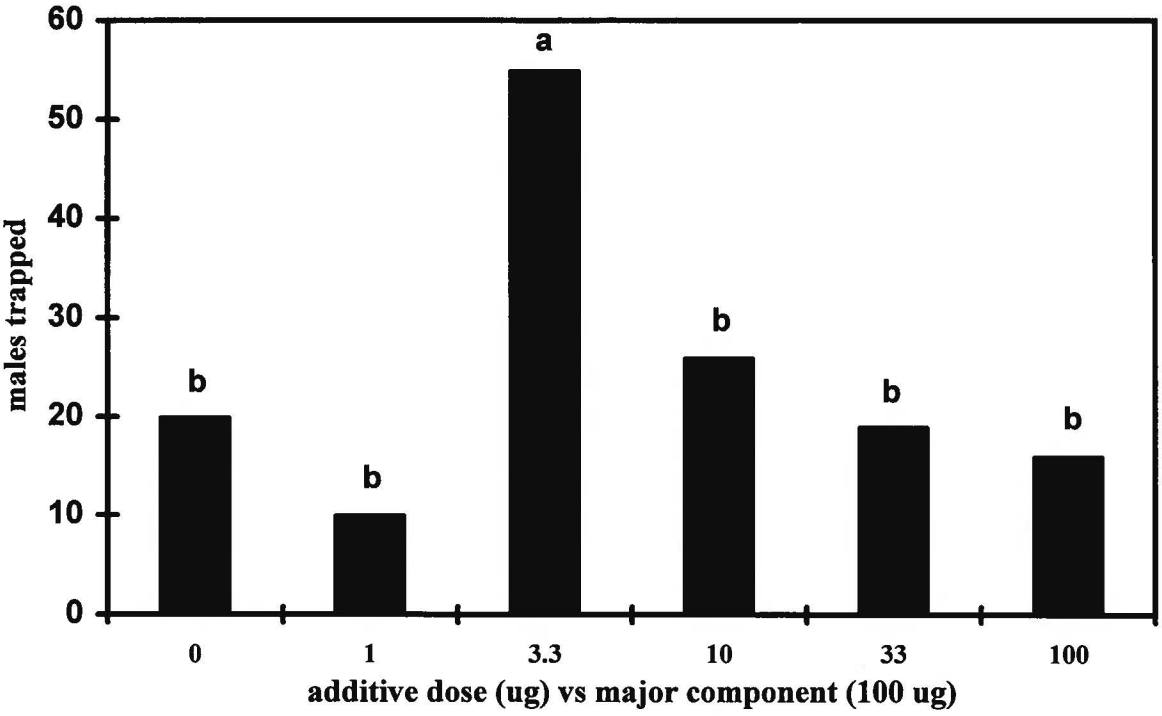
97-8
attractant blend

Fig 5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20
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males trapped
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0
1
3.3
10
33
100
additive dose (ug) vs major component (100 ug)