WHY PUFFERS WORK: DETERMINING THE EFFECTS OF RESIDUAL RELEASES ON CONTROL OF CODLING MOTH IN WALNUTS AND PEARS

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ABSTRACT

The effective area of a pheromone plume from a single puffer, a high-dose aerosol emitter, was shown to disrupt male location of pheromone traps completely for an area ca. 75 meters by 230 meters. The end of the puffer plume appears to have exceed the length of the orchard. Suppression of traps baited with virgin females showed a similar pattern with 100% suppression directly downwind of the puffer. Lateral suppression appears to occur for ca. 75 meters and the degree of suppression was a function of the position of the female baited traps relative to the center line of the plume. Leaves downwind of the puffer unit up to 60 meters appear to absorb pheromone from the plume and re-release the pheromone over time. In wind tunnel trials, moths would orient to plume from the pre-exposed leaves across the entire sample range. Thus, secondary release of pheromone may prove to be an important means by which pheromone levels are maintained at high levels within the tree canopy. The infrastructure for an enzyme-linked immunosorbent assay was established with our lab to look at movement by moth species in walnuts and pears. The technique was refined in the laboratory during most of the summer, and a late season field trial confirmed our ability to mark and capture moths treated with a protein marker, e.g. eggs. Thus wild codling moths can be marked using commercial insecticide application equipment and tracked for movement in response to the puffer pheromone plume.

INTRODUCTION

High dose aerosol pheromone emitters, “puffers”, have been used successfully for a number of years in pears and are emerging as a leading mating disruption strategy for control of codling moth in walnuts. While their success has been documented in both large acreage settings (Lake county pears or Stockton walnut orchards), the exact mechanism by which they operate has not been adequately studied. Understanding how this type of dispenser works may provide opportunities for improving program performance as well as decreasing overall costs. Therefore, a series of studies were initiated in 2009 which attempted to 1) define the effective area of the pheromone plume from a puffer 2) understand how position in the plume impacted with biological variables such as female mating location 3) determine codling moth flight responses to plumes from high emitting sources and 4) enhance our understanding of the effects of direct release of pheromone from the unit as well as secondary release from other parts

of the orchard, e.g. leaves exposed to a pheromone plume. More specifically, we are attempting to build layers of information across a 2 and/or 3 dimensional grid in an orchard such that we can integrate these findings into planning puffer distributions within orchards.

Objectives:

1. Determine secondary pheromone release rates from a) the pheromone cabinet and b) surrounding organic material (bark and leaves) within wind tunnel trials to determine actual biological activity as evidenced by codling moth ability to orient to the pheromone source.
2. Determine the correlation in space and time between secondary release rates and behavioral correlates of codling moth control: 1) trap suppression 2) female mating status 3) preliminary codling moth movement studies.

Experiment 1. Effect of a pheromone plume on suppression of female- and pheromone-baited traps.

Earlier work in our lab on the effects of a puffer plume used sterile codling moths provided by Agricultural Canada released uniformly within an orchard. From these plume studies, the effective area of a plume was estimated to be at least 1000 ft long by several hundred feet wide. In some cases, the effects were noted up to 1800 feet downwind with the end of the plume never observed before the edge of the orchard was reached. The 2009 studies focused not only on evaluating these findings using wild males within walnut and pear orchards but also added the second dimension of the plumes effect on preventing female location by the males. What was not clear at the start of the trial was the similarities or differences that might be expected between the effective size of the plume for 1) suppressing traps baited with a sex pheromone lure and 2) traps baited with virgin females.

Materials and methods

The effect of a single puffer on the suppression of female- and pheromone-baited traps was investigated for codling moth. The assay was conducted in an unfarmed pear orchard of approximately 7 ha, near Freeport (CA). A puffer loaded with Puffer® CM (Suterra LLC, Bend, OR) was placed around 4.5 m above ground at the southwest corner of the orchard (upwind end), and a total of 53 pheromone-baited and 42 female-baited delta traps (LPD, Suterra LLC) were distributed through the orchard following a fairly regular pattern (Figure 1), and hung at 2 m above ground. The pheromone lure used was CM 1X (1 mg, Suterra LLC). Recently emerged (less than 24 h) laboratory virgin females were used as female baits. These were kept inside aluminum net cages (4 x 4 x 4 cm^3 approx.), and provided with sugar water daily.

Traps were checked daily and the number of male captures was recorded. The experiment was replicated twice, from July 7th to 17th, and from July 23rd to 31st. A control replicate was also conducted from August 18th to 27th, by removing the puffer one week earlier. The duration of the different replicates (between 8 and 11 days) was determined by the lifespan of the females. For the two last replicates (one treatment and the control) a wind station was set-up, and wind speed and wind direction were recorded every 15 min.

Using the data recorded, three different parameters were calculated: a) average number of **captures per night**; b) **effectiveness**, proportion of nights with at least one capture out of the total number of nights (nights alive for females); and c) night of the **first capture**. Trap positions were georeferenced, and interpolation surfaces were created for the above parameters.

Results

Pheromone traps

In the absence of the puffer (control replicate) the number of captures per night did not show any clear pattern (Figure 2) as would be expected. Moths were captured through the whole orchard in a homogeneous way. The values of captures recorded in this replicate are low compared to those of the replicates with the puffer. The reason is that the control replicate was run very late in the season and the population of moth had dramatically decreased. However, the aim of the control replicate was to show the absence of spatial patterns in the captures of males, not to compare the number of captures itself among replicates. Similarly, there was no evidence of any spatial pattern for the other two parameters studied in the control replicate, effectiveness (Figure 3), and night of the first capture. For the latter, all pheromone traps had a value of 1, since they all caught moths on the first night of the trial.

On the other hand, in the two replicates with the puffer in the field, captures were totally suppressed in a large proportion of the orchard. The pattern observed for the number of captures in both replicates was almost identical, differing slightly in the magnitude of the captures (Figures 4 and 5). The wind data recorded during the second replicate confirmed that capture suppression was strongly correlated with the wind direction during the evenings, and that a gradient of suppression intensity perpendicular to the wind direction occurs. The area where captures were suppressed 100% was roughly 75 m in width, and reached the downwind end of the orchard (270 m in length). It is very likely, that the puffer influence extends to further distances, but our data cannot confirm that hypothesis.

The same trend was also observed for effectiveness (Figures 6 and 7), and night of first capture (Figures 8 and 9). In the second replicate, captures were never recorded in the central area of the plume, hence effectiveness was 0 around the center of the plume, and the first capture never occurred during the replicate (Figures 7 and 9). On the contrary, in the first replicate some central traps had effectiveness higher than 0 (Figure 7). Most of those traps failed in the same night. It is difficult to determine why traps captured that night. Unfortunately we do not have wind data for that replicate, but during the first replicate there were a couple of days of strong and changing wind. If this hypothesis is true, the occurrence of strong and changing winds can be a limitation for the maximum distance among puffers. However, in a uniform grid, issues of shifting winds would be compensated for by upwind puffers.

Female-baited traps

The results obtained with females widely agreed with the ones of pheromone-baited traps, as the same trends were observed. The most important difference between females and pheromone lures was their different attractiveness. Pheromone lures were much more attractive, catching always a higher number of males than their neighboring female-baited traps (Table 1).

Table1. Average (SEM) captures of males per trap per night at the different replicates.

Lure	Early-July	Late-July	Control
Females	1.69 (0.36)	2.27 (0.49)	1.19 (0.22)
Pheromone	5.47 (0.73)	6.73 (0.94)	4.16 (0.28)

In the control replicate (without the puffer), no clear spatial patterns were found for any of captures per night, effectiveness and first capture (Figures 10, 11 and 12, respectively) as would be expected.

When the puffer was in the field, not only did females capture fewer males than the neighboring pheromone lures, but also the area of total suppression was wider (Figures 13 and 14). Similarly, as a consequence of their lower attractiveness, female-baits also had a lower effectiveness (Figures 15 and 16), and a longer delay for the first capture (Figures 17 and 18) than the pheromone-baits. For all the parameters, the occurrence of a gradient in response from the center to the outside of the plume was clear.

The better performance of the pheromone lures can be due to several factors, including difference in emission rates of pheromone, and difference in timing of activity. Standard codlemone lures are known to emit higher amounts of codlemone than codling moth females, and some authors suggest that a 100 μg lure releases amounts of codlemone closer to the female emissions than the standard 1000 μg. The difference in timing would be related to the time of activity of males, which is usually longer than that of the females, while pheromone lures are continuously emitting.

Our results in trap suppression show that in a wide area around the puffer plume, two important reproductive parameters of codling moth are greatly affected. Those are the effectiveness, which can be interpreted as the probability of a female to successfully attract one male in a given night; and time to the first capture, which represents a delay on mating. Delay in mating may have a great importance, as reproductive capacity of females decreases as they age.

Further assays should be conducted to better assess the impact of wind speed and direction in puffer performance, leading to an optimization of the density of puffers, as well as providing tools for detection of failure risk due to unfavorable conditions.

Experiment 2. Upwind attraction of codling moth males to pear leaves exposed to a puffer under field conditions

Previous studies have shown that foliage exposed to pheromones will both absorb and release pheromone at levels detectable by insects (e.g. European grape moth in grapes, codling moth in apples). What was not known was the effect of the pheromone plume from a high-dose emitter and secondly, how does distance from the source affect secondary release rates. If leaves represent significant release sources after exposure to the pheromone plume, then perhaps the entire orchard becomes the effective release

device or at least an important secondary release source that helps to maintain pheromone levels within the tree canopies.

Materials and methods

We are currently testing attraction of codling moth males to leaves that have been exposed to the sex pheromone in orchards. Samples of leaves were collected from the same orchard in Experiment 1 at different distances and directions from a puffer (Figure 19). The puffer had been relocated to a new position on September 4th, and samples were taken at two different dates, September 27th and October 21st. In the first sampling date leaves were taken up to 60 m away from the puffer. After some preliminary assays, we decided to extend the data points to further distances, and a new group of samples was collected. In addition to the samples collected inside the plume of the puffer, samples were also taken at the northeast side of the orchard, which is out of the plume, and around 300 m away from the puffer. These last samples were considered as negative controls. Samples were stored in a freezer, until the day of their use.

Twigs bearing 6 to 7 leaves have been defined as experimental units. Batches of 15 to 20 codling moth males (replicate) were flown to each twig. Males were flown one-by-one, and they were allowed to respond for 3 min. The behaviors recorded are as follows: **oriented** (**Or**), the male finds the plume and starts the oriented flight; **F1**, upwind oriented flight up to one half of the wind tunnel (around 1 m); **F2**, upwind flight up to 4/5th of the wind tunnel length (around 170 cm); **close-in** (**Ci**), the male approaches the twig and zigzags closely (a few centimeters); **contact** (**Ct**), the male touches the twig; and **landing** (**La**), the male lands on the twig walking and wing-fanning excited.

Four replicates (accounting for between 74 and 78 individuals) have already been conducted for the samples from the first date of collections, but one or none have been for the samples from the second date. For that reason only the results for the first date will be shown in this document. Moreover, to simplify the data have been pooled by range (similar distance).

Proportions of males responding to leaves at the different ranges were analyzed using GLM. The minimal significant model was determined by step-wise simplification of the initial models. Additionally, a second approach for the comparison among ranges based on scores was performed. Every male assayed received a score depending on the further behavior exhibited: 0, no response; 1, oriented; 2, F1; 3, F2; 4, close-in; 5, contact; and 6, landing. Scores were analyzed by model simplification, but this time a linear mixed-effects model was used.

Results

Results obtained in the wind tunnel are summarized in Figure 20. Codling moth males responded to some extent to twigs of all the sampling ranges. The higher responses were recorded to twigs from the closest range (<1 m), achieving a 14.5 % of contacts. Complete flights were not only obtained for that range, but also with twigs from up to 30 m (Figure 20). Responses to the control twigs were lower than for the exposed ones, and none of the insects flown to the control twigs approached them closely (close-in behavior). No differences were found in general among ranges from 7 to 60 m. Furthermore, some contacts have been observed with twigs up to 175 m downwind from the puffer (data not shown, due to the low number of replicates currently conducted).

When scores were analyzed the same pattern was obtained (Figure 21). Twigs from the shortest range elicited the higher responses, followed by those collected at 7, 30 and 60 m. Finally control twigs elicited significantly lower responses than all the other twigs. The advantage of using scores instead of analyzing the behaviors separately is that all the information for each individual is given in a single value, and comparison among treatments becomes easier.

Our data shows that the amount of pheromone released from pear leaves exposed to a puffer is enough to elicit a behavioral response in codling moth males. However the effects under field conditions, where the amount of leaves is extremely large, are difficult to predict. Different experimental approaches are needed to further understand this effect.

Experiment 3. Upwind attraction of codling moth males to walnut leaves exposed to a puffer under field conditions.

A similar experiment to Experiment 2 was conducted in walnuts with the addition of a third dimension. Given the height of the walnut tree canopies, questions about the vertical distribution of the pheromone were also incorporated at 3 locations downwind.

Materials and methods

Samples of leaves exposed to a single puffer were collected from a walnut orchard near Winters (CA). The puffer was placed in a central position on November 12th, and leaves were collected one week later. An anemometer and a data logger were set up close to the puffer to determine average wind direction in the evenings (from 5:00 pm to 1:00 am).

Samples were taken at <1, 17, 50, 100, 135 and 170 m downwind from the puffer. Negative control leaves were collected more than 200 m away from the puffer on the opposite direction. For all sampling points leaves were collected at a height slightly lower to the puffer, and for the distances 17, 50, 170 m and control, additional samples were also taken at higher positions than the puffer. Samples were stored in a freezer, until the day of their use in wind tunnel.

Three leaflets were considered to represent a leaf surface similar to that of the pear samples (experiment 2), and were consequently chosen as experimental units. Wind tunnel procedure was analogous to that used with pear twigs.

At the time of this report, only two replicates (accounting for 40 individuals) have been conducted for the distances <1, 50 and control, and none for the remaining ones. These are the only results that we can show here. Due to the low number of replicates, no statistical analyses have been carried out. Additional samples will continue to be processed over the winter.

Results

Results obtained in the wind tunnel are summarized in Figure 22. The partial results obtained so far are in agreement with those of the pear twigs (Figure 20). Control leaves elicit upwind flight but no male reaches (or closely approaches) the leaves. The leaves at the shortest distance (<1 m) elicit some complete flights, including close leaf approach, contact and landing. At the intermediate distance (50 m) some close-in behaviors have been recorded.

It is still too soon to draw conclusion as this experiment is at a very early stage, but it seems that exposed leaves will yield higher behavioral scores than the control ones, and we expect to observe some complete flights for some of the intermediate or long distances.

Experiment 4. Movement of codling moth within walnut and pear orchards in response to high dose pheromone emitters.

The effects of high dose pheromone sources, puffers, on codling moth movement are unknown. Some researchers have speculated that moths may orient to the pheromone for a portion of the plume, with some moths showing arrested movement as the source is approached. This has not been the case for codling moth, but no studies have exposed them to a source with the release rate equivalent to a puffer. Because we are able to mark more than 1 species of moth with a single treatment, we are able to compare the responses of one species with reports of limited average movement (codling moth) compared to a species reported as highly dispersive (e.g. the navel orangeworm). What is also not clear is whether or not these potential differences in movement are part of the explanation for why some pests are more easily managed than others with pheromones.

Similarly, development and confirmation of our techniques in the laboratory were conducted in parallel for both navel orangeworm (NOW) and codling moth (CM), but the NOW portion of this project is funded by the USDA – ARS project. A walnut orchard in the Woodland area was used as the initial trial given the known populations, whereas a second attempt was made later in the year in pears, but the flights failed to continue in the field. The marking of wild males and female moths takes advantage of the existence of commercially available antibodies to a variety of proteins, eggs, milk, or soy. In 2009, we focused on eggs as the protein source for marking. A slurry of eggs are mixed within a sprayer and applied directly to the trees with high pressure sprayers. Moths in the trees are either covered with the spray or walk on the leaves contaminated with the egg residues. The moths are captured in traps after a few days or weeks and then assayed for the presence of the eggs. In essence, the moths are removed from traps, rinsed, and the rinse examined using a color assay for the presence of eggs. Antibodies for eggs bind with rinse wells and are treated with secondary antibodies that have a color induced marker. Moths that score positively are then known to have either originated from or at least visited the treated area of the orchards. The position of capture are noted in the orchard and measured relative to the site of treatment.

Methods:

Enzyme-linked immunosorbent assay:
Methods were followed according to Jones et al. 2006 with one modification. The three washes of phosphate buffered saline containing 2.3 g/liter sodium dodecyl sulfate (PBS-SDS) were replaced with two washes of phosphate buffered saline containing 0.09% Triton-X100 (PBST). We found that the SDS detergent was too harsh and significantly decreased our marking rate in controlled lab trials.

ELISA spray
On July 29, 2009, a field study was conducted within a 27.7 acre walnut orchard near Woodland CA to examine our ability to mark and track NOW, and CM using the above enzyme-linked immunosorbent assay (ELISA) (Fig 23). . A plot of 64 trees in the

center of the orchard with an area of 1.1 acres was sprayed with a 10% solution of reconstituted powdered egg whites (Honeyville Food Products, Rancho Cucamonga, CA) Traps for NOW were placed in a radiating design (Mills et al. 2006), radiating from the sprayed area to the edges of the orchard. These traps were placed at 23 locations, each location had 2 traps for a total of 46 traps. Twenty-four traps for CM were placed through the whole orchard. These traps included 12 baited with CM sex pheromone (1X, 1 mg, Suterra LLC), and 12 baited with a combination of sex pheromone and the pear ester (CM/DA Combo, Trécé Inc.) (Figure 23).

NOW traps were checked after 2 days, and then again weekly for 5 weeks. Traps were baited with three virgin female NOW housed in a fiber glass mesh cage that hung inside the delta trap (Suterra, Bend, OR). Virgin females were changed on a weekly basis as traps were checked. CM traps were checked weekly for 4 weeks. Collected trap bottoms were wrapped in saran wrap and stored at -20 ºC until ELISA assay could be performed. Saran wrap was used to prevent contamination between insects caught on sticky traps.

Wind Direction:
An anemometer was placed in the center of the orchard to determine overall wind direction and wind patterns. Wind has the potential to influence moth movement and therefore might be important to subsequent analyses.

Data analysis:
Moths were recorded as marked if the ELISA optical density readings were 4 standard deviations above the mean of unmarked control insects. Control insects of NOW were collected in Capay Valley, and laboratory reared insects were used in the case of CM.

Results:
Marked NOW moths were found for 4 weeks, after which we no longer detected any marking in the traps. In the first four weeks 589 NOW moths were caught, with 21 of those being marked. This constitutes a 3.6% marking percentage across the whole orchard. Although this is a fairly low marking rate, we have to consider that we sprayed 4.2% of the entire orchard. If we take the percent of the moths marked over the percent of the orchard sprayed, we see an 86% recapture rate. This suggests that our low marking rates are the product of a small sprayed area, rather than the ELISA being unsuccessful. Similar recapture rates for the egg protein within a egg sprayed area have been reported (Jones et al. 2006; recapture = 70.3%). There were no significant movement patterns across the orchard.

Thirteen out of the 24 traps for CM caught moths (the 12 baited with combo lures and one pheromone trap). A total of 366 CM individuals were caught, and 39 of them (10.7 %) were found marked by the egg protein. Most of the marked insects were captured downwind from the egg-sprayed area (Figure 23). Intuitively, one would expect moths to fly upwind, but in the orchard sprayed a mating disruption assay for CM was being conducted. This technique is suitable to future comparisons of CM movement between mating disrupted orchards and orchards under a different control strategy.

Figure 1. Scheme of trap display and puffer position in the trap suppression experiment. Distances are in meters from the puffer position in the south-north and west-east directions.

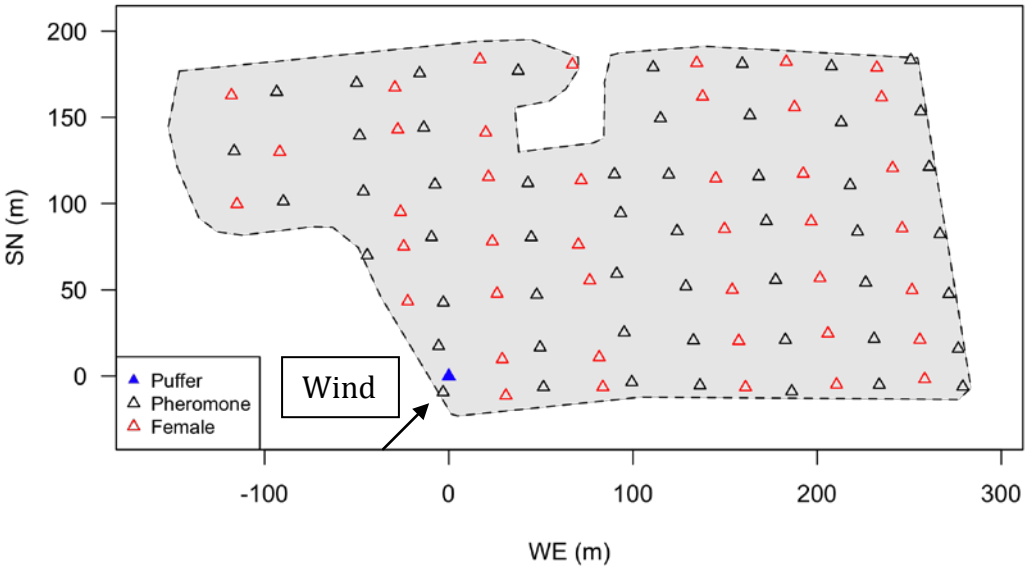

Figure 2. Interpolation for captures/night by *pheromone lures* in the absence of puffer (August 18th to 27th). Distances are in meters from the puffer position in the south-north and west-east directions. Arrow shows evening average wind direction.

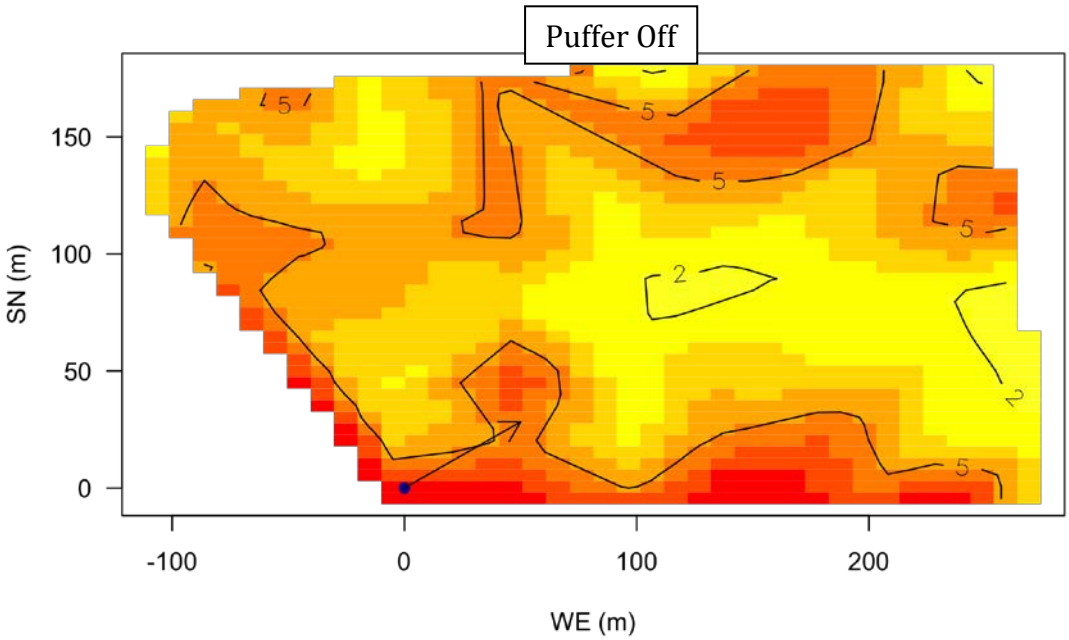

Figure 3. Interpolation for effectiveness (nights with captures/total nights) by *pheromone lures* in the absence of puffer (August 18th to 27th). Distances are in meters from the puffer position in the south-north and west-east directions. Arrow shows evening average wind direction.

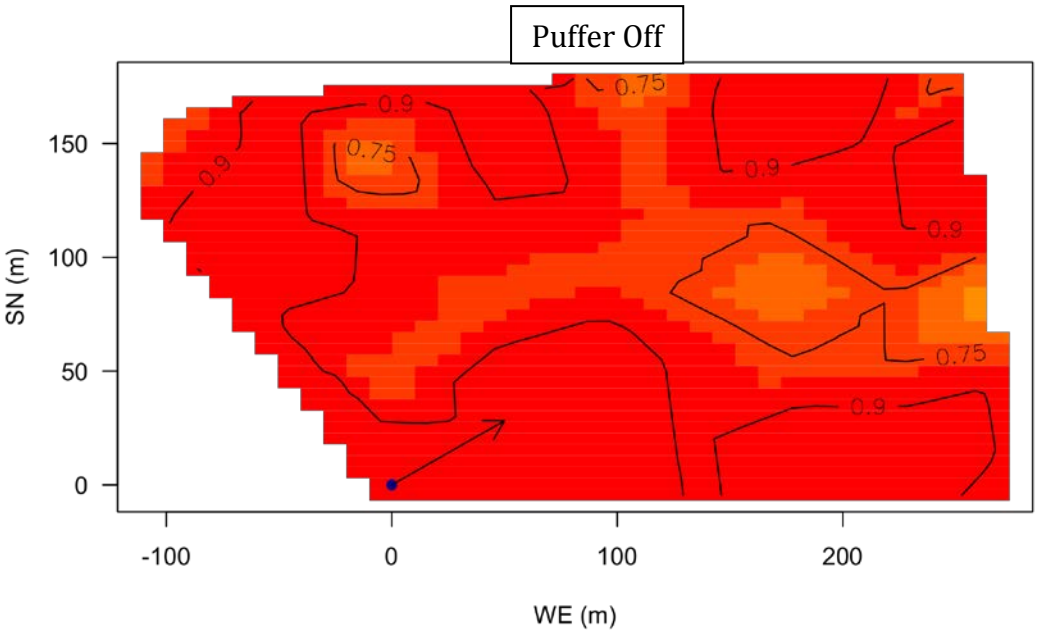

Figure 4. Interpolation for number of moths/night by *pheromone lures* from July 7th to 17th (puffer on). Distances are in meters from the puffer position in the south-north and west-east directions. Blue dot denotes puffer position.

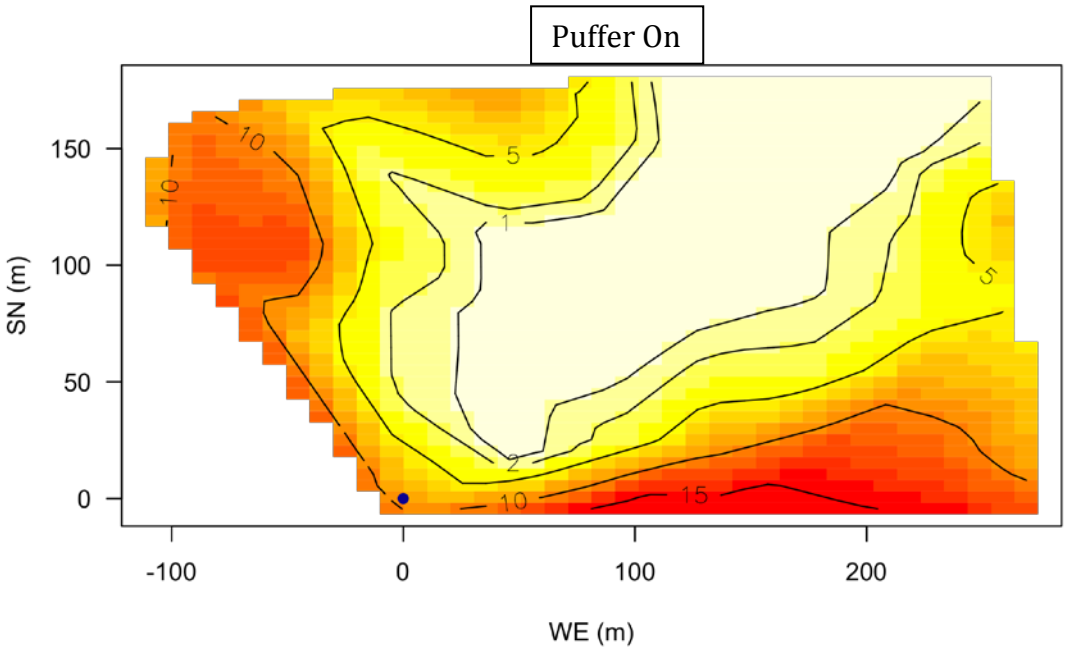

Figure 5. Interpolation for captures/night by *pheromone lures* from July 23rd to 31st (puffer on). Distances are in meters from the puffer position in the south-north and west-east directions. Blue dot denotes puffer position. Arrow shows evening average wind direction.

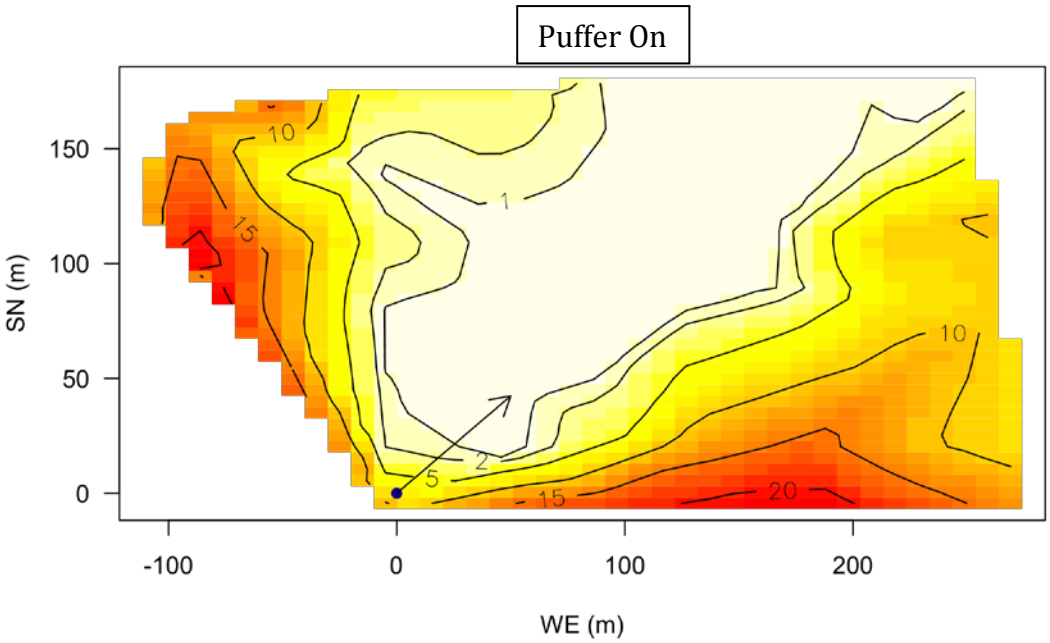

Figure 6. Interpolation for effectiveness (nights with captures/total nights) by *pheromone lures* from July 7th to 17th (puffer on). Distances are in meters from the puffer position in the south-north and west-east directions. Blue dot denotes puffer position.

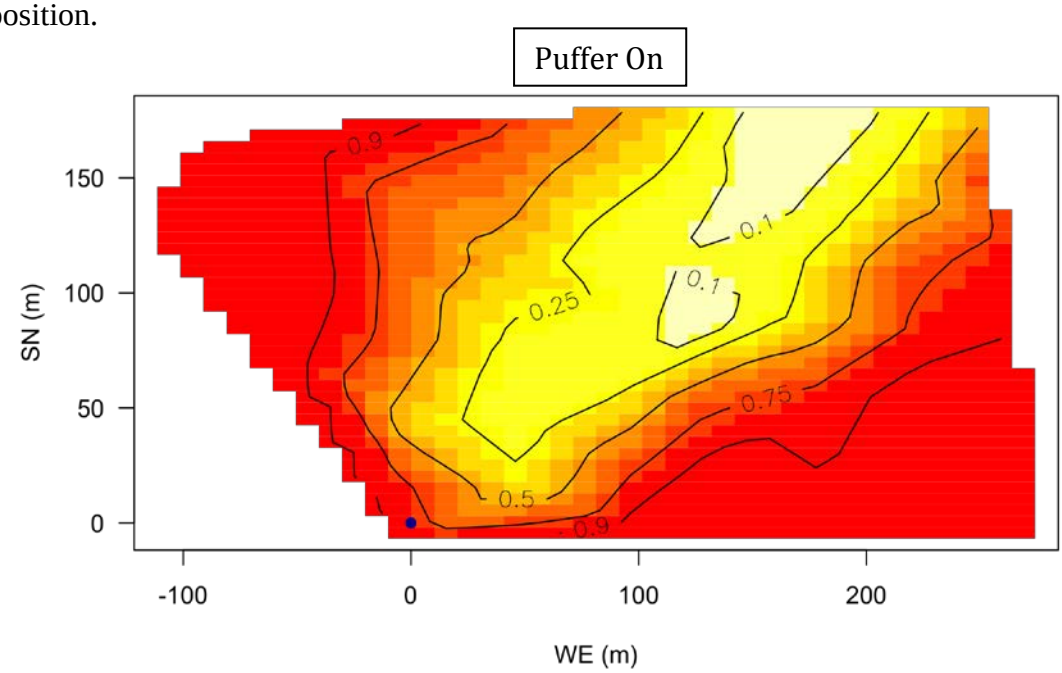

Figure 7. Interpolation for effectiveness (nights with captures/total nights) by *pheromone lures* from July 23rd to 31st (puffer on). Distances are in meters from the puffer position in the south-north and west-east directions. Blue dot denotes puffer position. Arrow shows evening average wind direction.

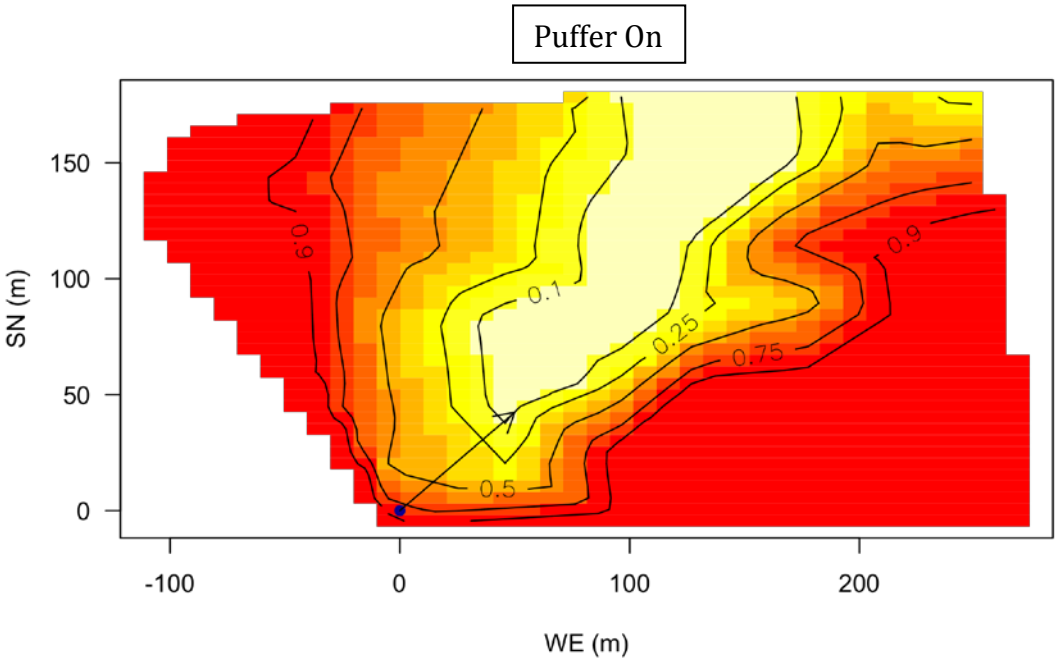

Figure 8. Interpolation for night of first capture by *pheromone lures* from July 7th to 17th (puffer on). Distances are in meters from the puffer position in the south-north and west-east directions. Blue dot denotes puffer position.

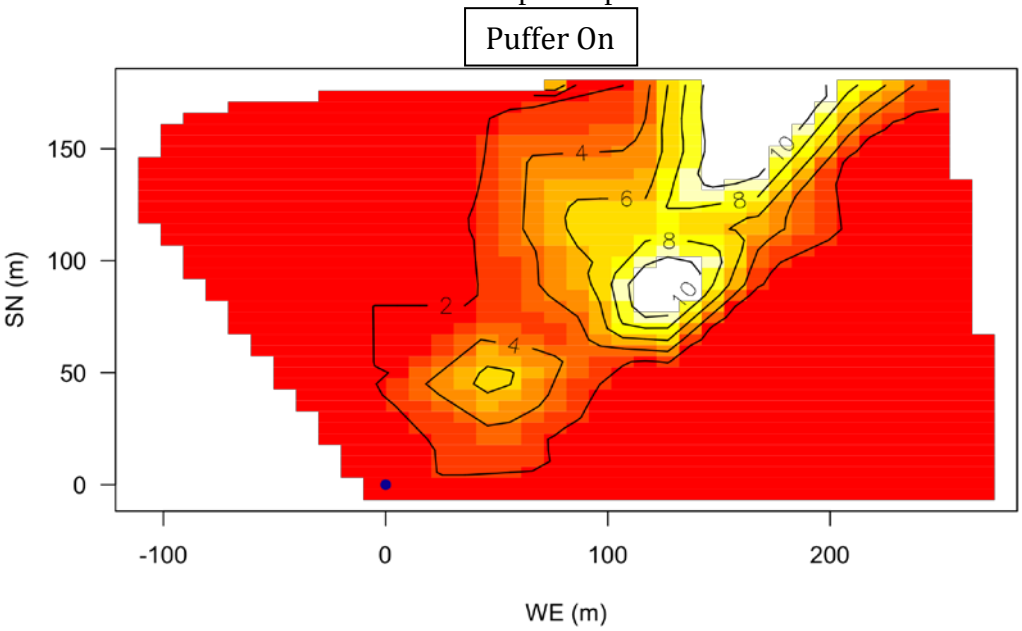

Figure 9. Interpolation for night of first capture by *pheromone lures* from July 23rd to 31st (puffer on). Distances are in meters from the puffer position in the south-north and west-east directions. Blue dot denotes puffer position. Arrow shows evening average wind direction.

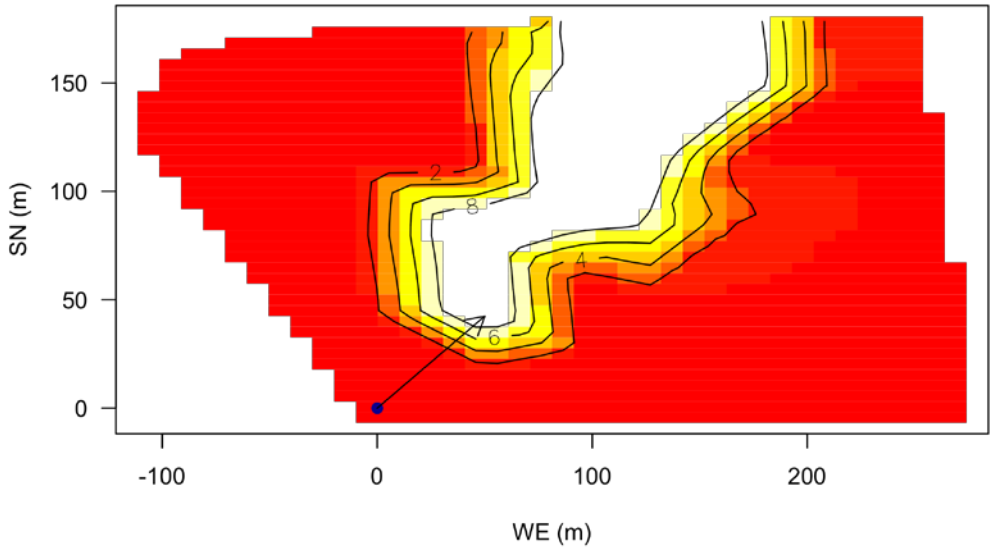

Figure 10. Interpolation for captures/night by *females* in the absence of puffer (August 18th to 27th). Distances are in meters from the puffer position in the south-north and west-east directions. Arrow shows evening average wind direction.

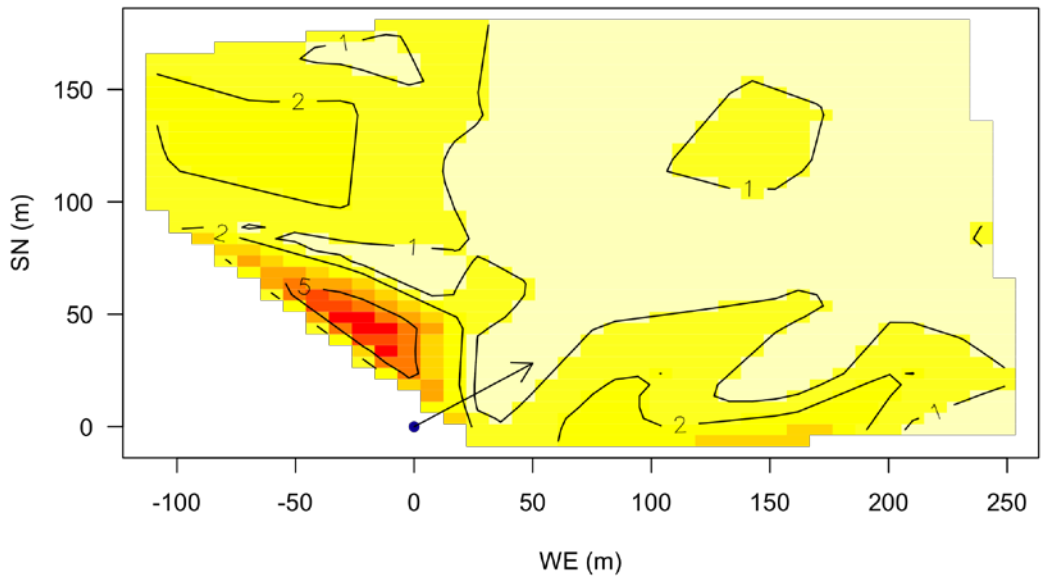

Figure 11. Interpolation for effectiveness (nights with captures/total nights) by *females* in the absence of puffer (August 18th to 27th). Distances are in meters from the puffer position in the south-north and west-east directions. Arrow shows evening average wind direction.

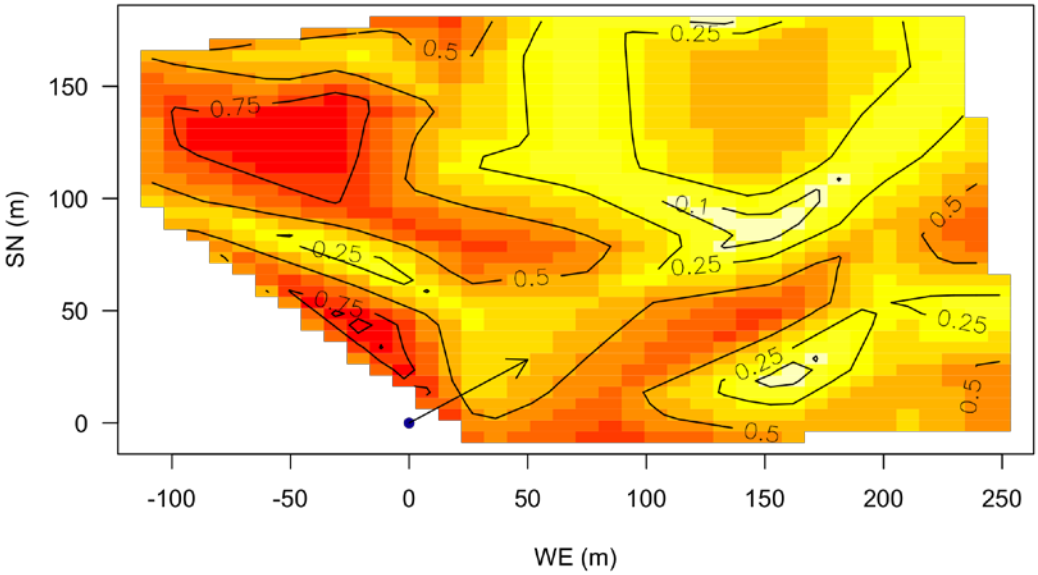

Figure 12. Interpolation for night of first capture by *females* in the absence of puffer (August 18th to 27th). Distances are in meters from the puffer position in the south-north and west-east directions. Arrow shows evening average wind direction.

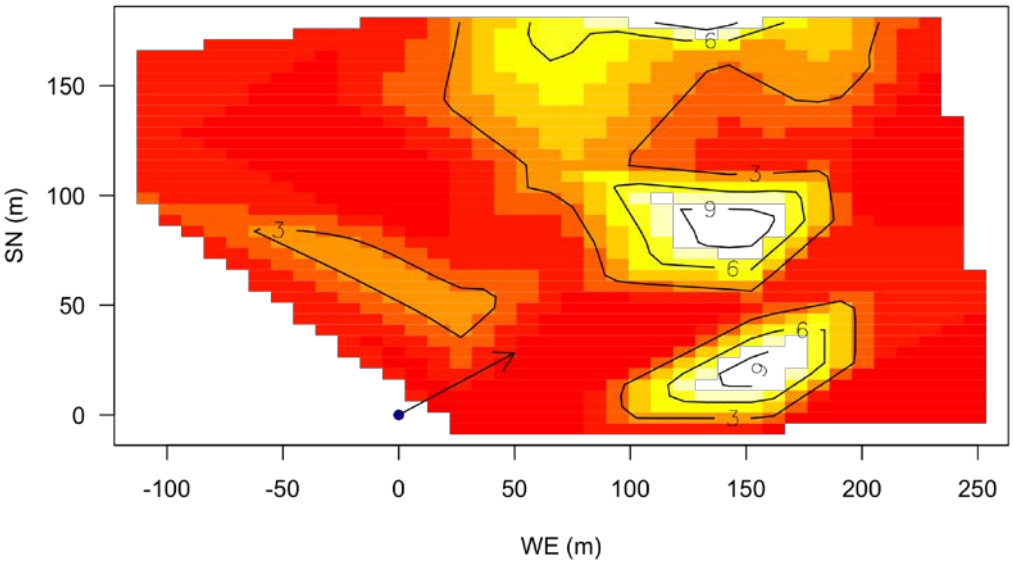

Figure 13. Interpolation for captures/night by *females* from July 7th to 17th (puffer on). Distances are in meters from the puffer position in the south-north and west-east directions. Blue dot denotes puffer position.

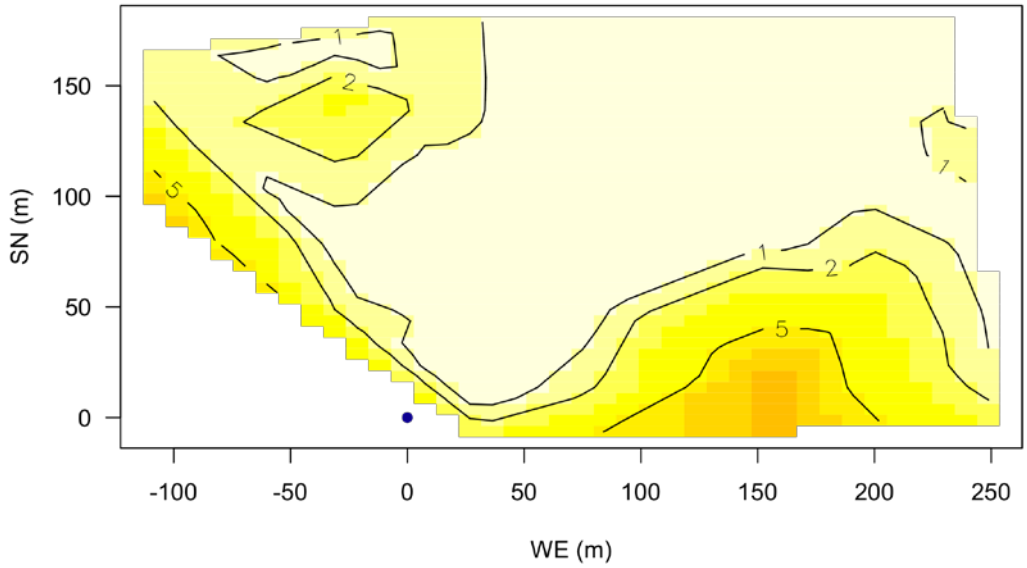

Figure 14. Interpolation for captures/night by *females* from July 23rd to 31st (puffer on). Distances are in meters from the puffer position in the south-north and west-east directions. Blue dot denotes puffer position. Arrow shows evening average wind direction.

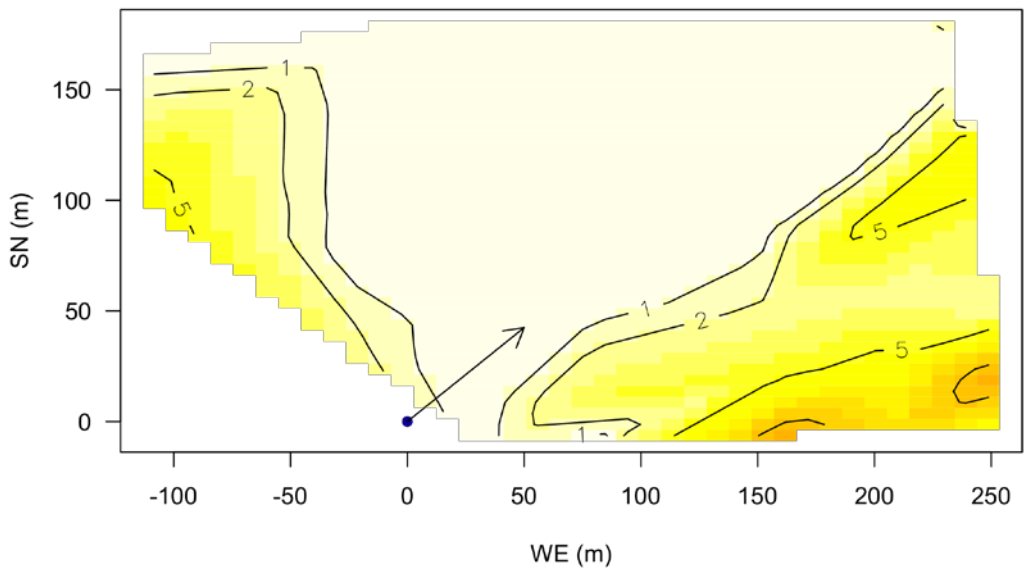

Figure 15. Interpolation for effectiveness (nights with captures/total nights) by *females* from July 7th to 17th (puffer on). Distances are in meters from the puffer position in the south-north and west-east directions. Blue dot denotes puffer position.

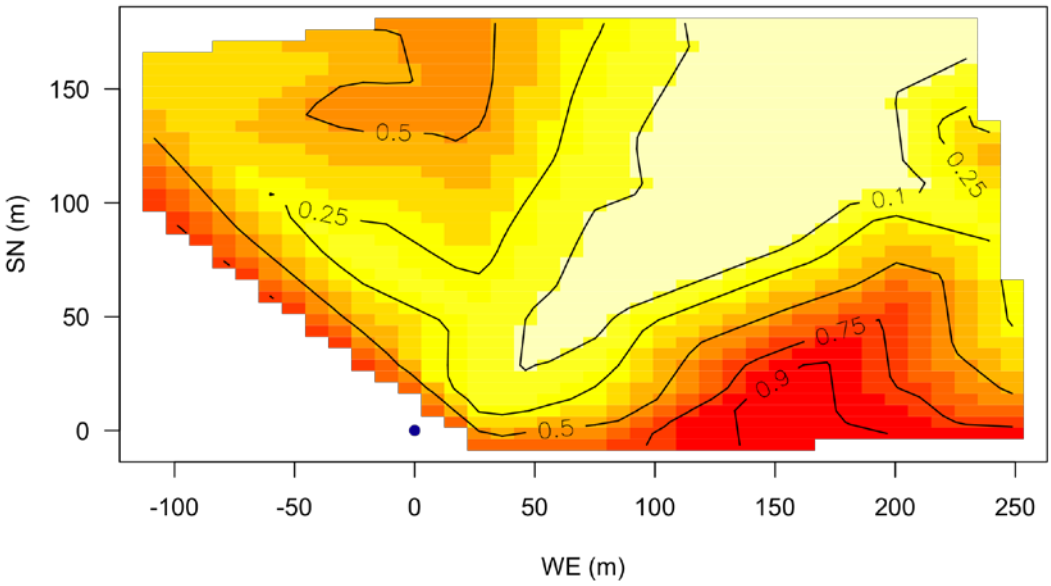

Figure 16. Interpolation for effectiveness (nights with captures/total nights) by *females* from July 23rd to 31st (puffer on). Distances are in meters from the puffer position in the south-north and west-east directions. Blue dot denotes puffer position. Arrow shows evening average wind direction.

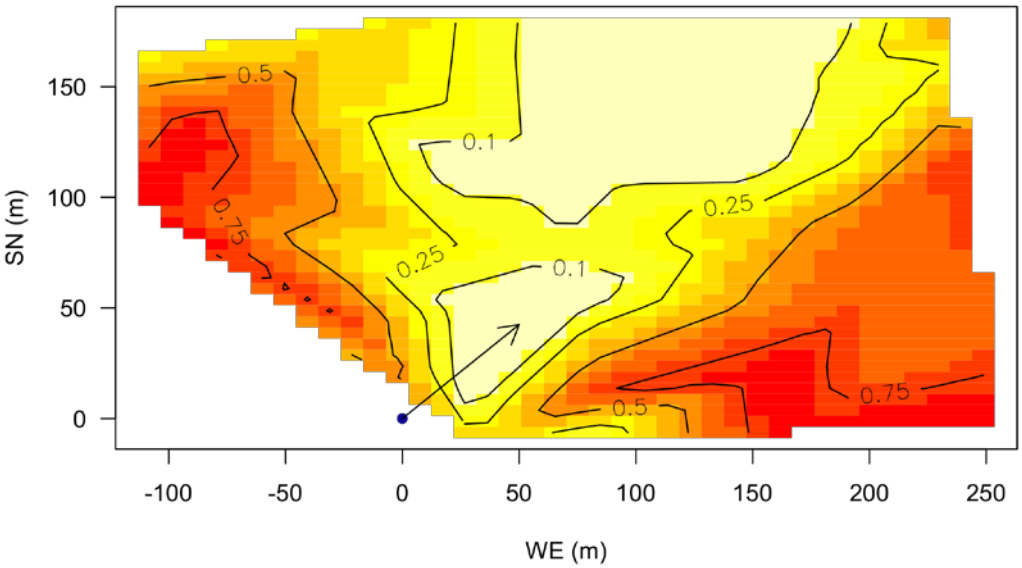

Figure 17. Interpolation for night of first capture by *females* from July 7th to 17th (puffer on). Distances are in meters from the puffer position in the south-north and west-east directions. Blue dot denotes puffer position.

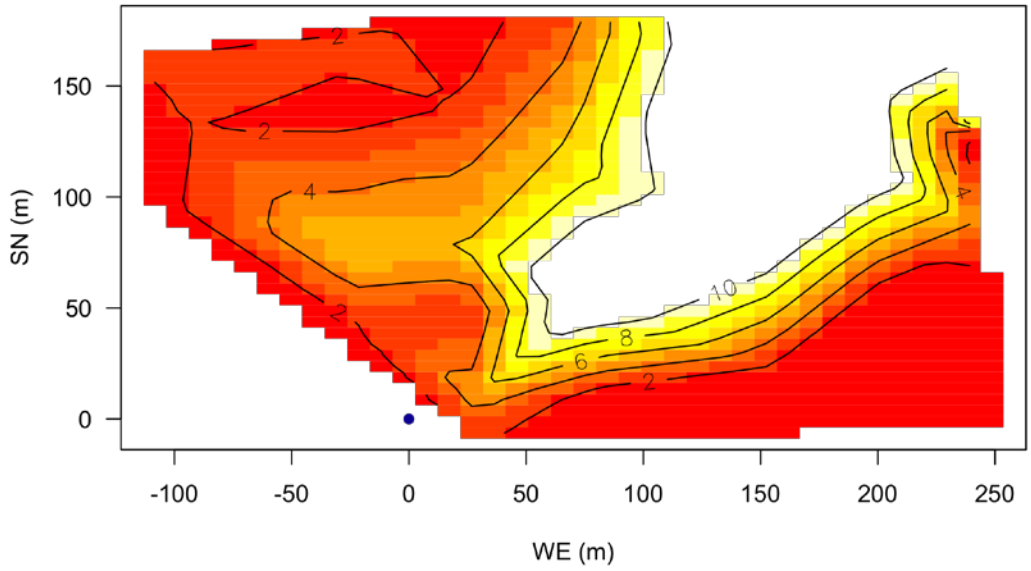

Figure 18. Interpolation for night of first capture by *females* July 23rd to 31st (puffer on). Distances are in meters from the puffer position in the south-north and west-east directions. Blue dot denotes puffer position. Arrow shows evening average wind direction.

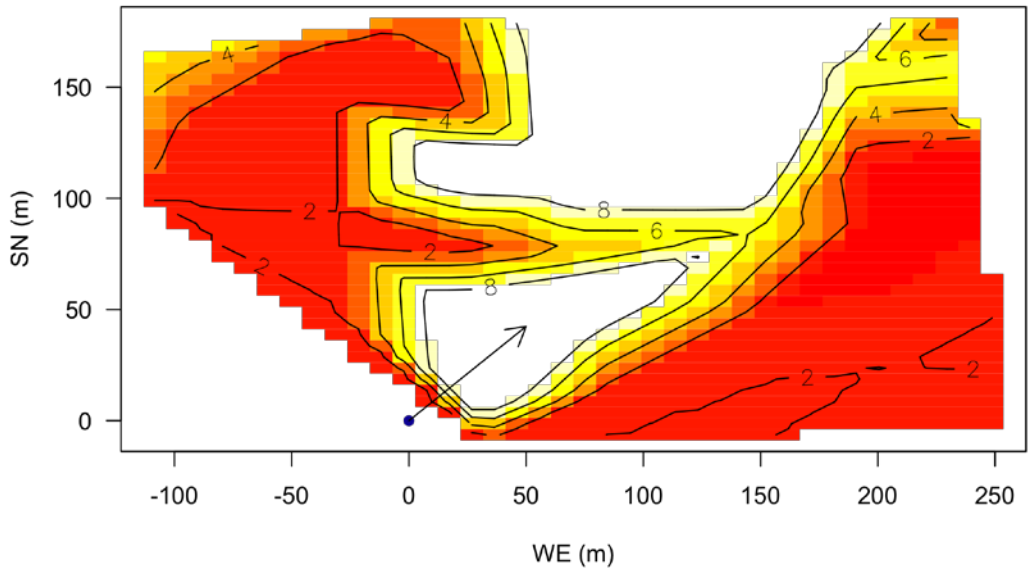

Figure 19. Scheme of sampling points and puffer position for the experiment on upwind attraction in wind tunnel. Distances are in meters from the puffer position in the south-north and west-east directions. Blue triangle denotes puffer position.

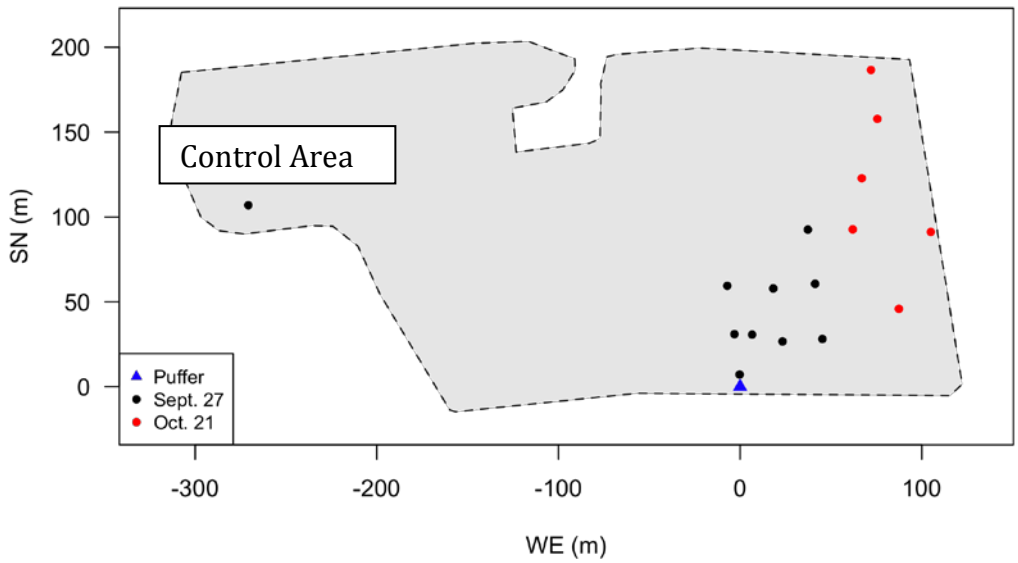

Figure 20. Average behavioral responses (± SEM) of males of codling moth to pear twigs, collected at different ranges from a puffer. Between brackets is the number of replicates (batches of males). Ranges with different letters within behaviors differed significantly.

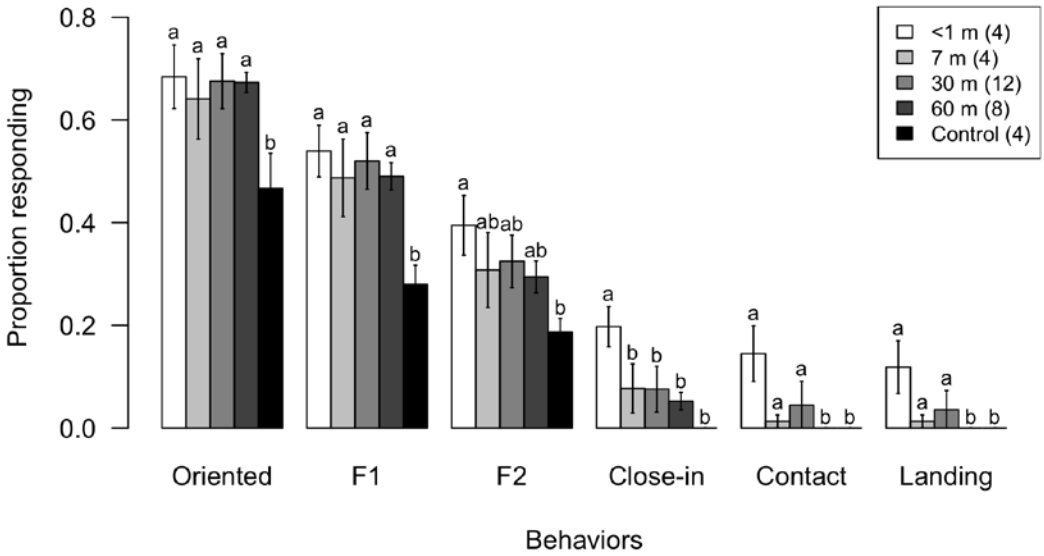

Figure 21. Average behavioral scores (± SEM) of males of codling moth in response to pear twigs collected at different ranges from a puffer. Between brackets is the number of replicates (individuals). Ranges with different letters differed significantly. The right axis is a reminder of the equivalence between score and behavior.

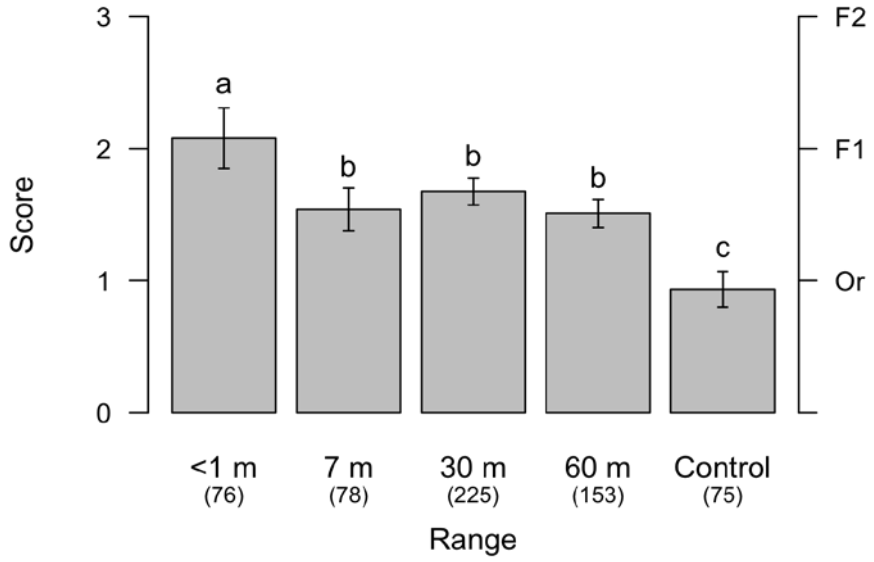

Figure 22. Average behavioral responses (± SEM) of males of codling moth to walnut leaves, collected at different distances from a puffer

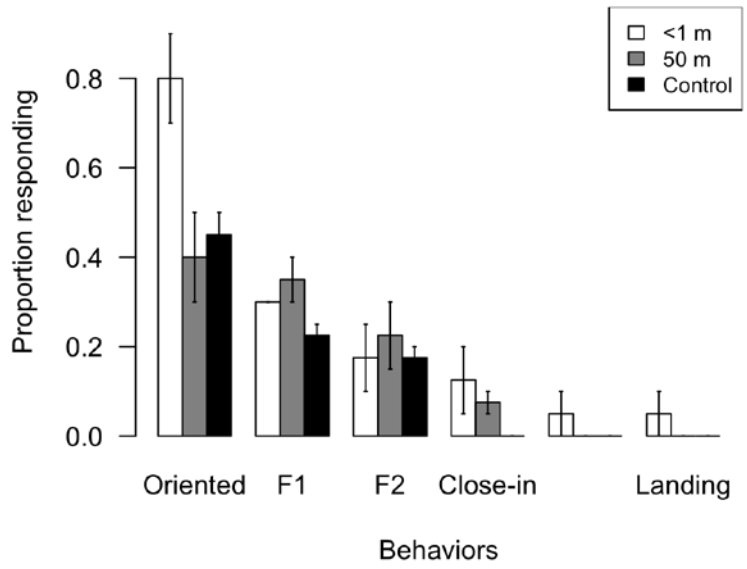

Figure 23. Scheme of trap display and egg-sprayed position. Distances are in meters from the center of the egg-sprayed area, in the south-north and west-east directions.

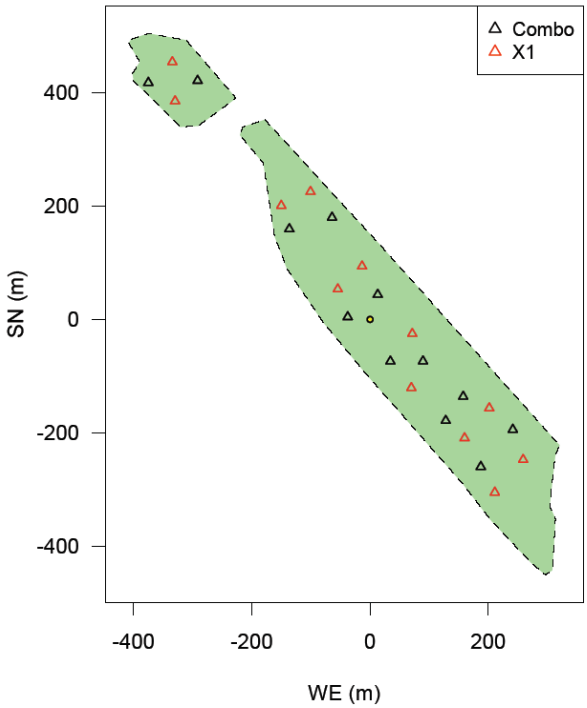

Figure 24. Total numbers of codling moth captures, and marked insects at the trap locations. Distances are in meters from the center of the egg-sprayed area, in the south-north and west-east directions. The arrow denotes average wind direction during the evening.