

Semiochemicals of *Ostrinia nubilalis*: diel response to sex pheromone and phenylacetaldehyde in open field

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Abstract

Ostrinia nubilalis (European corn borer, ECB) is a widely recognized pest of several crops over much of the northern hemisphere. Diel flight rhythms of this moth in response to the attractants phenylacetaldehyde (PAA) and sex pheromones were studied in field conditions. Trapping experiments during three seasons (2008, 2009, 2010) were conducted in an area of the Po valley (northern Italy) where maize cultivation is widespread and *O. nubilalis* E and Z strains live in sympatry. Both ECB males and females were captured by water pan traps baited with PAA and males alone with Z/E pheromone isomers (11-tetradecenyl acetate). Moths caught by traps were counted every three hours, from 18.00 to 06.00 h. Both ECB males and females showed no crepuscular habits. Adults were caught by traps before 21.00 h only at the end of August, when light values were almost zero. Most of females attracted by PAA were trapped early in the night (21.00-24.00 h). Almost all of those females had mated, suggesting that PAA attraction could be related to oviposition. Unlike females, males did not show a diurnal rhythm in response to PAA, i.e. male captures maintained a steady level all night long. Males behaved differently in response to both Z and E pheromone blends; captures were mainly recorded after 24.00 h. This finding is consistent with previous data that demonstrated a gradual increase of pheromone titer and calling behaviour by females during the scotophase, following a decrease of temperature and an increase of relative humidity.

Key words: European corn borer, adult attractants, E/Z strains, water pan traps, maize, Italy.

Introduction

The European corn borer (ECB), *Ostrinia nubilalis* (Hubner) (Lepidoptera Crambidae), is a widely recognized pest of economic importance. A great effort has been made by researchers to learn more about ECB population dynamics, life cycle and behaviour (Calcagno *et al.*, 2010) to improve control strategies (Lassance, 2010). The identification of sexual attractants produced by females (Klun, 1968; Klun *et al.*, 1975) provided an important tool for monitoring its life cycle (Maini and Burgio, 1999). Like other species of genus *Ostrinia* (Tabata and Ishikawa, 2011) ECB populations exhibit pheromone polymorphism (Peña *et al.*, 1988). They exist as two distinct sex pheromone races: ECB (E) females produce a 97:3 blend of E11- and Z11-tetradecenyl acetate, as against the 3:97 ratio of the isomers released by ECB (Z) females.

Differences in blends released by females and males response are controlled by at least three loci (Roelofs *et al.*, 1987; Dopman *et al.*, 2004; Lassance *et al.*, 2010). In lab conditions, the analysis of hybrid virgin females from the genotypes described above revealed that they secrete an isomer blend that approximates 35:65 Z:E (Klun and Maini, 1979). Hybridization can occur also in field conditions (Klun and Maini, 1979; Willet and Harrison, 1999; Coates *et al.*, 2013) but hybrids are usually less common than Z and E strains (Cardè *et al.*, 1978; Pelozuelo *et al.*, 2004). E and Z races can coexist (Calcagno *et al.*, 2007) and their proportion tends to change in relation to geographical distribution. The most common ECB phenotype in nature is the Z one, both in Europe (Leniaud *et al.*, 2006) and North America (Klun and Cooperators, 1975). In the Palearctic region Z populations tend to

prevail, except for Switzerland and Italy (Anglade *et al.*, 1984). In France, where ECB cycle is mainly monovoltine, Z and E phenotypes do not share maize as a common host: E strain populations feed upon mugwort (*Artemisia vulgaris* L.) and hop (*Humulus lupulus* L.), while Z populations (Bontemps *et al.*, 2004; Leniaud *et al.*, 2006; Malausa *et al.*, 2005; 2008) are hosted by maize, pepper (*Capsicum frutescens* L.) and cocklebur (*Xanthium* sp.). This race segregation is not a general rule: in Italy for example, where ECB is bivoltine, maize hosts both E and Z strains (Maini *et al.*, 1979).

An important, additional step of research on ECB semiochemicals was the discovery of the attractive power of phenylacetaldehyde (PAA) (Cantelo and Jacobson, 1979; Maini and Burgio, 1991; Burgio and Maini, 1995). The availability of PAA and sex pheromone was an important advance in monitoring techniques because it makes possible to better assess oviposition trends (Maini and Burgio, 1999).

Anyway, despite the great amount of data on those semiochemicals, the response of ECB adults to PAA and sex pheromone overnight in field conditions is still unclear, therefore a research was planned.

Materials and methods

The research was carried out during three seasons (from 2008 to 2010) in the Po river valley, the main Italian agricultural district. The study area was located in Bastida Pancarana 45°05'24"N 9°04'59"E. The agricultural landscape is dominated by arable land, where mainly maize, wheat, barley, sorghum and horticultural crops are grown.

Maize is widespread and is cultivated both for cattle-feed production and as a source of biomass for biogas generation. Together with *Diabrotica virgifera* LeConte, ECB is the most important pest of maize in the Po floodplain, where it usually develops two generations. Adults coming from the overwintering larvae (first flight adults) produce a summer generation of adults (second flight adults) which in turn generate larvae that overwinter in diapause. Under favourable conditions (warm weather and abundance of rain) a third partial generation can occur (Maini and Burgio, 1999). Like in the whole Po floodplain, in the study area males attracted by E isomer of sexual pheromone prevail on the race responding to Z one (Maini *et al.*, 1979).

Data on moth captures were collected since June, when adults arising from the ECB overwintering generation start flying. However, as it turned out that the number of adults attracted by PAA and Z isomer was low during this life cycle stage, the main focus of the research was shifted to the study of second flight adults. These are much more abundant than those arising from overwintering larvae and moth captures are therefore easier to obtain in July and August. Ten series of observations were dedicated to first flight adults: five in 2008 and five in 2009, from 28th May to 15th June. Fourteen series of observations were carried out on second flight adults, from 22nd July to 30th August: four in 2008, six in 2009 and four in 2010. In addition to sampling of adults at different moments of the night, the complete seasonal flight curves were determined in 2008 and in 2009.

EBC adults were caught by means of water pan traps, held on a pole 1.5 m high, on which a plastic plate (24 cm diameter) was fixed. This plate worked as a stand for an upper plate of the same size, filled with water and wetting agent (5%). The bait was tied over the plates by means of a steel wire linked to the pole, in such a way that PAA or pheromone dispensers were suspended on water surface.

Four replicates made of 4 traps for each one of samples (control, E, Z, PAA) were set up and the 16 traps were kept at least 40 m away from artificial lights in order to prevent any disturbance caused by light pollution.

Traps were placed at the border of maize fields where grass and wild plants work as aggregation sites (Showers *et al.*, 1974; Bailey *et al.*, 2007). Distance between traps was at least 25 meters. Trapped moths were counted every three hours, from 18.00 to 06.00 h. After counting, trapped specimens were brought to the lab and observed with a stereoscopic microscope. ECB females were dissected and the bursa copulatrix was extracted. The proportion of mated/virgin females was determined by the presence or absence of the sclerotized spermatophore in the bursa copulatrix (Showers *et al.*, 1974).

Before starting a new set of observations, the trap arrangement was randomly changed, and plates were filled with water and wetting agent to the brim just before 18.00 h, when observations started.

Pheromone was released by commercially available rubber septa (Isagro, Milan; loading 0.1 mg/lure). Two blends of 11-tetradecenyl acetate were used: E/Z 3:97 and E/Z 97:3. The attractant (PAA 370 mg), stabilized with an UV-screener and an antioxidant, was impregnated on a 25 × 25 mm filter paper (2.7 mm thick). Both

PAA filter papers and sex pheromone rubber septa baits were weekly renewed.

ECB flight was not surveyed in windy or rainy nights, since those conditions are not suitable for moth flight. Temperature, relative humidity and wind were measured by “testo 410-2”, an instrument consisting of an hygrometer and an NTC air thermometer.

An important factor affecting ECB flight is light level (Loughner and Brindley, 1971). For this reason, a luxmeter Delta Ohm HD2302 (resolution 0.01 lux) was used to record the illuminance around 21.00 h in order to monitor the summer sunset shift which deeply affects light levels.

Data coming from the research were elaborated using Biostat software. Data sets were first analyzed to test their distribution. As data were not normally distributed, the non-parametric tests χ^2 and Kruskal-Wallis were applied. The χ^2 statistic made it possible to investigate whether distributions of categorical variables (moth captures in different time ranges, in the case of study) differed among themselves. In addition, contingency tables were used to record and analyze the relation between two or more categorical variables.

Kruskal-Wallis test of variance by ranks - a method for assessing whether samples originate from the same distribution - was used to compare medians of more than two samples.

Results

ECB male flight curves related to 2008 and 2009 seasons are shown in figures 1 and 2. Z and E strains exhibited a similar phenology pattern. A first peak of flying males occurred at the end of May, due to adult emergence coming from the overwintering generation. Second flight moth males were observed starting from the first decade of July and the maximum number of captures occurred in the second half of August. The male flight trend recorded in 2009 also fits this pattern.

As expected, males population attracted by E isomer (70.97%) prevailed on Z population in 2008 (χ^2 test - $P < 0.01$). This trend (figure 2) is confirmed by captures by E isomer (73.7%) in 2009 (χ^2 test - $P < 0.01$).

Table 1 summarizes data (temperature and relative humidity) measured during the nights when trapped adults

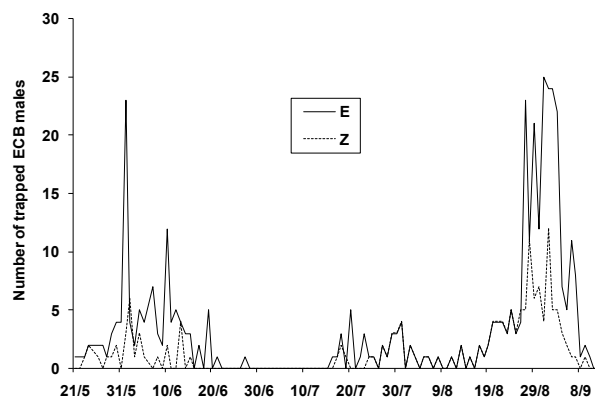


Figure 1. Trend of ECB males captured by water pan traps (E and Z isomers, season 2008).

Table 1. Temperature and relative humidity data (average \pm SD) recorded during the research.

ECB adult flight	Parameter	21.00 h	24.00 h	03.00 h	06.00 h
First	°C	21.7 (\pm 1.4)	18.9 (\pm 1)	17 (\pm 1.3)	17.4 (\pm 1.1)
	RH%	67.3 (\pm 10.8)	74.7 (\pm 10.9)	74.4 (\pm 9.9)	76.6 (\pm 7.6)
Second	°C	25.3 (\pm 1.7)	23.9 (\pm 2.4)	22 (\pm 2)	22.5 (\pm 2.2)
	RH%	64.9 (\pm 5.6)	67.4 (\pm 4.8)	67.1 (\pm 5.9)	66.8 (\pm 5.8)

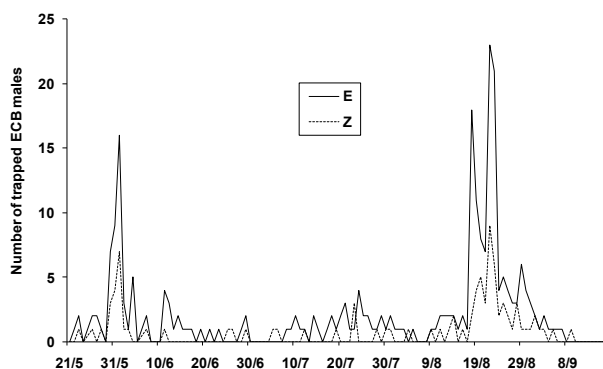


Figure 2. Trend of ECB males captured by water pan traps (E and Z isomers, season 2009).

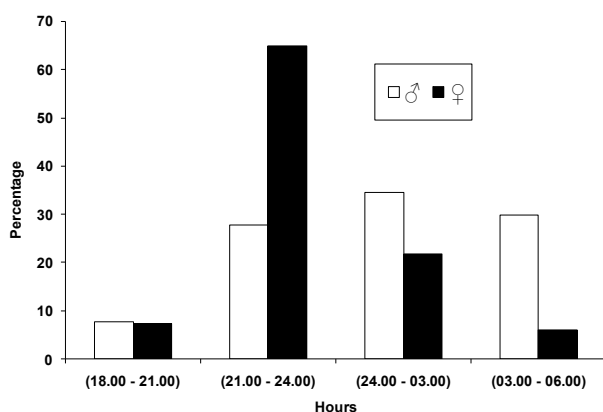


Figure 3. Frequency distribution of second flight ECB males (N = 194) and females (N = 165) caught by traps baited with PAA.

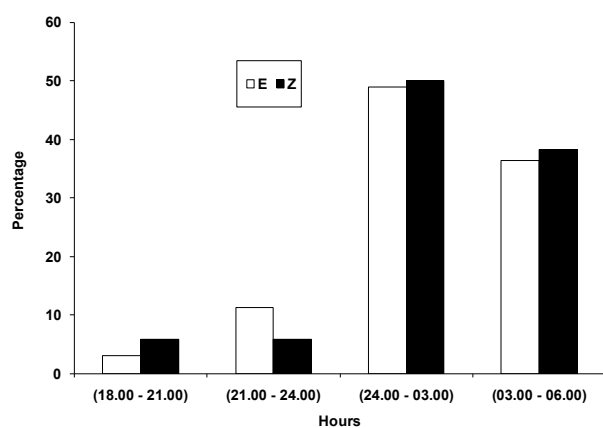


Figure 4. Frequency distribution of second flight ECB males caught by traps baited with Z isomer (N = 86) and E isomer pheromone (N = 159).

Table 2. Captures in different time ranges: statistical tests. (*Time range 18.00-21.00 h not considered).

Sample	χ^2 test	Kruskal Wallis
♀ PAA (second flight)	P < 0.01	P < 0.001
♂ PAA (second flight)	P < 0.01	P < 0.01
♂ PAA* (second flight)	P > 0.05	P > 0.05
♂ Z (second flight)	P < 0.01	P < 0.001
♂ E (second flight)	P < 0.01	P < 0.001
♂ E (first flight)	P < 0.01	P < 0.001

were monitored. Since no data were taken on stormy or rainy nights, both sets of values showed a quite regular trend.

ECB females were mainly captured in response to PAA in the early night, within the time range 21.00-24.00 h (figure 3). Captures were significantly different overnight, as proved by both statistical tests (table 2). Most specimens were mated females (90.9%).

Frequency distribution of males trapped in response to PAA is shown in figure 3. The difference is significant due to the small number of moths trapped before 21.00 h (table 2). Anyway, if the tests are referred to data collected from 21.00 to 06.00 h, no significant difference appears between captures in compared time ranges (table 2).

Male flight trend in response to Z pheromone isomer is displayed in figure 4. The occurrence of a significant difference between compared time ranges is supported by statistical tests (table 2).

Figure 4 also reports the trend of second flight males captures in response to E pheromone isomer. Before 21.00 h the number of specimens in the traps was negligible. Due to the sharp increase in captures recorded between 24.00 and 06.00 h, the difference between time ranges is significant (table 2).

Captures by traps baited with E and Z isomer are distributed overnight according to a similar pattern, as stated by the results of the contingency table test (P > 0.05).

With regard to first flight adults, both PAA and Z traps caught a small number of moths. Consequently, only data coming from traps baited with E isomer are reported here (figure 5). Difference in distribution of capture records is significant, such as the one of second flight males. Most of the males were trapped from 24.00 to 03.00 h (figure 5).

The pattern of captures overnight of second flight males does not significantly differ from that of first flight ones (contingency table - P > 0.05).

A common trait of the graphs showing capture trends overnight (figures 3, 4, 5) is the negligible proportion of adult moths caught before 21.00 h. Only at the end of August it was possible to trap some specimens within

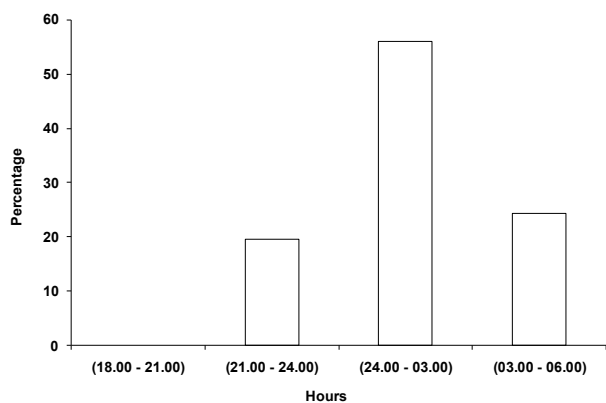


Figure 5. Frequency distribution of first flight ECB males caught by traps baited with E isomer pheromone (N = 82).

the time range 18.00-21.00 h, since, by then, darkness would set in before 21.00 h. Around the end of August 2009, for example, illuminance was < 0.1 lux at that time. From the last week of May (when ECB moths start flying) to middle August, at 21.00 h the light level was still so high as to inhibit ECB flight.

In all trapping seasons the unbaited water pan traps (control) never caught ECB moths.

Discussion and conclusions

Since 1949 was described the sensitivity of ECB to temperature and humidity (Vance, 1949), confirmed later by Sparks (1963). The production of sexual pheromone by females reared in lab conditions occurs during the scotophase, indicating that ECB behaves as a strictly nocturnal moth (Borek and Kalinova, 1991). The calling behaviour of virgin females is maximum when the females, provided with drinking water, are maintained for 24-96 h at 27 °C and then at 20 °C. The temperature shift and the simulation of a dew stimulate the release of sex pheromone (Klun and Maini, 1979).

In open field conditions trapping of ECB overnight confirmed this indication. Furthermore, it was demonstrated that neither males nor females exhibited any crepuscular habit in response to semiochemicals.

The trend of captures confirmed the coexistence of Z and E males already observed in the past in Po river valley (Maini *et al.*, 1979). According to Pelozuelo *et al.* (2007) assortative mating and assortative meeting are leading to the differentiation of the two strains which may acquire characters of species rank (semi species) or sibling species (Cardé *et al.*, 1978).

Hybridization can occur, but a large proportion of the males are inhibited from mating when exposed to the incorrect sex pheromone isomer ratio (Liebherr and Roelofs, 1975; Pelozuelo *et al.*, 2007). Furthermore it was observed that hybridized males from Z/E reciprocal crosses tend to fail the precopulatory behavioural sequence, regardless of the pheromone blend they are exposed to (Glover *et al.*, 1991).

Recent advances in research on ECB suggest that se-

lective coupling between males and females of the same strain is mediated by physiological reception mechanisms (Karpati *et al.*, 2010; Lassance *et al.*, 2011) based on a genetic determination (Dopman *et al.*, 2004; Olsson *et al.*, 2010; Yasukochi *et al.*, 2011).

Where races coexist, but do not share the same host plant, such as in northern France (Thomas *et al.*, 2003; Pelozuelo *et al.*, 2004) an additional mechanism which can support precopulatory isolation is the lack of a synchronization between life cycle of the strains. When E and Z strains can share the same host plants and behave the same way in terms of life cycle, it was suggested that the accumulation of genetic and phenotypic differences between Z and E strains could result in a preferential mating (Coates *et al.*, 2013).

One more potential factor supporting prezygotic isolation could be the different rhythm of response of males to pheromone released by females during the night. However, results from this research show that males tend to respond to both E and Z sex pheromone blends mainly between 24.00 and 06.00 h, according to a similar pattern. The attraction of males of both strains to baited traps could reasonably be the result of a synchronized response to female “calling behaviour”. By comparing pheromone titer and mating frequencies of E and Z strains in lab conditions, Karpati *et al.* (2007) found very similar trends of both strains in the diel fluctuation of calling behaviour and pheromone titer within the scotophase, as well as in timing and mating frequency. The titer of the respective main pheromone component gradually increased during the scotophase, in agreement with the trends of male captures in response to pheromone attraction in field conditions.

Lab research suggests that mating periodicity exhibits a diel rhythm that can be affected and modulated by photoperiod, temperature cycles (Loughner, 1972), relative humidity and dew (Loughner and Brindley, 1971; Royer and McNeil, 1991; 1993; Bailey *et al.*, 2007). A temperature decrease in the scotophase is important for the onset of ECB sexual activities (Loughner and Brindley, 1971), and so is the availability of free water as dew (DeRozari *et al.*, 1977). As relative humidity tends to rise, the onset of calling occurs earlier and the proportion of calling females is greater. At relative humidities of 50 and 75% calling behaviour frequency from E females is low in the first three hours after the beginning of the scotophase, but then tends to rise (Webster and Cardé, 1982). Such a trend could be consistent with the increase (figures 3, 4, 5) of male captures between 24.00 and 06.00 h, when relative humidity is higher, temperature tends to decrease and dew deposition is more likely to occur (table 1). The peak of ECB sexual activity between 24.00 and 01.00 h was reported by Showers *et al.*, 1976. The irrigation was another important factor influencing the ECB adults behaviour and dispersion (Hunt *et al.*, 2001).

Unlike the response to sex pheromone, the attraction of males to PAA traps did not significantly change across the scotophase. PAA given off by maize and wild flowers can interact with both sexes (mated and unmated) and males can respond even more strongly than females, indicating that for this species the attraction is not solely for oviposition (Cantelo and Jacobson, 1979; Valterová

et al., 1990; Maini and Burgio, 1991). Anyway, our research shows that the pattern of response is not the same for both sexes and given the lack of a specific response to PAA in terms of time, the biological meaning of PAA for ECB males cannot easily be analyzed.

Unlike males, ECB females preferably responded to PAA within a specific time range (24.00 to 03.00 h). Almost all trapped females had mated. Females can mate twice or more and the more females mate, the higher the number of egg complements is (Fadamiro and Baker, 1999). Also, volatile organic compounds released by maize plant can affect oviposition by gravid ECB female moths (Binder *et al.*, 1995; Leppik and Frérot, 2012; 2014). Furthermore the existence of repellent odours can affect the gravid female movements and possible dispersion flights (Schurr and Holdaway, 1970). Because the role of PAA as an attractant produced by maize has been proved (Cantelo and Jacobson, 1979), the recorded number of mated females may support the idea of the 24.00-03.00 h time range as the one when the insects mostly tend to move towards maize plants for laying egg masses. However, this runs counter to the fact that corn silk, that was identified as the main source of PAA, usually wither before the maximum occurrence of second-flight adults. Data obtained from the combined use of PAA and sex pheromones discussed here can form the basis of further research on moth behaviour during the scotophase and movements in field conditions. The setup of multibaited traps, as a monitoring tool in different crops attacked by ECB, could be made more effective by a knowledge of the diel adult response and of other environmental factors. Furthermore the application of mating disruption, using an appropriate blend of both attractant and suppressor pheromone components (Klun *et al.*, 1979), could be improved by tailoring it to the flight habits and dispersion of the ECB adults.

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