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Bactrocera oleae reproductive biology: new evidence on wintering wild populations in olive groves of Tuscany (Italy)

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Abstract

Bactrocera oleae (Rossi) (Diptera Tephritidae) is the most alarming pest of the olive tree. Nevertheless, the reproductive cycle of overwintering populations of this pest in the spring is still unclear. A better understanding of the reproductive cycle over the year can lead to testing new strategies in preventive adult treatment scheduling, thus reducing the populations in the following summer when fruits are set. Adult flights of overwintering populations of *B. oleae* have been described for two areas of Tuscany. A degree day model for the overwintering population of *B. oleae* was applied to adult emergence in spring. We monitored the reproductive cycle of this pest during two years. Insect morphology and oviposition activity of *B. oleae* females caught by traps in the two sampling areas in the spring were studied by dissecting and observing the whole mount reproductive apparatus under a stereomicroscope. The observation of ovaries containing follicular relics and the presence of sperm cells in most ovipositing females validated the occurrence of egg fertilization and, therefore, the presence of a complete generation in the spring. The tested degree-day phenological model for adult emergence from autumn oviposition was proved useful in studying the population dynamics of *B. oleae*. The 50% of cumulative captures (male plus female) was reached in the first half of April, with few days of difference between years and sites. Olive fruits collected in the spring, in coincidence with flight peak, showed between 10 and 20% of active infestation. Understanding the complex interactions between reproductive biology of *B. oleae* and the surrounding environment might lead to potential new approaches in integrated pest management programs.

Key words: *Bactrocera oleae* (Rossi), reproductive biology and morphology, degree day model, Integrated Pest Management.

Introduction

The olive fruit fly *Bactrocera oleae* (Rossi) (Diptera Tephritidae) is the most important pest of olive (*Olea europaea* L.) (Gutierrez *et al*., 2009; Danae and Johnson, 2010). *B. oleae* is an oligophagous and multivoltine species (Yokoyama, 2012), whose larvae feed on olive fruits and wild relatives (Dominici *et al*., 1986). The females lay their eggs just under the surface of the olive fruits and the first instar larva encloses in 2-3 days. The larval stage lasts from 10 to 15 days at 25 °C. In summer till mid-fall, eggs complete their development until adult stage in the olive fruit; while, later in fall, larvae leave the fruit and pupate in the soil (Tsitsipis, 1984; Tsitsipis and Loher, 1987).

Damages caused by *B. oleae* can reach very high percentages if left untreated. Understanding its life cycle and infestation trend is important for effective integrated management (Petacchi *et al*., 2015), and may help develop or improve innovative management techniques and strategies. Effective and sustainable management of *B. oleae* is a challenge, particularly in areas where high oil quality and environmental standards are simultaneously pursued, and requires a better understanding of the reproductive cycle over the year for accurate treatment scheduling. Indeed, targeting behaviours that relate to reproduction are among the most promising issues for reducing population of pest insects.

Olive fruits become susceptible to *B. oleae* oviposition at pit hardening (diameter of 7-8 mm), which, in Italy, generally takes place around mid-July (Fletcher *et al*., 1978; Girolami, 1979; Ricci and Ambrosi, 1981; Girolami *et al*., 1983). For this reason, studies on bioecology and pest control have been traditionally focused from this time of year, when the pest interacts with its known host, until harvest period in November. Little attention has been paid on *B. oleae* wild populations in winter until spring season, after fruit harvest and before the beginning of a new production cycle. Several authors have asserted that adult flies overwinter in a facultative reproductive-dormancy (Tzanakakis and Koveos, 1986; Koveos and Tzanakakis, 1990) that begins to break when fruits of increasing age become available (Fletcher *et al*., 1978; Fletcher and Kapatos, 1983). Nevertheless, flights of male and female adults have been observed in Italy during spring, March to May (Delrio and Cavalloro, 1977; Neuenschwander *et al*., 1986; Petacchi *et al*., 2015). These authors observed that, in this period, *B. oleae* adults emerged from the pupae in the soil, flying into olive tree groves. It is expected that, beginning from late winter-early spring to June-July, when fruits from the previous season are on olive trees and climatic conditions are favourable for pest development, *B. oleae* completes at least one generation.

Raspi *et al*. (1997; 2002) observed that in April about 60% of caught females had mature eggs, but, after mating, they entered in a reproductive diapause till the olive fruits became suitable for oviposition in summer. Ragaglini *et al*. (2004), monitoring adult flights in spring and summer at the area-wide scale, in Tuscany (Italy), noticed that there was a different "spatial organization" between spring and summer populations, suggesting the occurrence of a complete generation and a redistribution in olive tree groves, due to micro-climatic conditions and fruit availability. In the northeast of Portugal, Gonçalves and Torres (2011) detected a first flight at the end of winter, though the suppression of ovarian maturation occurred in late spring-early summer. In northern California, Burrack and Zalom (2008) found that the peak of *B. oleae* captures in food bait traps occurred in spring (May-June), and again in early fall (September).

The period of late winter-early spring is a "bottleneck" for *B. oleae*. We hypothesize that a strong selective pressure exists that would favour those individuals best synchronized with their limited host plant choices. *B. oleae* is forced to implement conservation strategies and its survival is at risk. The olives that are left on trees after the harvest or in abandoned olive tree groves, and even those that may have fallen and remain on the ground, probably serve as oviposition sites for the overwintering adults of *B. oleae*. An alternative hypothesis is that adults move, on a small scale, from olive tree groves, to feed on other host plants (Delrio and Cavalloro, 1977), though the only known host is the genus *Olea* with preference for *O. europaea* (Tremblay, 1994). Indeed, the theory of a secondary host has never been proved. In order to test whether *B. oleae* wild overwintering adults complete a generation season, are sexually mature, and mate in spring, and females oviposit, two scenarios should be compared: 1) new perspectives for managing *B. oleae* in prevention strategies and 2) novel standpoints in the bio-ecology of *B. oleae* in springtime, to understand survival and reproductive strategies (i.e., substitute hosts for oviposition).

In Italy, only female with eggs has been observed and described (Delrio and Cavalloro, 1977; Raspi, 2002). However, no studies have reported physiological status and ovarian development of wild overwintering populations. This study aims to demonstrate the hypothesis of a complete generation in spring season through: (i) describing wild adult flights in overwintering populations of *B. oleae* in Tuscany, testing the degree day phenological model for adult flights (Petacchi *et al.*, 2015); (ii) clarifying the reproductive biology of *B. oleae* female overwintering populations; (iii) reporting new experimental evidence on morphology and oviposition activity of wild *B. oleae* populations.

Materials and methods

Study area description and field experimental design

Field experiments were conducted in two sites representative of Tuscany: Monti Pisani (43°45'08.3"N 10°28'24.9"E, elevation ranging from 100 to 200 m asl, municipality of San Giuliano Terme, Pisa); Castagneto (43°11'26.4"N 10°37'25.6"E, elevation ranging from 0 to 50 m asl, municipality of Castagneto Carducci, Livorno).

In order to track *B. oleae* spring biology, we selected 5 and 3 olive groves respectively for Monti Pisani and Castagneto. At each site, the trees were 80-100 years old, 4-6 m tall and 4-5 m apart; the density was approximately 300-400 trees per ha. The cultivars were typical of the area, with a predominance of Frantoio and Leccino. The groves were rainfed. There was one agrometeorological station of the regional monitoring network in one of the groves at each site. In the olive groves, organic olive fruit fly management was implemented.

B. oleae adults were monitored during 2008 and 2009 with two types of trap: a) Dacotrap® (Isagro) with spiroketal pheromone lure (1,7-dioxaspiro-[5,5]-undecane) attractive for males, and b) Tephri Trap ecological® (Sorygar) baited with Tephry-lure® food lures attractive to both sexes.

Six traps per each site (3 Dacotrap and 3 Tephri Trap) were installed in both years at the end of February and removed at the end of July. The traps were checked weekly. During the second year of experiment (2009), time elapsed between the second and the third sampling has been four weeks, due to high-intensity biannual pruning of olive trees in this period. The flies were counted, removed, sexed, and lures were changed. In Dacotrap, the pheromone caps were replaced every 20 days.

The effectiveness of both traps in adult population density evaluation were tested in 2008, in the experimental sites.

Validation of CDD model for spring flight

In both years, CDD (Cumulative Degree-Day) model for the prediction of spring flight, described by Petacchi *et al.* (2015), was tested. The correspondence between the date of oviposition in the fall (overwintering generation) and the 50% of cumulative adult catches in early spring was checked, comparing the accumulation of obtained heat units. As start date for CDD, we selected 15 October according to Petacchi *et al.* (2015). The CDD required for the development from egg to adult is 379.01 °C ± 41.264 °C with a base temperature of 8.99 °C (Crovetti *et al.*, 1982).

To measure the predictive accuracy of the CDD model, we compared the deviation in emergence in actual days, between the current data set (i.e., observed) and those predicted through modelling, and on the basis of the lowest MAE (Mean Absolute Error).

Reproductive biology

B. oleae adult females collected by Tephri Traps during 2009 spring were analyzed (N = 61 Castagneto and N = 125 Monti Pisani) to study reproductive biology of the overwintering generation.

Samples collected in the field were stored in 70% ethanol or 0.1 M Phosphate buffer (PB) at 4 °C up to one week, or at −80 °C until subsequent analysis.

Samples were dissected under a stereomicroscope (25-40X) in PBS at room temperature to make observations on whole mount reproductive apparatus.

For each female examined, the number of ovary mature oocytes was recorded. When preservation status of the samples allowed it, the mating status of females was assessed by the presence of spermatozoa in the storage organs, spermathecae and fertilization chamber (Solinas and Nuzzaci, 1984; Marchini *et al.*, 2001). Spermathecae and fertilization chambers (finely dissected from the anterior vagina), or whole anterior vaginas were squashed and mounted on slides in PB plus 50% glycerol and observed by Nomarski optics.

Observations were also performed on reproductive apparatus samples fixed in 2.5% glutaraldehyde in PB overnight at 4 °C and accurate rinsing in PB. Images were kept by an Axiocam digital camera on a Olympus stereomicroscope or a Leica DMRB microscope equipped with Nomarski optics.

Females were ranked using a modified scale based on ovary observation as in Fletcher *et al*. (1978). Additional information on the morphological appearance of the accessory glands, including secretion filling, might permit a more accurate determination of the female sexual maturity. As in other Tephritids, the female trophic status parallels the ovary maturation (Manetti *et al*., 1997) and undergoes an aging cycle (Dallai *et al*., 1988):

- immature: undifferentiated or developing ovarioles, with no mature (choriogenic stage) egg. Accessory glands - non-trophic or not completely expanded or filled with secretion;
- mature: ovaries containing at least one mature egg. Accessory glands - trophic, expanded, completely or partially filled with secretion. Larger size than in immature stage;
- post-ovipositing: ovaries containing follicular relics or other changes, indicating they had contained mature eggs. Accessory glands - similar size as that of mature females, but less expanded and partially filled with secretion.

Infestation data

Just further proving that *B. oleae* completes one generation in spring season, in addition to data on flight and fertility, we carried out observations in olive groves on non-harvested and residual olives in spring. During the experimental years, we sampled non-harvested olive fruits that were dissected under a stereomicroscope; therefore, oviposition scars (stings), living larvae, and pupae or larval/adult exit holes were counted.

Results

Environmental conditions

Average monthly minimum and maximum temperatures from October to July in the two experimental sites are shown in figure 1. In general, 2009 was warmer than 2008, especially in daily minimum values and during fall, at both sites, with positive deviations up to 5-6 °C. In Castagneto, differences in minimum and maximum daily temperatures between years were rather marked in the first 7 months of the year (with the exception of January, which was relatively cold in 2009). Monti Pisani, had a relatively warm winter (January and February) in 2008, and cooler April in 2009.

Monitoring campaign

In 2008, flights of overwintering adults started in late February, at both sites (figure 2). In 2009, adult catches began in the second decade of March, at both sites. The 50% of cumulative captures (male plus female) was reached at a different date between years: in 2008, on 1 April (DOY, day of the year, 92) in Monti Pisani, and on 2 April (DOY 93) in Castagneto; in 2009, in mid April (DOY 102) in Monti Pisani, and in early April (DOY 92) in Castagneto. In Castagneto, an early starting of adult flight was observed in 2009.

CDD model with start date set at 15 October predicted the 50% of cumulative spring emergence, at both sites. The difference in days between predicted and observed values was 12 in Monti Pisani and 22 in Castagneto, in 2008; while the difference was only 2 days in Monti Pisani and −14 days in Castagneto, in 2009. Positive numbers indicate that the emergence date predicted by the model occurred earlier than observed; conversely, negative numbers indicate that it occurred later than observed. The MAE varied from 2 (at Monti Pisani in 2009) to 22 (at Castagneto in 2008).

Infestation data

Olive fruits collected in the spring, in coincidence with flight peak, showed 20.3% (at Castagneto in 2009) and 10.5% (at Monti Pisani in 2009) of active infestation (eggs, living 1st and 2nd instar larvae; Belcari *et al*., 2005), and demonstrated the capability of *B. oleae* females to originate new generation.

Reproductive biology

The analysis of reproductive biology of *B. oleae* females collected from March to May 2009 in olive groves of Castagneto and Monti Pisani are shown in figure 3.

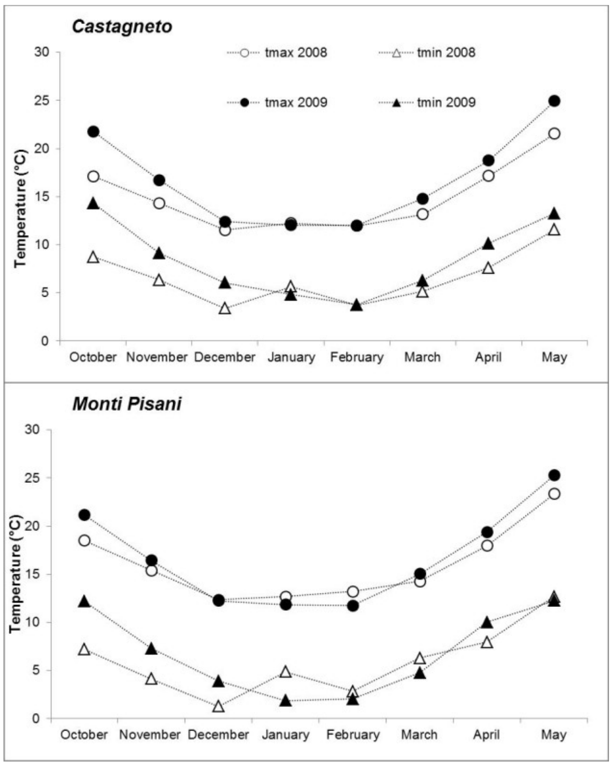

Figure 1. Maximum and minimum average monthly temperatures in the two experimental sites from October to April during 2007-2008 and 2008-2009. Parameters and years are referred to by the legend in the figure.

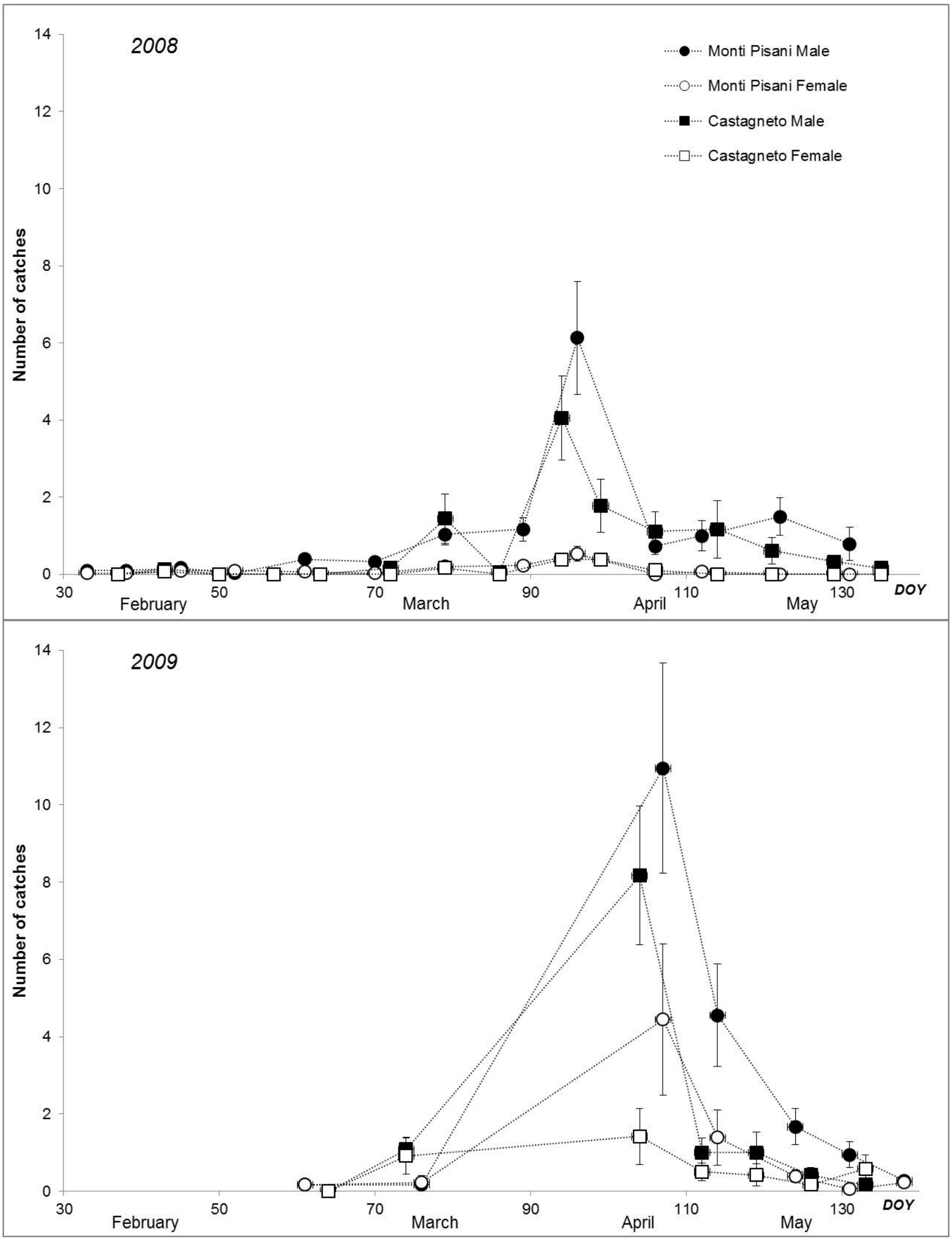

Figure 2. Number of adults (males and females) captured in pheromone traps from February to May in both experimental sites in 2008 and 2009. For each day of the year (DOY), data are the mean of observations ± standard error (n = 3).

At Monti Pisani, the percentage of immature females was maximum (100% of the total observed), in the first sampling (DOY 61, 2 March 2009); in the following sampling (DOY 76, 17 March 2009), the percentage of immature females decreased to 75%, with 25% of mature females having eggs. In the sampling of DOY 107 (17 April), the percentage of immature females decreased to 11%, while mated females were 56% of the total, and post-ovipositing females 33%. The two samplings of May showed only post-ovipositing females.

At Castagneto, in the first sampling (DOY 64, 5 March), no flies were sampled. In the second sampling (DOY 74, 15 March), a predominance of immature females (82%) was found, with 18% of mature females. In the subsequent sampling (DOY 104, 14 April), 56% of the captured females were mature and 44% were in post-ovipositing stage. After a week, the captured flies were all mature. At the end of April, 80% of immature

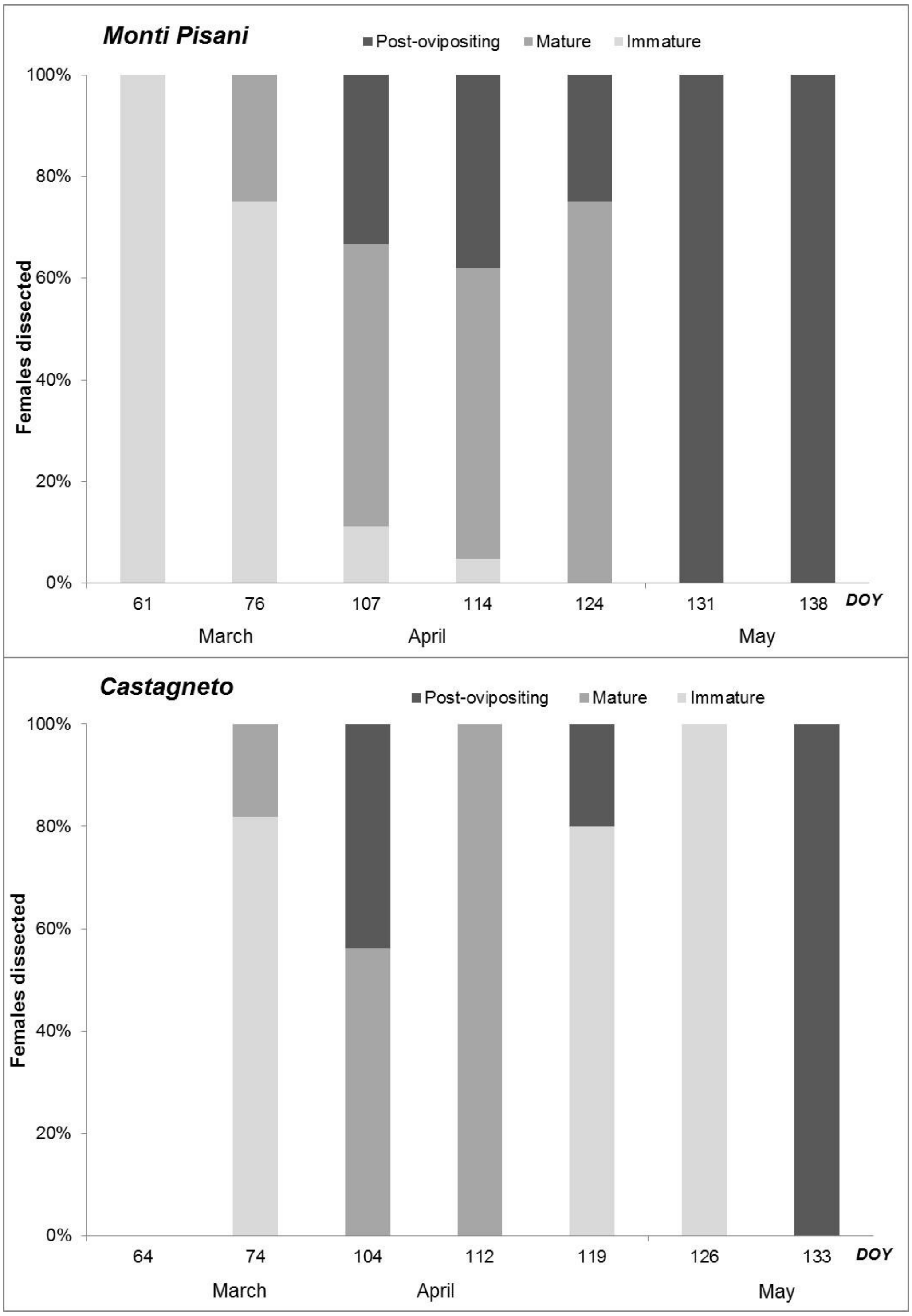

Figure 3. Mating status of *B. oleae* females collected in Castagneto and Monti Pisani in March-May 2009. Immature (undifferentiated or developing ovarioles with no mature egg), mature (ovaries containing at least one mature egg) and post-ovipositing (ovaries containing follicular relics or other changes indicating they had contained mature eggs).

females of the new generation were captured, while 20% were post-ovipositing females. The second spring generation was completed (100% post-ovipositing females) by the end of May.

The presence of sperm cells in the spermathecae and fertilization chamber was observed in an increasing number of females from March (46%, n = 13) to half May (75%, n = 8); whereas, on 1 June, the examined females were all virgin (n = 5). Although mating was also observed in immature females, the higher percentage of mated females appeared positively related to the ovary maturation. On the other hand, the females with no evidence of sperm in the storage organs, captured in June, displayed ovaries at a lower degree of maturation, in comparison with those sampled in March (figure 4 and 5).

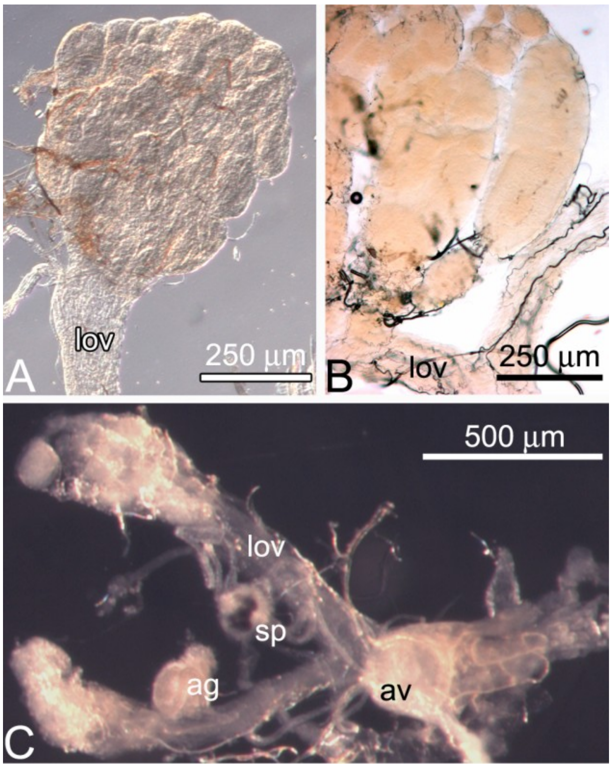

Figure 4. A-B-C. Nomarski optics (A, B) or stereomicroscope (C) images of ovaries from immature *B. oleae* females (captured in March), at early (A, C) or advanced stage of oocyte maturation (B). Legend: lov, lateral oviduct; sp, spermatheca; ag, accessory gland; av, anterior vagina.

Discussion and conclusions

This study added new evidence on reproductive biology of overwintering wild populations of *B. oleae*, showing that females of this pest complete one generation in early spring. We also demonstrated the possibility of using CDD to predict *B. oleae* flights in spring season. This outcome endorses previous experimental observations in Tuscany (Ragaglini *et al*., 2005). Observations of adult populations in the spring and long-term monitoring activities conducted in the same region provide support to the CDD modelling approach developed by Petacchi *et al*. (2015), which predicts the complete development of one generation of *B. oleae*, resulting from oviposition of previous fall.

Accurate estimates of emergence are required for studying the population dynamics of insects (Haavik *et al*., 2013). Higley *et al*. (1986) found an acceptable error of 10-15% in predicting insect emergence when degree-day models were used to provide pest management recommendations. In Petacchi *et al*. (2015), the errors in the CDD model, used for predicting 50% of cumulative emergence, were below these thresholds.

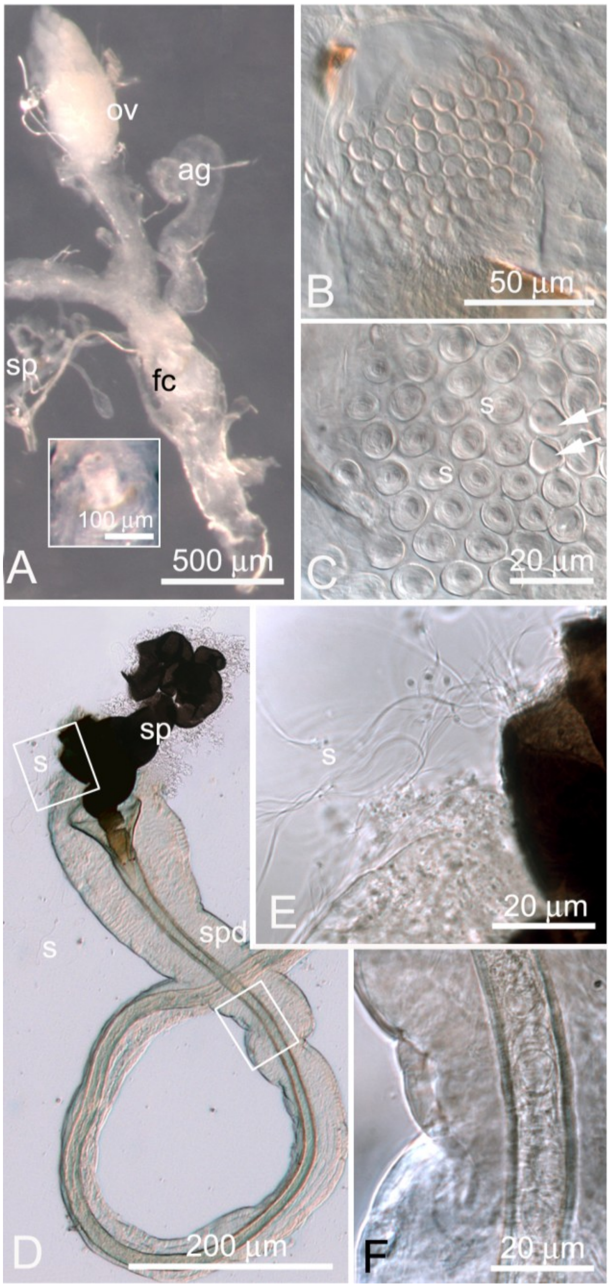

Figure 5. A. Steromicroscope micrograph showing the reproductive apparatus (ventral view) of a *B. oleae* immature female. Note that ovary (ov) contains some developing oocytes but any mature egg. The accessory glands (ag) are not completely filled with secretion and only partially expanded. The fertilization chamber (fc) is closed up in the inset. **B-C.** Nomarski optics of the fertilization chamber of virgin and mated females, respectively. Mating status is ascertained by the presence of sperm cells (s) rolled in the alveoli (C). Arrows indicate two alveoli devoid of sperm cells. **D-E-F.** Spermatheca of a mated female. Sperm cells (s) are coming out the receptacle broken by dissection pins (E) and are also visible moving into the spermathecal duct (spd), (F).

Clarifying the reproductive biology of females of *B. oleae* overwintering populations is important for implementing morphological observations and oviposition activities of wild populations of this pest in treatment plans based on CDD-based phenology models, thus improving their prediction potential. Indeed, the observation of ovaries containing follicular relics or other changes indicated the preceding occurrence of mature eggs, in turn suggesting that females originated new generation. The presence of sperm cells in most ovipositing females might also validate the occurrence of egg fertilization and, therefore, the presence of a complete generation in the spring. This information and the infestation of not harvested fruits in the spring clarified the strategy adopted by *B. oleae* to overcome the winter-spring "bottleneck", before new fruit production in summer.

We confirmed previous observations by Raspi *et al.* (2002 and 2005), in which *B. oleae* females with eggs were claimed to occur in March and April, when about 60% of caught females showed mature eggs. However, the same authors speculated about the role of the constant and variable photoperiod on ovarian maturation, hypothesizing reproductive diapauses in spring. They also affirmed that females emerged in spring season survive until the following summer. By contrast, our observations are in accordance with the hypothesis of a complete *B. oleae* spring generation, originally mentioned by Delrio and Cavalloro (1977). However, the latter authors did not report robust information in support of this hypothesis, except for a spring flight in 1975, in one experimental site in the coastal area of Liguria.

For an effective management of *B. oleae*, particularly in prevention strategy, a deeper knowledge of seasonal reproductive biology and nutritional ecology is fundamental. Results of the present study open new perspective for applying preventive adulticide treatments in the spring. Preventive treatments should be carried out in area-wide olive groves (at least 5 ha contiguous) by means of attract and kill techniques with canopy traps (i.e., EcoTrap) or bait traps (i.e., SpintorFly). Spring treatments are not aimed at protecting the olive fruits, but rather to reduce the *B. oleae* populations and also the reproductive activity of adults. Consequently, a low number of individuals in the following summer generation would be expected. Again, the presence of not harvested fruit samples with source of infestation in the spring provides new element to model, at landscape scale, the risk of summer infestation of this pest, taking into account climatic data (Marchi *et al*., 2016). Further studies warrant to verify and monitor the occurrence of a complete *B. oleae* generation in the spring, and to clarify biological aspects of spring populations and impacts on olive tree productivity, as well as test the effectiveness of preventive treatments, specifically organic control agents.

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