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Olive cultivar and maturation process on the oviposition preference of *Bactrocera oleae* (Rossi) (Diptera: Tephritidae)

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Abstract

The olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), is a key-pest in the main olives producing areas worldwide, and displays distinct preference to different olive cultivars. The present work intended to study oviposition preference towards three Portuguese cultivars (Cobrançosa, Madural, and Verdeal Transmontana) at different maturation indexes. Multiple oviposition bioassays (multiple-choice and no-choice) were conducted to assess cultivar preference. No-choice bioassays were conducted to assess the influence of different maturation indexes (MI 2; MI 3, and MI 4) in single cultivars. The longevity of olive fly adults according to the cultivar in which its larvae developed was also evaluated through survival assays.

Cultivar and maturation are crucial aspects in olive fly preference. Field and laboratory assays revealed a preference towards cv. Verdeal Transmontana olives and a lower susceptibility to cv. Cobrançosa olives. A higher preference was observed for olives at MI 2 and MI 3. The slower maturation process in cv. Verdeal Transmontana (still green while the other cultivars are reddish or at black stage) seems to have an attractive effect on olive fly females, thus increasing its infestation levels. Olive fly adults from both sexes live longer if emerged from pupae developed from cv. Verdeal Transmontana fruits and live less if emerged from cv. Cobrançosa. Therefore, olive cultivar and maturation process are crucial aspects in olive fly preference, also influencing the longevity of adults.

Keywords: adult longevity, cultivar preference, olive fly, olive ripening, oviposition

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Introduction

The olive fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) is one of the most important pest of olive crops worldwide and a key-pest in the Mediterranean region. The impact of the olive fly is considerably high, causing production losses due to pulp spoilage, fruit drop, and contamination of olives with microorganisms (Iannota *et al.*, 2012), compromising the commercial classification of olive products, reducing olive oil quality, composition and functional properties (Pereira *et al.*, 2004), and causing an overall unprecedented economic impact (Malheiro *et al.*, 2015*a*). Nevertheless, this impact varies considerably among cultivars. Some cultivars are considerably less susceptible to olive fly attacks, while in others, the production can be totally lost in years of high infestation levels (Navrozidis *et al.*, 2007; Burrack & Zalom, 2008).

The interaction between olive cultivars and olive fly regarding oviposition preference has been studied in order to understand the parameters involved in such phenomena. The studies conducted were mainly based on physical

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(Rizzo *et al.*, 2012), chemical (Kombargi *et al.*, 1998), and molecular factors (Corrado *et al.*, 2012; Imperato *et al.*, 2012). In the case of physical parameters, studies were conducted in order to establish correlations between the olive size and hardness and the olive fly oviposition preference (Gonçalves *et al.*, 2012), as well as its colour (Katsoyannos & Kouloussis, 2001; Malheiro *et al.*, 2015*b*). The olive colour is intrinsically correlated with fruit maturation, during which olives undergo modifications induced by metabolic processes, but in each olive cultivar, the maturation process is characteristic. Some cultivars have a fast ripening process, rapidly changing from green to black and passing through the so-called 'cherry-phase', while others remain green for quite long periods. Therefore, the colour of olives in the different maturation indexes (MI) influences olive flies' choice towards a specific olive cultivar (Malheiro *et al.*, 2015*b*). Some authors verified a higher attraction of olive fly females to red colours (Katsoyannos & Kouloussis, 2001), while others report the preference of the olive fly for green olives (Vlahov, 1992).

In Portuguese cultivars from the Trás-os-Montes region, the olive fly oviposition preference is also observed (Gonçalves *et al.*, 2012), particularly for cvs. Verdeal Transmontana and Madural, while cv. Cobrançosa reported, every year, to have low infestation levels.

Therefore, the main objective of the present work is to study the influence of olive cultivar and MI under laboratory conditions, in Portuguese cultivars with different susceptibility degrees. The effect of the cultivar on olive fly adult survival was also evaluated.

Material and methods

In the present work, all olive samples and insects used were collected from an Integrated Production olive grove located in Paradela (Mirandela – 41°32′35.72″N; 7°07′27.17″W), Trás-os-Montes region (Northeast of Portugal) in the years of 2013 and 2014. The study focused on three of the main cultivars of the Trás-os-Montes region, namely Cobrançosa, Madural, and Verdeal Transmontana.

Infestation level and MI determination

From each olive cultivar, five trees (approximately 60 years-old; 4 m high; rain-fed; no control treatments) were selected and marked to determine the infestation level and MI. Both parameters were assessed fortnightly from 27 August to 6 November 2013 (last possible date to be assessed prior to olives harvest).

In order to assess infestation level, 40 random handpicked fruits were collected from each olive tree (five trees per cultivar; 200 fruits; morphological characterization of the fruits from each cultivar can be found at Malheiro *et al.*, 2015*a*) in the mentioned periods and inspected in a binocular stereomicroscope for signs of infestation (oviposition sites or exit holes). The infestation level was expressed as the percentage of infested olive fruits.

Simultaneously, fruits were collected per tree for calculation of the MI as described by Hermoso *et al.* (2001). Briefly, samples of 100 olive fruits (20 fruits per tree) were separated in eight maturation categories based on epidermis and pulp colour (0–7). Therefore, the fruits were classified as 'MI 0' if the epidermis was green; 'MI 1' for yellowish green; 'MI 2' if the epidermis showed red spots in less than half the fruit; 'MI 3' if the epidermis was red or purple in more than half the fruit; 'MI 4' for black epidermis and white pulp; 'MI 5' if the epidermis was black and less than half the pulp is purple; 'MI 6' if the epidermis is black and more than half pulp purple (without reaching the stone); 'MI 7' if the epidermis was black and the total pulp purple (reaching the stone). The MI was calculated as follows:

$$\mathrm{MI} = \frac{(n \times 0 + n \times 1 + n \times 2 + n \times 3 + n \times 4 + n \times 5 + n \times 6 + n \times 7)}{100}$$

where n is the number of fruits in each MI of classification considered.

Bactrocera oleae *collection and rearing*

Olives with signs of olive fly infestation were collected from the trees, separated and spread in trays, larvae and pupae were collected daily and transferred to rearing boxes. Once hatched from pupae, adults were separated daily (for age control purposes) into new rearing boxes. Larvae, pupae, and adults were maintained under the following conditions: 26 ± 1°C, 70 ± 10% of relative humidity, with a photoperiod of 16L:8D. Adults were fed *ad libitum* with a honey solution (10% w/v), artificial diet (sucrose and yeast extract at a ratio of 4:1) and water, and the diet changed every 2 days.

Oviposition bioassays

For all the oviposition assays, 15-day-old olive fly adults (both males and females) were used in order to ensure that all females were gravid. The olives used in all oviposition bioassays were collected from marked trees in each cultivar. Once in the laboratory, all olives were inspected one by one so as to select only perfectly healthy olives. Olives with signs of diseases were discarded and olives with signs of olive fly infestation were used for survival assays (in more detail in the section '*Survival bioassays*').

The conditions in which all oviposition bioassays were conducted and the diet given to the flies are the same as those described in the section '*Bactrocera oleae collection and rearing*'.

After the bioassays, each set of olives was observed under a binocular stereomicroscope in order to count the number of oviposition sites on each olive. Subsequently, the olives were maintained for 1 month under the same conditions previously described so that the number of pupae and/or adults emerged could be collected and registered. The following parameters were considered: number of ovipositions; number of olives recovered without ovipositions; number of olives recovered with ovipositions; number of ovipositions per assayed olive; number of ovipositions per infested assayed olive; number of pupae/adults collected; percentage of recovered pupae/adults to the total amount of ovipositions.

Oviposition bioassays based on olive cultivar

In order to verify the cultivar oviposition preference of the olive fly, two types of bioassays were carried out:

(i) *Multiple-choice oviposition assays*: olive fly males and females (10 pairs) were placed in cages (0.05 m^3) and maintained without the presence of olives for 24 h. After this period, 60 olives (20 per olive cultivar) were exposed to the flies for 24 h for oviposition, and immediately replaced by a new set of 60 olives. This operation

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was repeated during 10 consecutive days in five independent cages ($n = 5$), totalizing 1000 olives assessed per cultivar.

(ii) *No-choice oviposition assays*: The no-choice oviposition bioassays were replicated under same procedure and conditions applied in multiple-choice oviposition bioassays, however, in this case, 60 olives of a single cultivar are exposed to the flies during 24 h for 10 consecutive days. Five independent bioassays per cultivar were developed, totalizing 3000 olives assessed per cultivar.

In both the multiple-choice and no-choice oviposition bioassays, the olives from cvs. Cobrançosa and Madural were at an MI of 3–4, and the olives from cv. Verdeal Transmontana were at an MI of 2–3.

Oviposition bioassays based on MI

In order to verify the impact of MI on the olive fly oviposition preference, an oviposition bioassay similar to the no-choice oviposition bioassay was developed. In this case, three MI were tested per olive cultivar: MI 2, MI 3, and MI 4. The olives used were separated according to the scale of Hermoso *et al*. (2001) (see section 2.1. for further details). The replicates were limited to the number of available healthy fruits in each MI, so five replicates were conducted at MI 2 and MI 3 for cvs. Cobrançosa and Madural (3000 olives per cultivar and MI); four replicates were conducted at MI 2 and MI 3 for cv. Verdeal | Transmontana (2400 olives assessed per MI); three and two replicates were conducted at MI 4 for cvs. Cobrançosa and Madural, respectively (1800 and 1200 olives assessed, respectively). Due to the slower maturation process of cv. Verdeal Transmontana, it was impossible to collect enough amounts of healthy olives prior to the harvest date in order to implement oviposition bioassays at MI 4 for this cultivar.

Survival bioassays

The olives destined for oviposition bioassays, but with signs of olive fly infestation were separated by cultivar. These fruits were spread in trays and larvae and pupae were collected daily and then transferred to three rearing cages, one per cultivar. Rearing cages were inspected daily (every 8 h) for signs of adults. Once emerged, olive fly adults were separated by sex and maintained in groups of a maximum of ten individuals in smaller cages, only with water (no diet supplied), and with diet (as mentioned in the section '*Bactrocera oleae collection and rearing*'). The cages were inspected at least every 8 h to remove and register possible dead individuals. For each olive cultivar and sex (without and with diet), 100 individuals were monitored. The most approximate date and hour of emergence and death were recorded so as to calculate the proximate survival period in days. Temperatures, relative humidity, and photoperiod applied were the same as previously described.

Statistical analysis

Analysis of variance (ANOVA)

An ANOVA with Type III sums of squares was performed using the GLM (General Linear Model procedure) of the SPSS software, version 21.0 (IBM Corporation, New York, USA). The fulfilment of the ANOVA requirements, namely the normal distribution of the residuals and the homogeneity of variance, were evaluated by means of the Kolmogorov–Smirnov with Lilliefors correction (if $n > 50$) or the Shapiro–Wilk's test (if $n < 50$), and Levene's tests, respectively. All the dependent variables were analyzed using a one-way ANOVA with or without Welch correction, depending on whether the requirement of the homogeneity of variances was fulfilled or not. The main factors studied were: longevity of olive fly males and females whose larvae developed in different olive cultivars. If a statistical significant effect was found, the means were compared using Tukey's honestly significant difference multiple comparison test or Dunnett T3 test, also depending on whether equal variances could be assumed or not. All statistical tests were performed at a 5% significance level.

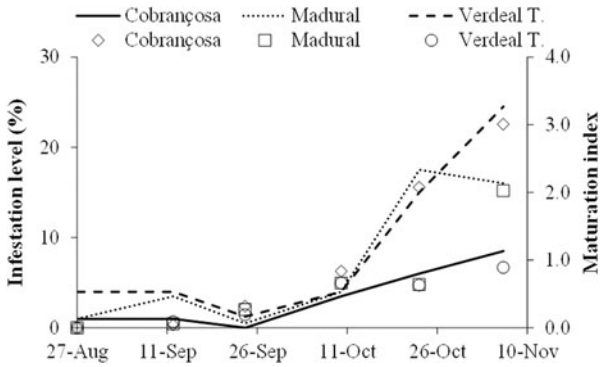

Fig. 1. Infestation levels (%) (lines) and maturation index (markers) of olives from cvs. Cobrançosa, Madural, and Verdeal Transmontana.

Survival curves were represented for each treatment with Kaplan–Meier estimates using the Surv and survfit functions from the 'survival' package (Therneau, 2014) in R software. Four separated Cox Proportional Hazard Models using the coxph function from the same package were fitted for: (i) unfed females, (ii) fed females, (iii) unfed males, (iv) fed males. This was done in order to analyze the effect of the larva development of the olive fly inside different olive cultivars (Cobrançosa, Madural, and Verdeal Transmontana) on the survival of adults (females and males), both unfed and fed on an artificial diet.

Results

Infestation levels and maturation

The infestation levels registered remained low (<5%), in the three olive cultivars until 10 October (fig. 1). After that, the olive fly infestation increased in all cultivars, with a higher increment in cvs. Madural (17.5%) and Verdeal Transmontana (15.0%). In the last sampling date (6 November), infestation levels of 24.5% for cv. Verdeal Transmontana were observed, followed by cv. Madural (16.0%), and at last by cv. Cobrançosa (8.5%). Significant differences were found between cv. Verdeal Transmontana and cv. Cobrançosa ($P = 0.030$) in the last sampling date.

Olives from cv. Verdeal Transmontana reported a very slow maturation process, being completely green at the beginning of the study (MI = 0) and still yellow-green (MI = 0.89)

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Table 1. Parameters evaluated in multiple-choice oviposition bioassays during 10 consecutive days, with olives from cvs. Cobrançosa, Madural, and Verdeal Transmontana (mean values ± standard error; $n = 5$).

	Days assayed										
Parameter	1	2	3	4	5	6	7	8	9	10	Σ
Ovipositions (*n*)											
Cobrançosa	1	1	2	6	19	26	27	21	37	44	184 ± 8 a
Madural	1	2	2	4	17	28	22	24	29	41	170 ± 21 a
Verdeal Transmontana	1	3	2	5	14	24	23	29	52	54	207 ± 24 a
Olives not attacked (*n*)											
Cobrançosa	19	19	18	15	9	5	6	8	4	3	106 ± 3 a
Madural	19	19	18	16	9	5	4	6	6	3	105 ± 5 a
Verdeal Transmontana	19	18	18	16	10	7	5	4	4	2	103 ± 7 a
Olives attacked (*n*)											
Cobrançosa	1	1	2	5	11	15	14	12	16	17	94 ± 3 a
Madural	1	1	2	4	11	15	16	14	14	17	95 ± 5 a
Verdeal Transmontana	1	2	2	4	10	13	15	16	16	18	97 ± 7 a
Ovipositions per olive											
Cobrançosa	0.04	0.04	0.11	0.31	0.95	1.28	1.36	1.04	1.86	2.22	0.92 ± 0.04 a
Madural	0.04	0.08	0.10	0.20	0.85	1.38	1.10	1.18	1.43	2.04	0.85 ± 0.11 a
Verdeal Transmontana	0.06	0.13	0.10	0.24	0.70	1.21	1.17	1.45	2.60	2.68	1.04 ± 0.12 a
Ovipositions per infested olive											
Cobrançosa	0.40	0.40	0.80	1.33	1.70	1.72	1.90	1.71	2.29	2.61	1.96 ± 0.04 a
Madural	0.40	0.40	0.80	0.85	1.59	1.86	1.35	1.65	1.94	2.36	1.79 ± 0.17 a
Verdeal Transmontana	0.60	0.64	0.65	1.08	1.26	1.73	1.56	1.77	3.07	3.00	2.13 ± 0.23 a
Collected pupae/adults											
Cobrançosa	0	0	2	4	10	16	13	14	25	31	115 ± 6 a
Madural	0	2	1	1	12	16	15	13	19	29	108 ± 4 a
Verdeal Transmontana	0	1	1	3	10	17	19	17	33	33	134 ± 8 a
Ratio pupae/stings (%)											
Cobrançosa	0	0	100	66.7	52.6	61.5	48.1	66.7	67.6	70.4	62.5 ± 2.6 a
Madural	0	100	50.0	25.0	70.6	57.1	68.1	54.2	65.5	70.7	63.5 ± 3.5 a
Verdeal Transmontana	0	33.3	50.0	60.0	71.4	70.8	82.6	58.6	63.5	61.1	64.7 ± 5.4 a

at the end of it, coinciding with the olive harvest for olive oil extraction. For cv. Madural, at the end of the study, the olives were already in the cherry-stage, becoming reddish (MI = 2.03). Regarding the olives from cv. Cobrançosa, they reported a faster maturation rate, reporting an MI = 2.08 on 23 October and an MI = 3.01 on the last assessed date, being completely reddish by then. Therefore, the olive fly can simultaneously find olives with green, yellow green and reddish colouration in the field but shows a clear preference towards green or yellow-green olives, as verified by our data (fig. 1).

Effect of cultivar in olive fly oviposition

Multiple-choice oviposition bioassays

In all the three olive cultivars, it was noted that the number of ovipositions progressively increased as the days assessed passed by, increasing from 1 oviposition *n* day 1–44, 41, and 54 ovipositions (day 10) for cvs. Cobrançosa, Madural, and Verdeal Transmontana, respectively (table 1).

The olives from cv. Madural reported the lowest oviposition average, 170 ± 21 (in 200 fruits overall), followed by cv. Cobrançosa with 184 ± 8, while cv. Verdeal Transmontana reported the highest number of ovipositions, with 207 ± 24 (table 1). Meanwhile, no statistical differences were observed in the number of ovipositions in the three cultivars ($P = 0.373$). Regarding the number of attacked and non-attacked olives, the three cultivars showed similar values without statistical differences ($P = 0.836$), with the number of attacked olives varying between 103 ± 7 and 106 ± 3 (table 1).

In general, the olives from cv. Verdeal Transmontana reported a higher number of ovipositions per fruit (1.04 ± 0.12), and when reporting ovipositions only to the attacked olives, the number increased to 2.13 ± 0.23, against 1.96 ± 0.04 and 1.79 ± 0.17 for cvs. Cobrançosa and Madural, respectively ($P = 0.344$). From the 207 ovipositions made in cv. Verdeal Transmontana olives, 134 pupae/adults were collected (table 1). In the cases of cvs. Cobrançosa and Madural, 115 ± 6 and 108 ± 4 pupae/adults were recovered, respectively. No statistical differences were verified in the number of pupae/adults recovered ($P = 0.285$).

No-choice oviposition bioassays

In the no-choice oviposition bioassays, a single olive cultivar was presented to the olive fly females, therefore a real choice is not in question. The results obtained in this type of bioassay were concise and clear: the olives from cv. Verdeal Transmontana are highly preferred to oviposit, followed by cvs. Madural and Cobrançosa olives (table 2).

Ovipositions were significantly higher in cv. Verdeal Transmontana ($P < 0.001$), with 1073 ± 77 ovipositions (in 600 olives overall). Only 133 ± 7 out of 600 olives were not infested, reporting an overall number of ovipositions per olive of 1.79 ± 0.13 (table 2). Regarding cv. Madural, 577 + 12 ovipositions were recorded, nearly half of those reported by cv. Verdeal Transmontana. An average of 0.96 ± 0.02 ovipositions per olive was observed, with a rise to 1.63 ± 0.04 per infested olive. The olives from cv. Cobrançosa were less attacked by the olive fly, with 450 ± 23 ovipositions overall, less than 1

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Table 2. Parameters evaluated in no-choice oviposition bioassays during 10 consecutive days, with olives from cvs. Cobrançosa, Madural, and Verdeal Transmontana (mean values ± ; $n = 5$).

	Days assayed										
Parameter	1	2	3	4	5	6	7	8	9	10	Σ
Ovipositions (*n*)											
Cobrançosa	16	60	26	45	17	29	36	82	55	84	450 ± 23 a
Madural	62	60	90	39	51	79	34	89	18	55	577 ± 12 a
Verdeal T.	42	148	96	111	140	107	135	85	125	84	1073 ± 77 b
Healthy olives (*n*)											
Cobrançosa	47	26	41	28	44	40	32	21	24	26	329 ± 17 a
Madural	11	29	12	33	31	16	35	14	44	22	247 ± 8 b
Verdeal T.	28	4	25	9	10	14	12	15	5	11	133 ± 7 c
Attacked olives (*n*)											
Cobrançosa	13	34	19	32	16	20	28	39	36	34	271 ± 17 a
Madural	49	31	48	27	29	44	25	46	16	38	353 ± 8 b
Verdeal T.	32	56	35	51	50	46	48	45	55	49	467 ± 7 c
Ovipositions per olive											
Cobrançosa	0.27	1.00	0.43	0.75	0.28	0.48	0.60	1.37	0.92	1.40	0.75 ± 0.04 a
Madural	1.03	1.00	1.50	0.65	0.85	1.32	0.57	1.48	0.30	0.92	0.96 ± 0.02 a
Verdeal T.	0.70	2.47	1.60	1.85	2.33	1.78	2.25	1.42	2.08	1.40	1.79 ± 0.13 b
Ovipositions per infested olive											
Cobrançosa	1.23	1.76	1.37	1.41	1.06	1.45	1.29	2.10	1.53	2.47	1.66 ± 0.11 a
Madural	1.27	1.94	1.88	1.44	1.76	1.80	1.36	1.93	1.13	1.45	1.63 ± 0.04 a
Verdeal T.	1.31	2.64	2.74	2.18	2.80	2.33	2.81	1.89	2.27	1.71	2.30 ± 0.15 b
Collected pupae/adults											
Cobrançosa	8	41	12	29	5	5	17	50	33	30	230 ± 14 a
Madural	104	36	78	24	23	17	22	70	11	34	361 ± 17 b
Verdeal T.	35	99	81	80	77	48	71	45	42	79	657 ± 16 c
Ratio pupae/stings											
Cobrançosa	50.0	68.3	46.2	64.4	29.4	17.2	47.2	61.0	60.0	35.7	51.1 ± 5.3 a
Madural	74.2	60.0	86.7	61.5	45.1	21.5	64.7	78.7	61.1	61.8	62.6 ± 2.2 a
Verdeal T.	83.3	66.9	84.4	72.1	55.0	44.9	52.6	52.9	33.6	94.0	61.2 ± 5.3 a

oviposition per olive assayed (0.75 ± 0.04) and an average of 1.66 ± 0.11 ovipositions per infested fruit (table 2).

Concerning the number of pupae/adults recovered, 657 ± 16, 361 ± 17, and 230 ± 14 were collected from cvs. Verdeal Transmontana, Madural, and Cobrançosa, respectively. This means a percentage of recovery from the total number of ovipositions of 61.2 ± 5.3, 62.6 ± 2.2, and 51.1 ± 5.3%. Besides being the least preferred by olive fly females to oviposit, the olives from cv. Cobrançosa reported a lower ratio between pupae/adults recovered and the number of ovipositions. In this case, it means that 39.8, 37.4, and 48.9% of the ovipositions made were unable to lead to adults' formation in cvs. Verdeal Transmontana, Madural, and Cobrançosa, respectively.

Maturation process and cultivar in olive fly oviposition preference

The results obtained showed that besides the olive cultivar, the maturation stage is a preponderant factor influencing the olive fly oviposition. At the sameMI, MI 2, the olives from cv. Verdeal Transmontana were the most infested, reporting an average of 778 ± 195 ovipositions against 614 ± 74 and 594 ± 19 in cvs. Cobrançosa and Madural, respectively (fig. 2). No significant differences were reported among cultivars at MI 2 ($P = 0.457$).

The number of ovipositions decreased slightly from MI 2 to MI 3, mainly in cv. Verdeal Transmontana, while in cv. Madural a significant increase ($P < 0.001$) was observed (fig. 2), to 948 ± 73. In fig. 2 it is possible to observe that both olive cultivars tested at MI 4 reduced the number of ovipositions significantly: cv. Cobrançosa decreased from 621 ± 128 at MI 3 to 229 ± 40 at MI 4 ($P = 0.007$), while cv. Madural decreased from 948 ± 73 to 608 ± 100 ($P = 0.003$). It is clear that the olive fly prefers green or reddish olives rather than black olives to oviposit. Another interesting aspect observed in the maturation oviposition bioassays was the percentage of collected pupae/adults in respect to the mean number of ovipositions. At MI 4, only 7 ± 0.7% of cv. Cobrançosa pupae/adults were recovered, which means that about 93% of ovipositions made were sterile punctures or eggs and larvae that died inside the drupe of cv. Cobrançosa. At MI 2 and 3, the percentage of recovery was, respectively, 52.1 ± 4.4 and 59.7 ± 3.1% (table 3). The same happened in olives from cv. Madural. The percentage of recovery of pupae/adults was 40.7 ± 3.8 and 41.4 ± 1.8% at MI 2 and MI 3, but it decreased to 23.2 ± 0.8% at MI 4. These observations clearly state that besides the lower attraction of olive flies to black olives, the ovipositions carried out are highly unsuccessful to maintain this pest populations.

Survival of olive fly adults

The cultivar influences significantly the longevity of olive fly adults (fig. 3), with no significant differences found between males and females in each cultivar ($P = 0.423$; $P = 0.374$; and $P = 0.868$; respectively, for cvs. Cobrançosa, Madural, and Verdeal Transmontana). However, the adults which emerged from pupae developed in cv. Verdeal Transmontana olives lived longer than those from cvs. Cobrançosa and Madural. On average, a male emerged from cv. Verdeal Transmontana lived for 3.31 ± 0.07 days while

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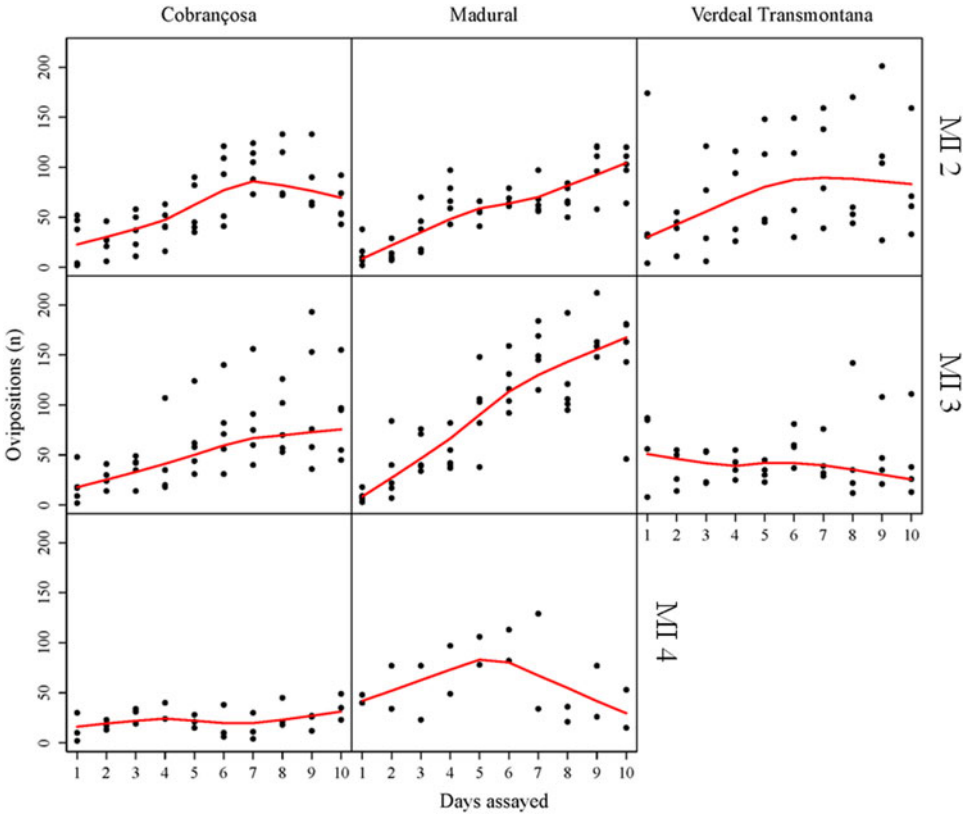

Fig. 2. Number of ovipositions (mean values; number of replicates available in Table 3) made by olive fly females in olives from cvs. Cobrançosa, Madural, and Verdeal Transmontana at different maturation indexes (MI = 2; MI = 3; and MI = 4).

females lived for 3.29 ± 0.06 days. In cv. Madural, males and females lived for 3.20 ± 0.11 and 3.08 ± 0.07 days, while in cv. Cobrançosa they lived for 2.98 ± 0.08 and 2.89 ± 0.08 days, respectively (fig. 3). Survival curves are represented in fig. 4.

The death hazard of unfed males and females was significantly different according to the olive cultivar ($P < 0.001$ for both sexes). Particularly, the death hazard was lower in unfed females developed in Verdeal Transmontana [1.99 times lower than in Cobrançosa ($P < 0.001$), and 1.48 times lower than in Madural ($P = 0.017$)]. The death hazard of unfed adult males born from larvae developed in cvs. Cobrançosa and Madural were significantly higher (1.47 times, $P = 0.026$) and slightly higher (1.34 times, $P = 0.059$), respectively, than the ones developed in cv. Verdeal Transmontana. Furthermore, the death hazard for males which died after day 4.1 was lower when larvae had developed in cv. Madural than when fed in cv. Cobrançosa (4.129 times, $P = 0.014$) and in cv. Verdeal Transmontana (5.058 times, $P < 0.001$).

The diet of tephritids, and specially the *Bactrocera* species considerably influence their survival (Jaleel *et al.*, 2017). The larva development in different olive cultivars did not significantly affect the adult females and males' survival when fed on the artificial diet ($P = 0.399$ and $P = 0.083$ for females and males respectively). In general, males and females from cv. Verdeal Transmontana lived significantly longer than those from cv. Cobrançosa ($P = 0.008$ and $P < 0.001$ respectively), approximately 11.1% longer for males, while females lived 13.8% longer.

Discussion

Infestation levels and maturation

The results obtained regarding the infestation level followed a trend observed in other years, indicating that cvs. Madural and Verdeal Transmontana are more susceptible to the olive fly infestation than cv. Cobrançosa (Gonçalves *et al.*, 2012; Malheiro *et al.*, 2015*b*, *c*). It was interesting to observe that infestation levels start rising at the exact moment when maturation begins (fig. 1). This period also coincides with the decrease of the high temperatures recorded during July and August, which are not favourable to a fast development of olive fly populations and causes high mortality of the laid eggs (Gonçalves *et al.*, 2012). The higher preference of olive flies to oviposit in cv. Verdeal Transmontana olives may be related to the maturation process since these olives remain green for longer periods. Some authors refer that olive fly females prefer to oviposit in greener and reddish olives (Vlahov, 1992; Katsoyannos & Kouloussis, 2001). This could be one explanation for the high levels of infestation in olives from cv. Verdeal Transmontana comparatively to the other two cultivars in different years. This hypothesis is further

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Table 3. Parameters evaluated in one-choice oviposition bioassays during 10 consecutive days at different maturation stages, with olives from cvs. Cobrançosa, Madural, and Verdeal Transmontana (mean values; number of replicates displayed in each row with maturation index).

		Days assayed										
Parameter		1	2	3	4	5	6	7	8	9	10	Σ
Healthy olives (*n*)	MI[1]											
Cobrançosa	2 (*n* = 5)	37	39	33	28	20	13	9	13	13	17	222 ± 33 aA
	3 (*n* = 5)	45	40	35	33	21	18	15	11	10	12	240 ± 34 aA,B
	4 (*n* = 3)	49	47	40	41	43	47	50	41	45	35	438 ± 23 bB
Madural	2 (*n* = 5)	47	49	34	16	20	12	11	9	11	14	223 ± 10 a,bA
	3 (*n* = 5)	52	38	25	24	12	7	4	7	7	10	186 ± 24aA
	4 (*n* = 2)	36	27	35	25	17	18	19	40	28	37	282 ± 29 bA
Verdeal T.	2 (*n* = 4)	33	37	32	29	22	22	22	30	15	24	266 ± 59 aA
	3 (*n* = 4)	25	34	34	32	38	25	30	31	27	34	310 ± 18 aB
Attacked olives (*n*)												
Cobrançosa	2 (*n* = 5)	23	21	27	32	40	47	51	47	47	43	378 ± 33 bA
	3 (*n* = 5)	15	20	25	27	39	42	45	49	50	48	360 ± 34 bA,B
	4 (*n* = 3)	11	13	20	19	17	13	10	19	15	25	162 ± 23 aA
Madural	2 (*n* = 5)	13	11	26	44	40	48	49	51	49	46	377 ± 10 aA
	3 (*n* = 5)	8	22	35	36	48	53	56	53	53	50	414 ± 24 aB
	4 (*n* = 2)	24	33	25	35	43	42	41	20	32	23	318 ± 29 aB
Verdeal T.	2 (*n* = 4)	27	23	28	31	38	38	38	30	45	36	334 ± 59 aA
	3 (*n* = 4)	35	26	26	28	22	35	30	29	33	26	290 ± 18 aA
Ovipositions per olive												
Cobrançosa	2 (*n* = 5)	0.48	0.43	0.60	0.71	0.97	1.38	1.68	1.56	1.38	1.05	1.02 ± 0.12 aA
	3 (*n* = 5)	0.31	0.41	0.61	0.72	1.06	1.27	1.41	1.36	1.72	1.49	1.04 ± 0.21 aA,B
	4 (*n* = 3)	0.23	0.29	0.47	0.49	0.36	0.30	0.25	0.47	0.36	0.59	0.38 ± 0.07 aA
Madural	2 (*n* = 5)	0.24	0.23	0.62	1.15	0.92	1.12	1.14	1.14	1.69	1.65	0.99 ± 0.03 aA
	3 (*n* = 5)	0.15	0.57	0.87	0.85	1.59	2.01	2.54	2.05	2.80	2.38	1.58 ± 0.12 bB
	4 (*n* = 2)	0.73	0.93	0.83	1.22	1.53	1.63	1.36	0.48	0.86	0.57	1.01 ± 0.17 aB
Verdeal T.	2 (*n* = 4)	1.01	0.63	0.97	1.14	1.48	1.46	1.73	1.36	1.85	1.35	1.30 ± 0.32 aA
	3 (*n* = 4)	0.98	0.60	0.63	0.66	0.55	0.98	0.73	0.88	0.88	0.78	0.77 ± 0.09 aA
Ovipositions per infested olive												
Cobrançosa	2 (*n* = 5)	1.23	1.20	1.31	1.31	1.42	1.72	1.99	1.95	1.76	1.47	1.62 ± 0.09 aA
	3 (*n* = 5)	1.15	1.23	1.42	1.47	1.53	1.72	1.81	1.62	1.95	1.80	1.72 ± 0.17 aA
	4 (*n* = 3)	1.17	1.38	1.41	1.55	1.28	1.19	1.38	1.43	1.42	1.38	1.41 ± 0.04 aA
Madural	2 (*n* = 5)	1.09	1.21	1.35	1.53	1.36	1.42	1.41	1.35	2.04	2.12	1.58 ± 0.04 aA
	3 (*n* = 5)	1.13	1.44	1.47	1.42	1.93	2.25	2.71	2.32	3.22	2.67	2.29 ± 0.14 bB
	4 (*n* = 2)	1.80	1.60	1.84	2.03	2.10	2.31	1.79	1.38	1.50	1.40	1.91 ± 0.14 a,bB
Verdeal T.	2 (*n* = 4)	1.70	1.56	1.78	2.02	2.22	2.20	2.67	2.75	2.27	2.16	2.33 ± 0.19 bB
	3 (*n* = 4)	1.58	1.33	1.39	1.40	1.56	1.69	1.43	1.54	1.45	1.62	1.59 ± 0.09 aA
Collected pupae/adults												
Cobrançosa	2 (*n* = 5)	11	13	21	28	33	44	51	47	44	28	320 ± 47 bA
	3 (*n* = 5)	13	14	20	27	46	45	51	53	57	45	371 ± 87 bA
	4 (*n* = 3)	3	1	3	2	1	1	1	1	0	3	16 ± 5 aA
Madural	2 (*n* = 5)	6	8	19	39	20	26	33	30	30	31	242 ± 17 aA
	3 (*n* = 5)	5	15	24	29	41	52	66	54	73	34	393 ± 40 bA
	4 (*n* = 2)	8	16	11	26	34	24	13	1	6	2	141 ± 18 aA
Verdeal T.	2 (*n* = 4)	32	23	31	23	34	32	43	41	40	30	329 ± 105 aA
	3 (*n* = 4)	32	15	13	22	11	33	25	32	29	19	231 ± 39 aA

Continued

Table 3. (*Cont.*)

Parameter		Days assayed										Σ
		1	2	3	4	5	6	7	8	9	10	
Ratio pupae/stings												
Cobrançosa	2 (n = 5)	32.2	46.1	55.6	63.9	54.1	53.3	50.6	52.6	54.6	41.0	52.1 ± 4.4 aA
	3 (n = 5)	75.4	60.4	52.3	66.0	72.9	57.6	58.2	63.8	55.3	50.6	59.7 ± 3.1 aB
	4 (n = 3)	24.4	6.8	14.1	4.7	2.4	8.2	2.2	7.1	1.3	11.3	7.0 ± 0.7 bA
Madural	2 (n = 5)	28.9	48.1	49.4	57.7	38.4	40.0	49.4	43.0	30.5	32.5	40.7 ± 3.8 bA
	3 (n = 5)	47.2	38.7	44.6	56.2	44.3	44.1	42.6	43.0	42.2	22.7	41.4 ± 1.8 bA
	4 (n = 2)	18.5	27.5	25.0	33.9	36.5	26.1	9.7	1.4	11.6	3.8	23.2 ± 0.8 aB
Verdeal T.	2 (n = 4)	60.3	62.0	54.9	40.2	42.8	33.5	41.6	46.5	40.7	34.0	42.3 ± 3.8 aA
	3 (n = 4)	57.5	45.8	34.3	54.1	33.3	55.3	54.1	51.6	50.7	39.5	50.0 ± 2.6 aA

[1]Maturation index based on Hermoso *et al.* (2001).

Fig. 3. Boxplot of longevity (days; n = 100) of olive fly adults emerged from pupae developed under cvs. Cobrançosa, Madural, and Verdeal Transmontana olives ((a) – only with water; (b) – with solid diet).

discussed ahead in the section '*Maturation process and cultivar in olive fly oviposition preference*'.

Effect of cultivar on olive fly oviposition

Multiple-choice oviposition bioassays

No differences were found in the results of the multiple-choice bioassays for all the parameters analyzed. Several factors influence the oviposition behaviour of tephritids. However, according to Aluja & Mangan (2008), the most important factors in artificial laboratory assays are: (i) ovarian dynamics and oviposition drive (motivation); (ii) learning; (iii) age and concomitant aculeus wear in females; (iv) social context; and (v) genetic and rearing background (wild flies versus laboratory reared). Nevertheless, volatiles and olfactory stimuli play an important role in the behaviour, recognition, and attraction of tephritids towards hosts (Light & Jang, 1996; Thibout *et al.*, 2007). Our hypothesis is that presenting the three cultivars at the same time in the same assay may cause confusion in the behaviour of the fly. Each cultivar has its own distinct volatile profile (Malheiro *et al.*, 2015*c*), which may exert important attractive and repellent actions in the olive grove. However, in laboratory bioassays, with olives

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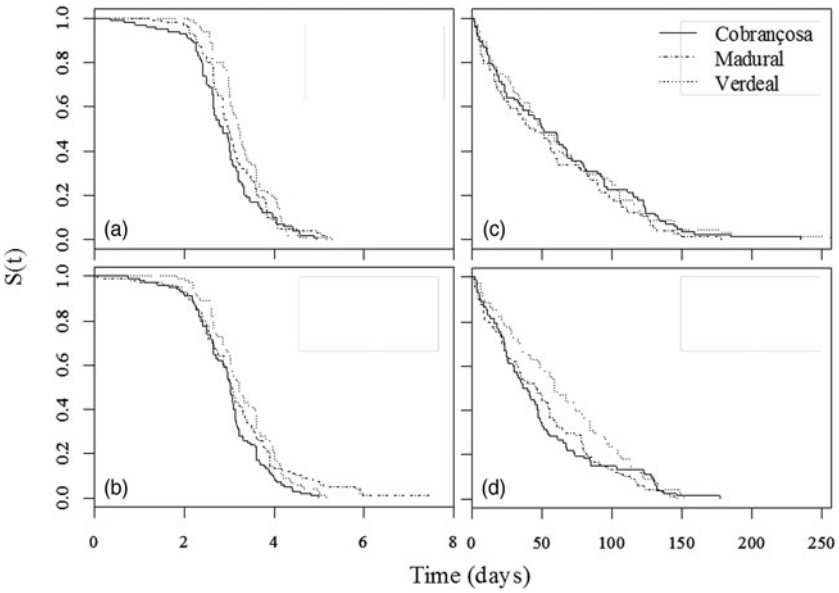

Fig. 4. Survival curves obtained by Kaplan –Meier estimates of unfed ((a) females; (b) males) and fed ((c) females; (d) males) olive fly adults emerged from pupae developed under cvs. Cobrançosa, Madural, and Verdeal Transmontana olives.

within similar maturation stages, the preference is highly reduced, and the volatiles action may be reduced. On the other hand, considering that all the available fruits had similar MI and were freely mixed, it was difficult for the females to select different cultivars. Therefore, we implemented a new bioassay with only one option, the no-choice oviposition bioassay.

No-choice oviposition bioassays

The results obtained from the no-choice oviposition bioassays were clear, showing a higher preference of olive fly females towards cv. Verdeal Transmontana, followed by cv. Madural, and cv. Cobrançosa, which showed to be the least susceptible cultivar (table 2). These results are in accordance with the infestation levels recorded in the field as well as with previous works carried out with the same cultivars (Gonçalves *et al.*, 2012; Malheiro *et al.*, 2015*b*, *c*).

In these bioassays, besides being the least preferred, cv. Cobrançosa also registered the lowest percentage of pupae/adults recovered from the olives within the three cultivars (51.1%). This suggests that in 48.9% of the ovipositions made in cv. Cobrançosa olives, the eggs did not hatch or the larvae or pupae died inside the fruit. Three aspects could be related to these observations: (i) a high number of sterile ovipositions, in which olive fly females perforate the fruit epidermis but no egg is laid inside; (ii) the egg is laid but it is unable to develop correctly and no larvae hatch; (iii) the larvae are capable of hatching but die inside the olive pulp. In the three scenarios presented, olive volatiles and phenolic compounds may have a crucial role. In the case of sterile ovipositions, when the olive fly introduces the ovipositor and the olive tissues are disrupted, it causes a series of enzymatic mechanisms (lipoxygenase pathway (LOX) and β-glucosidase activity) that yield deterrent volatiles (Scarpati *et al.*, 1993). Another aspect is the structural degradation caused in the aculeus tip due to excessive firmness of the drupe (Jones & Kim, 1994), thus increasing the number of sterile ovipositions. Olives from cv. Cobrançosa report a significantly higher firmness comparatively with cvs. Madural and Verdeal Transmontana (Gonçalves *et al.*, 2012).

In the second and third hypothesis raised, phenolic compounds may have a direct role in the low rate of pupae/adults collected in cv. Cobrançosa. Indeed, once an olive is attacked by the olive fly, a complex internal defense mechanism is triggered which yield highly reactive toxic molecules (Spadafora *et al.*, 2008), with strong protein-denaturing properties (Koudounas *et al.*, 2015). This enzymatic mechanism is more pronounced in less susceptible olive cultivars and reports a low expression in highly susceptible cultivars, as observed in cvs. Carolea (highly susceptible) and Cassanese (low susceptibility) (Spadafora *et al.*, 2008). These toxic molecules may abort eggs in the olive pulp, since a higher accumulation of these structures are found around the oviposition site (Spadafora *et al.*, 2008). Nevertheless, even if the egg is viable and larvae hatch, the amounts of these compounds during larvae development as well compromise its normal development, thus leading to death due to toxicity.

Maturation process and cultivar in olive fly oviposition preference

A considerable decrease in ovipositions from olives at MI 3 (reddish-green olives) to those at MI 4 (black olives) was verified. Some authors claim that the dark colouration of olive drupes at advanced ripening stages may confuse the olive fly females, preventing them from recognizing the olives and consequently reducing the risk of infestation (Iannotta & Scalercio, 2012). Also, the infestation odds of black olives are clearly low comparatively with red or green olives (Rizzo *et al.*, 2012). Katsoyannos & Kouloussis (2001) results highlight that olive fly females are especially attracted to red colour sphere traps. Reddish spheres catch three times more females compared to McPhail traps, the most common trap for monitoring olive flies in Greece (Katsoyannos & Kouloussis, 2001). Another aspect that could contribute to the reduction of

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ovipositions at MI 4 is the penetrability. The physical efforts necessary to oviposit influence host choice (Aluja & Mangan, 2008). Olives at MI 4 report a lower skin break force, but higher skin elasticity associated with a lower firmness (Gonçalves *et al*., 2012), a combination of factors which could difficult the oviposition. Furthermore, these physical changes in the fruits can lead to their rejection, since the chemical composition and characteristics of the fruit skin change and tephritids lose the ability to recognize host surface chemicals with their tarsal receptors (Städler *et al*., 1987).

Another interesting result obtained was the very low percentage of recovery of pupae/adults in olives from MI 4, mainly in cv. Cobrançosa, the least susceptible olive cultivar. These results could be ascribed to the accumulation of toxic molecules (Appel, 1993) of glutaraldehyde-type structures that may remain in the olive pulp during maturation, an hypothesis in accordance with the decrease of oleuropein in olives from the three cultivars (Sousa *et al*., 2014, 2015). Sharma & Sohal (2013) also verified that phenols, namely gallic acid, are toxic to tephritids (*B. cucurbitae*), thus critically influencing larval survival and emergence in a concentration-dependent manner. Our results point out that maturation reduces the ovipositions of olive flies, but can also naturally control their population by affecting the development of eggs and larvae.

Survival of olive fly adults

We verified that the adults emerged from cv. Verdeal Transmontana olives live longer. Besides being more susceptible to the olive fly, the olives from this cultivar also confer a higher life span to olive flies. From an ecological point of view, this is an important asset for the species survival, since flies can live longer even if they hatch in a place with food scarcity. In this case, lipid reserves sustain the survival of the individual for some days, as observed in our work and also in other Tephritidae species, such as *Anastrepha serpentine* (Jacome *et al*., 1995). *A. serpentine* individuals reduce their lipid reserves by 75% 4 days after hatching, only with the supply of water (Jacome *et al*., 1995). Therefore, we assume that olives fat content influenced the survival of both sexes of the olive fly. Verdeal Transmontana olives possess a higher fat content than the remaining cultivars (Gonçalves *et al*., 2012). Since pupae feed on olive pulp, they store higher amounts of fat in their body, which will influence adults' longevity, providing them with higher lipid reserves. Another aspect related to the oviposition preferences of tephritids is the learning process and previous experience with hosts (Prokopy *et al*., 1993; Robacker & Fraser, 2005). Olive flies may know that cv. Verdeal Transmontana may confer higher survival odds to their progeny.

Based on our results, olive cultivar and maturation are crucial parameters in the oviposition preference of olive fly females. Both in the field and in laboratory bioassays, olive fly females have a clear preference for olives from cv. Verdeal Transmontana, followed by cv. Madural and the least preferred was cv. Cobrançosa. The MI influenced olive fly oviposition preference, with results showing a clear reduction in oviposition from green (MI 2) and reddish (MI 3) to black olives (MI 4). Advanced MI may cause high levels of mortality of eggs/larvae and absorptive ovipositions. Since each olive cultivar has different maturation pathways, the slower process in olives from cv. Verdeal Transmontana has a highly attractive action over olive fly females since olives remain greener for longer periods. It was also concluded that olive fly adults live longer and the death hazard is lower if larvae develop in olives from cv. Verdeal Transmontana, a fact probably related to insects' lipid reserves.

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