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RESEARCH ARTICLE

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Detection of *Bactrocera oleae* (Diptera: Tephritidae) DNA in the gut of the soil species *Pseudoophonus rufipes* (Coleoptera: Carabidae)

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Abstract

Pest control service provided by natural enemies of *Bactrocera oleae*, the key pest of the olive tree, is nowadays recognized as fundamental. *B. oleae* has developed resistance to common insecticides, and negative effects both on consumers' health and non-target species are the major drawbacks of conventional control strategies. Carabid beetles are potential *B. oleae* pupae predators, but their predation on field still need to be assessed. We tested adult *Pseudoophonus rufipes*, a species known to be active in olive orchard when pest pupae are abundant in the soil, in order to detect *B. oleae* pupae consumption at different post feeding times for both male and female carabids. An already existing protocol was used for detecting *B. oleae* mtDNA sequences of the *cytochrome oxidase subunit I* gene in carabids' gut, and its versatility improved. *B. oleae* mtDNA was detected up to 20 h after pupa ingestion with a high percentage of success, without significant differences between sexes and pair primers used. Prey DNA extraction was tested from both dissected and non-dissected carabids, obtaining comparable results. The trapping system used to collect carabids for molecular assays and the new elements introduced in the protocol represent cost-effective solutions that may be beneficial for future laboratory trials and, mostly, for the analysis of field-collected predators. Fostering the investigation of soil predators in olive orchard may increase the design of conservation control strategies against *B. oleae*.

Additional keywords: fruit fly; gut content; olive; PCR; biological control; pitfall trap; post feeding time.

Authors' contributions: Conceived and designed the experiments: AA, SAPS, PB. Performed the experiments and analyzed the data: AA, FM. Contributed reagents/materials/analysis tool: TLN, PB, JAP. Wrote the manuscript: AA. Revised the manuscript: RP, JAP, SAPS, PB.

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Introduction

The olive tree (*Olea europaea* L.) is one of the main crops in the Mediterranean basin, where the region alone produces 91.2% and consumes 72% of the world's olive oils (International Olive Oil Council, http://www.internationaloliveoil.org, data updated to 2015/2016 crop year). Losses up to 80% of the oil value and 100% of some table cultivars are caused by the obligate olive key pest, the olive fruit fly *Bactrocera oleae* (Rossi, 1790) (Diptera: Tephritidae) (Daane & Johnson, 2010; Malheiro *et al.*, 2015). The pest indirectly damages the crop by ovipositing inside the fruit and feeding upon the pulp, until reaching the pupal stage. Historically confined to the Mediterranean Basin, *B. oleae* has spread in almost every country where olive is cultivated for commercial purposes (Augustinos *et al.*, 2002). In addition, a predicted 2°C global warming in the Mediterranean Basin between 2030 and 2060 (Giannakopoulos *et al.*, 2009) is alerting bioeconomics and producers, as interactions between olive and *B. oleae* are expected to be enhanced (Ponti *et al.*, 2014). For the conservation of olive orchard agroecosystem, economy and cultural heritage, holistic strategies are urgently needed (Ponti *et al.*, 2016; Marchini *et al.*, 2017).

Conventional control strategies against *B. oleae* have proved to have a negative impact both on consumers' health and non-target species (Vickerman & Sunderland, 1977; Amvrazi & Albanis, 2009), not to mention the development of resistance by the pest to frequently used insecticides (Daane & Johnson, 2010; Pavlidi *et al.*, 2018). Initial inroads have been made in understanding the role of resident natural enemies of *B. oleae*. Indeed, the assessment of pest control ecosystem service would be of utmost importance in the design of environmental-friendly management plans. Literature on conservation biological control of *B. oleae* has been addressing at the interactions between potential soil predators and pre-imaginal stages of the pest (Orsini *et al.*, 2007; Santos *et al.*, 2007; Dinis *et al.*, 2016). The third instar larvae of the overwintering generation leave the drupe to pupate in the first centimeters of the soil (Dimou *et al.*, 2003). Here, they spend several months, being thus exposed to a guild of soil predators and abiotic factors, until they emerge as adults (Bateman, 1972; Cavalloro & Delrio, 1975).

DNA-based techniques have been providing valuable results in unraveling these interactions between the pest and its natural enemies, mostly when direct observation of predation in the field are impractical (González-Chang *et al.*, 2016). Polymerase chain reaction (PCR)-based analysis of unique prey DNA sequences has proven to be a highly sensitive, specific and cost-effective tool for detecting prey's remains in the predator's gut (Symondson, 2002). Several factors affect the probability of detecting prey DNA sequences, such as the identity and physiology of the predator, the collection method, the preservation of the specimens and the choice of the molecular markers, and some methodological issues still need to be optimized (Juen & Traugott, 2006; Weber & Lundgren, 2009). Since the likelihood of detecting the prey target sequences decreases with post feeding time, one of the main ecological issues is inspecting which is the maximum time that prey consumption can be detected, in order not to underestimate predation (Greenstone *et al.*, 2007). While in laboratory trials the post feeding time can be controlled, this issue is particularly relevant in field analyses, where predators are collected after an unknown post feeding time. In addition, predator identity (*e.g.* taxonomy identity and sex) may affect post feeding prey DNA detection intervals (Zaidi *et al.*, 1999; Sheppard *et al.*, 2005). For a given specific prey, calibratory feeding trials are therefore required to assess the robustness and versatility of the PCR-based diagnostic assay for a broad range of predators (King *et al.*, 2008; Aebi *et al.*, 2011).

Despite the accumulating evidence that carabid beetles (Coleoptera: Carabidae) are a dominant component in the predatory fauna of olive orchard (*e.g.*, Lasinio & Zapparoli, 1993; Gonçalves & Pereira, 2012), a better understanding of the chance to molecularly detect the olive pest in adult carabid's gut is required. Recently, Rejili *et al.* (2016) designed *B. oleae* specific primers based on mtDNA sequences of *cytochrome oxidase subunit I* (*COI*) gene to successfully detect the prey consumption by the carabid *Pterostichus globosus* (Fabricius, 1792) up to 16 h after prey's ingestion, in laboratory conditions. To validate the versatility of the above PCR-based diagnostic assay, we tested *Pseudoophonus rufipes* (=*Harpalus rufipes*) (De Geer, 1774), a medium-size (11-16 mm length) carabid species, generalist feeder, with a wide (Palearctic) distribution, preferring open habitats (Thiele, 1977; Honek *et al.*, 2013). It is among the most active species in arable lands (Lövei & Sárospataki, 1990; Thomas *et al.*, 1997; Avgin & Luff, 2009) being mentioned as a biocontrol agent of several pests (Kromp, 1999; Sunderland, 2002; Boreau de Roincé *et al.*, 2012). Its life cycle has been detailed described (Matalin, 1997) and the species has showed peaks of activity during the period of highest abundance of *B. oleae* pupae (Albertini *et al.*, 2017). In this study, we tested the post feeding detection of *B. oleae* within *P. rufipes* gut up to 20 h in both female and male predators, to assess if *B. oleae COI* fragments were still detectable in the carabids' gut. Finally, we extracted prey's DNA both from dissected and not dissected carabid specimens, aiming at improving Rejili *et al.* (2016) protocol in terms of efficiency and user-friendly features.

Specifically, this study was designed to compare: i) *B. oleae* mtDNA detectability at different post feeding time intervals; ii) carabid female and male differences in prey detection time; iii) the sensitivity of two different pair primers designed to amplify two fragments of mtDNA *COI* gene of *B. oleae* and iv) the efficiency of the use of two different sample types for extracting DNA ("gut" *vs* "whole body" samples).

The PCR-based assay optimization here presented may allow accurate analyses by means of molecular tools, prompting the development of quantitative measurements of predation in-field by the natural enemies of *B. oleae* pre-imaginal stages.

Material and methods

Insect collection

Adult carabid beetles were collected from July to August 2016 in a maize field in Monte Pisano area (43°40'06.8"N, 10°37'05.9"E), Pisa, Italy. Specimens were collected alive, avoiding the use of preservatives

in the traps, such as acid acetic and formaldehyde, since these compounds are known to inhibit PCR (Gurdebeke & Maelfait, 2002; King *et al.*, 2008). We used "semi-dry" pitfall traps described in Albertini *et al.* (2018), a method that combines the use of an attractant without the risk of compromising the subsequent molecular analysis. The traps were dug into the soil and levelled with the soil surface. Most of ground-dwelling carabids are active during the night (Thiele, 1977) therefore traps were activated in the afternoon and collected in the morning after. Carabids were brought into the laboratory and identified to species and sex using a binocular stereomicroscope.

According to a preliminary feeding trial performed in November 2015 in orchards located in Monte Pisano area using the above described "semi-dry" pitfall traps, we opted to run experiments on adult *P. rufipes* species as it is: i) easy to collect, without sex bias, by means of the described traps; ii) easy to rear and feed, being vital after 48 h of starvation and generally eating the offered prey in a short time (less than 24 h) and iii) active in those months when *B. oleae* pupae are more abundant in the soil.

Bactrocera oleae pupae were collected from infested olives of orchards located in Monte Pisano area during Summer 2016. *Ceratitis capitata* (Wiedemann, 1824) (Diptera: Tephritidae) pupae were obtained from a laboratory culture maintained at the University of Pisa, Italy. *C. capitata* was used in order to test PCR primers specificity, since it is one of the main fruit flies widely distributed in the Mediterranean basin together with *B. oleae*, and the soil predators may feed on both pupae species. Pupae of *B. oleae* and *C. capitata* were stored in the fridge at 2°C till they were used in feeding trials.

Feeding trials

Adults of *P. rufipes* were individually placed in a plastic vessel (85 mm in diameter at the opening and 120 mm height), provided with water and a wet sponge shelter, maintained at room temperature and 16:8 h L:D from the beginning to the end of the experiments. Specimens were starved for 48 hours prior to experiments. Each *P. rufipes* was fed with one pupa that was introduced in each vessel, except those specimens used as negative control, where no food was provided.

In the experiments with *B. oleae* pupae, 116 carabids were randomly assigned to groups with a different post feeding time: 2, 4, 6, 8, 9 to 15 and 16 to 20 h (16 to 20 carabids were used for each time period). In the experiments with *C. capitata*, only 2 h of post feeding time was considered (n=6). In the negative control experiments, carabids were not fed and were frozen after the starvation period (*i.e.*, 48 h) (n=6). In all the trials, sex ratio of carabids was 1:1.

For each time, carabids were observed feeding. When they started to eat the pupae, they generally consumed it at once firstly piercing the cuticle and then eating the internal parts. We assumed that the prey was eaten after 90% of the pupa had been consumed, by the visual inspection of its volume, then we removed the remains from the vessel and started counting the post feeding time. After the post feeding time carabids were transferred individually into vials with ethanol 70% and frozen at -80°C until molecular analyses.

DNA extraction

The detection of *B. oleae* DNA in the gut of *P. rufipes* along the time was studied using two different approaches: (1) the "gut trial", where carabid guts were removed by dissection from 84 carabids (6 females and 6 males for each time period; 3 females and 3 males both for the experiments with *C. capitata* and for the negative control) and DNA was extracted from the dissected gut, and (2) the "whole body trial" (*i.e.* non-dissected specimens) where head, legs and elytra of 24 carabids, 13 female and 11 males (2 females and 2 males for each time period, except for the 9 to 15 h period where 1 female and 3 males were tested), were removed, and the rest of the body was ground to a fine powder in liquid nitrogen. The powder was used for DNA extraction. In both approaches, Ron's Tissue DNA Mini Kit (Boiron GmbH, Germany) was used to extract DNA, following the manufacturer's instruction, except that the incubation time was 24 h in our procedure instead of overnight.

VWR® mySPEC microvolume Spectrophotometer Twin 732-2535 was used to evaluate DNA concentration and purity by calculating A260/A280 ratio. After quantification, DNA pools from two individuals were prepared using 1:1 DNA ratio and further used for amplifications. DNA integrity was verified by gel electrophoresis in 1% (w/v) agarose gel, stained with GelRed Nucleic Acid Gel Stain (Biotium, USA), at 90 V for 20 min (constant voltage) and visualized under UV light using Stratagene Eagle Eye® II video system (BioSurplus, USA).

DNA amplification

The pair primers SBo1-F/SBo1-R and SBo2-F/SBo1-R, developed by Rejili *et al.* (2016) specifically for the detection of *B. oleae*, were used to amplify two regions (108 bp and 214 bp, respectively) of the mitochondrial DNA *cytochrome oxidase I* (mtDNA *COI*) gene of *B. oleae*. Conditions of the used PCR protocol were described by Rejili *et al.* (2016). All PCRs included positive control (*B. oleae* DNA), negative

control (the DNA extracted from the guts of starved specimens or fed with *C. capitata*) and no template control (PCR reaction without template DNA). All carabids were screened using singleplex PCR analysis.

PCR products were run on 2% (w/v) agarose gel, stained with GelRed Nucleic Acid Gel Stain (Biotium, USA), at 60 V for 60 min (constant voltage) along with a DNA ladder as molecular weight marker, and visualized under UV light using Stratagene Eagle Eye® II video system (BioSurplus, USA).

Data analysis

The success of the detection of *B. oleae* DNA in the gut of predators was assessed comparing the percentage of positive amplifications for i) different post feeding times; ii) the two pair primers; iii) females and males and iv) "gut" and "whole body" samples. In the tests, post feeding times from 2 h to 8 h were grouped in a unique class in order to compare a short time interval (*i.e.*, until 8 h) with medium (from 9 to 15 h) and larger (from 16 to 20 h) post feeding time. Chi-squared test with Yates's correction was used to compare the variables in 2x2 contingency tables. When sample size was small and/or expected frequencies were low, Fisher's exact test was used (McDonald, 2009). Significance was reported at the level of $p < 0.05$.

To evaluate the efficiency of the use of two different sample types for extracting DNA, both the quantity ([DNA] µg/µL) and the quality (estimated by 260/280 purity ratio) of the extracted DNA were compared. Normality was assessed by Shapiro-Wilk test ($p < 0.05$). Being the assumption of normality not respected, and with an unequal sample sizes, Mann-Whitney U test (also known as Wilcoxon rank-sum test) was applied to determine whether difference occurred between "gut trial" and "whole body trial". Significance was reported at the level of $p < 0.05$. All the analyses were carried out using R software (R Core Team, 2016).

Results

A specific amplification with the predicted products of *B. oleae COI* gene fragments (108 and 214 bp) was generated by the use of both pairs of primers up to 20 h after prey ingestion for both female and male predators (Fig. 1). No amplification was revealed when DNA extracted from carabids fed with *C. capitata* or starved carabids were used (Fig. 2).

For all the post feeding treatments, 160 PCRs were carried out in total, 146 of which showed specific amplification. For all sample types, 90.36% showed positive amplifications with the first pair primers (SBo1-F/SBo1-R, 83 PCRs in total) and 92.21% with the second pair (SBo2-F/SBo1-R, 77 PCRs in total). No significant differences were found when compared the overall success of the two pair primers and when

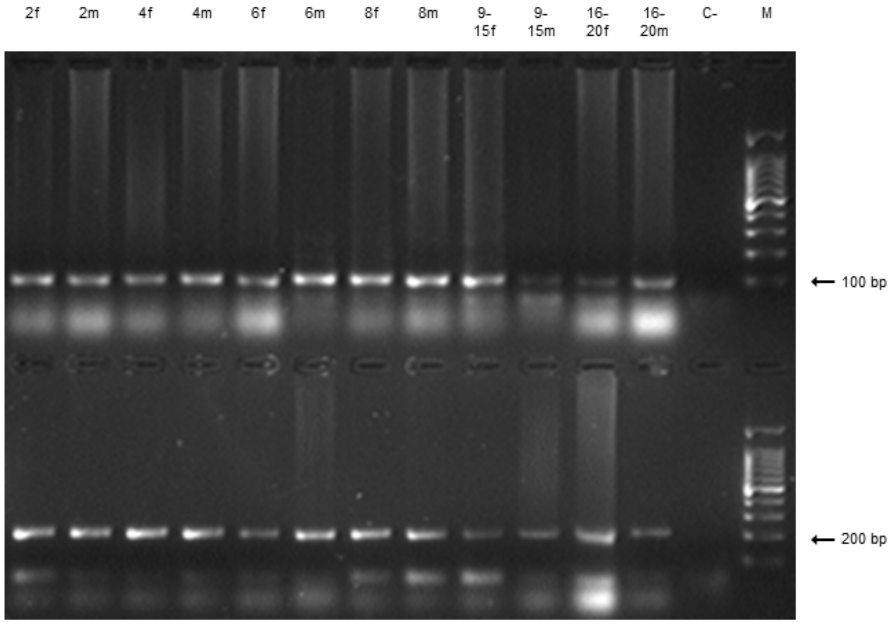

Figure 1. Agarose gel electrophoresis for the "gut trial" of *Pseudoophonus rufipes*, using *Bactrocera oleae* specific primer pairs SBo1-F/SBo1-R (upper lanes) and SBo2-F/SBo1-R (lower lanes). Female (f) and male (m) 2h (2), 4h (4), 6h (6), 8h (8), 9-15h (9-15) and 16-20h (16-20) post feeding. Lane M is a 100 bp DNA ladder and the C- lane is no template control.

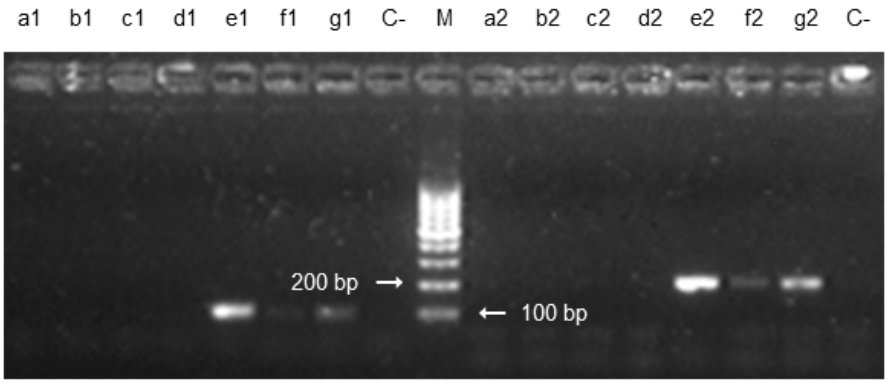

Figure 2. Agarose gel electrophoresis using *B. oleae* specific primer pairs SBo1-F/SBo1-R (lanes left to marker - M) and SBo2-F/SBo1-R (lanes right to marker). a: male *P. rufipes* fed with *C. capitata*. b: female *P. rufipes* fed with *C. capitata*. c: male *P. rufipes* not fed. d: female *P. rufipes* not fed. e, f, g: females 2 h post feeding. C-: no template control. M: 100 bp DNA ladder. 1: first pair primers SBo1-F/SBo1-R. 2: second pair primers SBo2-F/SBo1-R.

compared short, medium and large time intervals (Table 1).

Female carabids obtained 89.29% of positive amplifications, male carabids 93.42% and no statistically significant differences were found between sexes (Table 1). Considering "gut" and "whole body" samples, 89.84% and 96.86%, respectively, responded positively to *B. oleae* detection, differences being again not statistically significant (Table 1).

For the "gut trial" samples (128 PCRs), *B. oleae* DNA fragments were detected in all the post feeding time treatments, ranging from 79.41% to 100% of positive amplifications. Higher rates were found for 2, 4, 6 and 8h post feeding (from 83.33% to 100%). Nine-15 and 16-20 h treatments had, respectively, 79.41% and 89.29% of successful amplifications. In comparing the total percentage of successful amplifications using the two pair primers, the first pair had 87.5% of positive results and the second 92.19% (Fig. 3). Females had 88.06% of successful amplifications and males 91.80%. Comparisons among the efficiency of the two pair primers and any differences among sexes revealed no statistically significant differences (Table 1).

For the "whole body trial" samples (32 PCRs), the percentage of positive amplifications was 100% for all post feeding time treatments, except for 16-20 h treatment where there was 80% of success (4 positive samples out of 5 assays). The first pair of primers gave 100% of positive results, the second pair 92.31% (12 positive samples out of 13 assays) (Fig. 3). Females had 94.12% of positive results and males 100%. Comparisons among the efficiency of the two pair primers and among

Table 1. Results of Chi-squared test with Yates' correction (df=1) and Fisher's exact test (*p*-values) for comparisons between primers, feeding times, sexes and sample types.

	χ^2 test	Fisher's exact test
General comparisons		
Primers	0.894	
–short post feeding time		1.000
–medium post feeding time		0.405
–long post feeding time		0.601
Sexes	0.519	
Sample types		0.305
"Gut" samples		
Primers	0.558	
Sexes	0.684	
"Whole body" samples		
Primers		0.406
Sexes		1.000

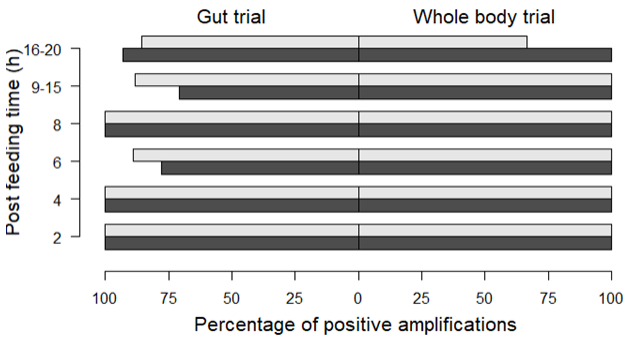

Figure 3. Positive amplifications (%) for detecting *B. oleae* in the gut of *P. rufipes* by comparing the "gut trial" and the "whole body trial" samples at different post feeding times. PCRs were performed using two pair primers: SBo1-F/SBo1-R (dark grey bars) and SBo2-F/SBo1-R (light grey bars).

sexes revealed no statistically significant differences (Table 1).

When comparing the quantity and quality of the extracted DNA, the average value was 457.90 (SE=31.80, n=58) µg/µL and the ratio 260/280 was 2.11 (SE=0.02, n=58) for the "gut trial" while for the "whole body trial" values were 586.74 (SE=84.81, n=12) µg/µL and 2.10 (SE=0.03, n=12) respectively. Differences were statistically not significant for DNA quantity (p=0.10) nor quality (p=0.37).

Discussion

Our laboratory study has demonstrated that it is possible to detect *B. oleae* consumption in carabids' gut at least up to 20 h since pupa ingestion, for both female and male predators that consumed one *B. oleae* pupa only. There are new elements compared to Rejili *et al.* (2016), which confirm the validity of their protocol and prove its versatility and sensitiveness. There is a general increasing interest in the tested species *P. rufipes*, which has already been proposed both as pest control agent and useful seed consumer (Wallinger *et al.*, 2015). Since it is also active in olive orchards when pest pupae are more abundant in the soil (Albertini *et al.*, 2017), *P. rufipes* may be used as a good model species for testing its efficiency as *B. oleae* natural enemy.

DNA-based diet analyses may allow verifying prey consumption on field, once the calibratory feeding trials have been carried out in laboratory. The chance to detect the DNA of a prey for a given predator can differ significantly and refers specifically to that prey-predator system (King *et al.,* 2010). The laboratory feeding trial are a needed step for the meaningful interpretation of the field results and the extrapolation of the prey-detection period from one prey-predator system to another. In field experiments, traps are checked the day after their activation, as most carabids have nocturnal predatory habits. Zaidi *et al.* (1999) suggested that covering an interval of at least 12 h between supposed prey consumption during the night and predator collection the next morning is an adequate amount of time for subsequent molecular analysis. Since *B. oleae* consumption can be detected up to 20 h post feeding time, this may allow researchers to activate the traps for a longer time still obtaining reliable results. Moreover, recent studies have proved for *P. rufipes* a DNA detectability over 30 h for other preys (seeds included) (Monzó *et al.*, 2011; Wallinger *et al.*, 2015). In addition, since Cornic (1973) reported for *P. rufipes* seasonal variations in the type of ingested food, this element should be taken into consideration both for field and laboratory experiments.

Recently Lantero *et al.* (2017) detected *B. oleae* consumption in two Mediterranean carabid species up to 72 h, however sex bias was not tested. Predator sex is frequently considered a factor affecting food intake and thus prey detection (Sunderland, 1975) and for carabids results are contradictory. We did not find any statistically significant difference among sexes in prey detection, confirming the results obtained by Zaidi *et al.* (1999). However, there could be behavioural differences in the field, being males generally more active and females showing diet seasonality (Sheppard *et al.*, 2005; Šerić Jelaska *et al.*, 2014; Šerić Jelaska & Symondson, 2016). Therefore, we still suggest considering sex identity for future analysis.

Meal size and amount have been often shown to affect the DNA detectability in the predator's gut (King *et al.*, 2008), however responses are not homogeneous. For example, Zaidi *et al.* (1999) demonstrated that there is no correlation between the number of preys consumed by the predators and the likelihood of successful prey DNA amplification, while King *et al.* (2010) found a strong effect of the meal size. In addition, it has been hypothesized that feeding the starved individuals with a large amount of prey, if not *ad libitum*, may bias towards longer post ingestion detection (Aebi *et al.*, 2011). In our test only one pupa was provided to each carabid, its consumption being detected with high percentage of success. Unless feeding trials with specific purposes should be carried out, we suggest providing the tested predators with the minimum amount of food, necessary and sufficient for a reliable molecular analysis. In such way there are three main advantages: it is a cost-effective solution, it reflects better the prey availability and accessibility in the field for a given predator, and it can be helpful when prey population size is controlled in experimental settings.

We compared the efficiency of two pair primers, amplifying for a shorter (108 bp) and a longer (214 bp) mtDNA region. Comparisons were made considering the overall reactions and reactions for specific post feeding time, and no statistically significant differences were found. Fragment size can affect DNA detectability, and short fragments (< 300 bp) are usually suggested (King *et al.*, 2008), even if for *P. rufipes* also medium-sized fragments allowed long prey DNA detection intervals (Waldner *et al.*, 2013). Besides both pair primers we tested proved to be highly efficient and specific for *B. oleae*, it is still preferable to use both of them, especially for field-collected carabids, where the probabilities to obtain positive results are lower than in laboratory-controlled experiments (Sheppard *et al.*, 2005; Aebi *et al.*, 2011). In fact, for field-collected predators

there is no *a priori* knowledge whether predation has occurred, and a number of additional factors, including PCR-inhibitory substances that edaphic arthropods may enter in contact with, may affect successful amplification of prey DNA (Juen & Traugott, 2006). A less time-consuming approach may be the use of a multiplex PCR (Harper *et al.*, 2005), instead of two separate singleplex PCRs, one for each pair primer.

No significant differences were found in the success of prey detection between carabids used in the "gut trial" and those used in the "whole body trial". Nor DNA quantity nor DNA purity were affected by the two sample types. The choice in dissecting or not dissecting a specimen has been discussed in King *et al.* (2008). The carabid species we used was relatively large in size, thus dissection was not impeded by its body dimension. It has to be stated that prey DNA extraction from the predator's gut relies on a certain expertise of the researcher in opening the gut and selecting the target carabid's material. In this case, the preparation of each dissected sample for the DNA extraction usually requires, as a rule of thumb, 20 min on average, before the 24 h of the sample incubation. On the opposite, DNA extraction from the whole body is a faster and more user-friendly procedure, as the beetle is not manipulated (except for bigger and chitinous appendices removal). However, its efficiency strongly relies on the body grinding procedure with liquid nitrogen, as a fine powder is needed. Because of this procedure, we estimate that the preparation of each non-dissected sample requires again 20 min on average. Being the two methods almost comparable for a predator of medium to large body size, the choice of the most convenient method may be driven by laboratory's equipment and expertise level.

In conclusion, we used the protocol of Rejili *et al.* (2016) introducing relevant modifications prior to the step of DNA extraction. The new protocol still ensures the extraction of quality DNA and offers many practical advantages such as cost-effective feeding trials and easy handling of specimens. The protocol is particularly suitable for field experiments, in which we suggest the collection of predators by means of "semi-dry" pitfall traps that allow carabids collection without the use of PCR inhibitors. This trapping system, together with the protocol, has been recently used for field-collected staphylinids in olive orchards, successfully demonstrating *B. oleae* consumption in *Ocypus olens* (Müller, 1764) (Coleoptera: Staphylinidae) (Albertini *et al.*, 2018). The assessment of *B. oleae* consumption by field predators may be particularly advantageous in developing biological control strategies against the most harmful pest of olive orchards.

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References

Aebi A, Brown PM, De Clercq P, Hautier L, Howe A, Ingels B, Ravn HP, Sloggett JJ, Zindel R, Thomas A, 2011. Detecting arthropod intraguild predation in the field. BioControl 56: 429-440. https://doi.org/10.1007/s10526-011-9378-2

Albertini A, Pizzolotto R, Petacchi R, 2017. Carabid patterns in olive orchards and woody semi-natural habitats: first implications for conservation biological control against *Bactrocera oleae.* BioControl 62: 1-13. https://doi.org/10.1007/s10526-016-9780-x

Albertini A, Marchi S, Ratti C, Burgio G, Petacchi R, Magagnoli S., 2018. *Bactrocera oleae* pupae predation by *Ocypus olens* detected by molecular gut content analysis. BioControl 63: 227-239. https://doi.org/10.1007/s10526-017-9860-6

Amvrazi EG, Albanis TA, 2009. Pesticide residue assessment in different types of olive oil and preliminary exposure assessment of Greek consumers to the pesticide residues detected. Food Chem 113: 253-261. https://doi.org/10.1016/j.foodchem.2008.06.073

Augustinos AA, Stratikopoulos EE, Zacharopoulou A, Mathiopoulos KD, 2002. Polymorphic microsatellite markers in the olive fly, *Bactrocera oleae.* Mol Ecol Notes 2: 278-280. https://doi.org/10.1046/j.1471-8286.2002.00222.x

Avgin SS, Luff ML, 2009. Biodiversity of carabid beetles (Coleoptera: Carabidae) from crops in Turkey. Proc Entomol Soc Wash 111: 326-334. https://doi.org/10.4289/0013-8797-111.2.326

Bateman MA, 1972. The ecology of fruit flies. Annu Rev Entomol 17: 493-518. https://doi.org/10.1146/annurev.en.17.010172.002425

Boreau de Roincé C, Lavigne C, Ricard JM, Franck P, Bouvier JC, Garcin A, Symondson WOC, 2012. Predation by generalist predators on the codling moth versus a closely- related emerging pest the oriental fruit moth: a molecular analysis. Agric Forest Entomol 14: 260-269. https://doi.org/10.1111/j.1461-9563.2011.00564.x

Cavalloro R, Delrio G, 1975. Osservazioni sulla distribuzione e sopravvivenza delle pupe di *Dacus oleae* Gmelin nel terreno. Redia 56: 167-175.

Cornic JF, 1973. Etude du régime alimentaire de trois espèces de carabiques et de ses variations en verger de pommiers. Ann Soc Entomol Fr 9: 69-87.

Daane KM, Johnson MW, 2010. Olive fruit fly: managing an ancient pest in modern times. Annu Rev Entomol 55: 151-169. https://doi.org/10.1146/annurev.ento.54.110807.090553

Dimou I, Koutsikopoulos C, Economopoulos AP, Lykakis J, 2003. Depth of pupation of the wild olive fruit fly, *Bactrocera* (*Dacus*) *oleae* (Gmel.) (Dipt., Tephritidae), as affected by soil abiotic factors. J Appl Entomol 127: 12-17. https://doi.org/10.1046/j.1439-0418.2003.00686.x

Dinis AM, Pereira JA, Pimenta MC, Oliveira J, Benhadi-Marín J, Santos SAP, 2016. Suppression of *Bactrocera oleae* (Diptera: Tephritidae) pupae by soil arthropods in the olive grove. J Appl Entomol 140: 677-687. https://doi.org/10.1111/jen.12291

Giannakopoulos C, Le Sager P, Bindi M, Moriondo M, Kostopoulou E, Goodess CM, 2009. Climatic changes and associated impacts in the Mediterranean resulting from a 2º C global warming. Glob Planet Change 68: 209-224. https://doi.org/10.1016/j.gloplacha.2009.06.001

Gonçalves MF, Pereira JA, 2012. Abundance and diversity of soil arthropods in the olive grove ecosystem. J Insect Sci 12: 1-14. https://doi.org/10.1673/031.012.2001

González-Chang M, Wratten SD, Lefort MC, Boyer S, 2016. Food webs and biological control: A review of molecular tools used to reveal trophic interactions in agricultural systems. Food Webs 9: 4-11. https://doi.org/10.1016/j.fooweb.2016.04.003

Greenstone MH, Rowley DL, Weber DC, Payton ME, Hawthorne DJ, 2007. Feeding mode and prey detectability half-lives in molecular gut-content analysis: an example with two predators of the Colorado potato beetle. Bull Entomol Res 97: 201-209. https://doi.org/10.1017/S0007485307004975X

Gurdebeke S, Maelfait JP, 2002. Pitfall trapping in population genetics studies: finding the right "solution". J Arachnol 30: 255-261. https://doi.org/10.1636/0161-8202(2002)030[0255:PTIPGS]2.0.CO;2

Harper GL, King RA, Dodd CS, Harwood JD, Glen DM, Bruford MW, Symondson WOC, 2005. Rapid screening of invertebrate predators for multiple prey DNA targets. Mol Ecol 14: 819-827. https://doi.org/10.1111/j.1365-294X.2005.02442.x

Honek A, Martinkova Z, Jarosik V, 2013. Ground beetles (Carabidae) as seed predators. Eur J Entomol 100: 531-544. https://doi.org/10.14411/eje.2003.081

Juen A, Traugott M, 2006. Amplification facilitators and multiplex PCR: Tools to overcome PCR-inhibition in DNA-gut-content analysis of soil-living invertebrates. Soil Biol Biochem 38: 1872-1879. https://doi.org/10.1016/j.soilbio.2005.11.034

King RA, Read DS, Traugott M, Symondson WOC, 2008. Molecular analysis of predation: a review of best practice for DNA- based approaches. Mol Ecol 17: 947-963. https://doi.org/10.1111/j.1365-294X.2007.03613.x

King RA, Vaughan IP, Bell JR, Bohan DA, Symondson WOC, 2010. Prey choice by carabid beetles feeding on an earthworm community analysed using species- and lineage- specific PCR primers. Mol Ecol 19: 1721-1732. https://doi.org/10.1111/j.1365-294X.2010.04602.x

Kromp B, 1999. Carabid beetles in sustainable agriculture: a review on pest control efficacy, cultivation impacts and enhancement. Agric Ecosyst Environ 74: 187-228. https://doi.org/10.1016/S0167-8809(99)00037-7

Lantero E, Matallanas B, Ochando MD, Pascual S, Callejas C, 2017. Specific and sensitive primers for the detection of predated olive fruit flies, *Bactrocera oleae* (Diptera: Tephritidae). Span J Agric Res 15 (2): e1002. https://doi.org/10.5424/sjar/2017152-9920

Lasinio PJ, Zapparoli M, 1993. First data on the soil arthropod community in an olive grove in central Italy. In: Soil biota, nutrient cycling, and farming systems; Coleman DC, Foissner W, Paoletti MG (eds.). pp: 113-121. CRC Press, Boca Raton.

Lövei GL, Sárospataki M, 1990. Carabid beetles in agricultural fields in Eastern Europe. In: The role of ground beetles in ecological and environmental studies; Stork NE (ed.). pp: 87-93. Intercept, Andover.

Malheiro R, Casal S, Baptista P, Pereira JA, 2015. A review of *Bactrocera oleae* (Rossi) impact in olive products: from the tree to the table. Trends Food Sci Technol 44: 226-242. https://doi.org/10.1016/j.tifs.2015.04.009

Marchini D, Petacchi R, Marchi S, 2017. *Bactrocera oleae* reproductive biology: new evidence on wintering wild populations in olive groves of Tuscany (Italy). Bull Insectology 70: 121-128.

Matalin AV, 1997. Specific features of life cycle of *Pseudoophonus* (s. str.) *rufipes* Deg. (Coleoptera, Carabidae) in Southwest Moldova. Izv Akad Nauk Ser Biol 4: 371-381.

McDonald JH, 2009. Handbook of biological statistics. Sparky House Publ, Baltimore, MD, USA. 313 pp.

Monzó C, Sabater-Muñoz B, Urbaneja A, Castañera P, 2011. The ground beetle Pseudophonus rufipes revealed as predator of *Ceratitis capitata* in citrus orchards. Biol Control 56: 17-21. https://doi.org/10.1016/j.biocontrol.2010.09.004

Orsini MM, Daane KM, Sime KR, Nelson EH, 2007. Mortality of olive fruit fly pupae in California. Biocontrol Sci Technol 17: 797-807. https://doi.org/10.1080/09583150701527359

Pavlidi N, Kampouraki A, Tseliou V, Wybouw N, Dermauw W, Roditakis E, Nauen R, Van Leeuwen T, Vontas J, 2018. Molecular characterization of pyrethroid resistance in the olive fruit fly *Bactrocera oleae*. Pestic Biochem Physiol 148: 1-7. https://doi.org/10.1016/j.pestbp.2018.03.011

Ponti L, Gutierrez AP, Ruti PM, Dell'Aquila A, 2014. Fine-scale ecological and economic assessment of climate change on olive in the Mediterranean Basin reveals winners and losers. Proc Natl Acad Sci 111: 5598-5603. https://doi.org/10.1073/pnas.1314437111

Ponti L, Gutierrez AP, Altieri MA, 2016. Preserving the mediterranean diet through holistic strategies for the conservation of traditional farming systems. In: Biocultural Diversity in Europe; Agnoletti M, Emanueli F (eds.). pp: 453-469. Springer Int Publ. https://doi.org/10.1007/978-3-319-26315-1_24

R Core Team, 2016. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna. https://www.r-project.org/

Rejili M, Fernandes T, Dinis AM, Pereira JA, Baptista P, Santos SAP, Lino-Neto T, 2016. A PCR-based diagnostic assay for detecting DNA of the olive fruit fly, *Bactrocera oleae*, in the gut of soil-living arthropods. Bull Entomol Res 106: 1-5. https://doi.org/10.1017/S000748531600050X

Santos SAP, Cabanas JE, Pereira JA, 2007. Abundance and diversity of soil arthropods in olive grove ecosystem (Portugal): Effect of pitfall trap type. Eur J Soil Biol 43: 77-83. https://doi.org/10.1016/j.ejsobi.2006.10.001

Šerić Jelaska, Symondson WOC, 2016. Predation on epigeic, endogeic and anecic earthworms by carabids active in spring and autumn. Period Biol 118: 281-289. https://doi.org/10.18054/pb.2016.118.3.4709

Šerić Jelaska L, Franjevic D, Jelaska SD, Symondson WOC, 2014. Prey detection in carabid beetles (Coleoptera: Carabidae) in woodland ecosystems by PCR analysis of gut contents. Eur J Entomol 111: 631.

Sheppard SK, Bell J, Sunderland KD, Fenlon J, Skervin D, Symondson WOC, 2005. Detection of secondary predation by PCR analyses of the gut contents of invertebrate generalist predators. Mol Ecol 14: 4461-4468. https://doi.org/10.1111/j.1365-294X.2005.02742.x

Sunderland KD, 1975. The diet of some predatory arthropods in cereal crops. J Appl Ecol 12: 507-515. https://doi.org/10.2307/2402171

Sunderland KD, 2002. Invertebrate pest control by carabids. In: The agroecology of carabid beetles; Holland JM (ed.) pp: 165-214. Intercept, Andover.

Symondson WOC, 2002. Molecular identification of prey in predator diets. Mol Ecol 11: 627-641. https://doi.org/10.1046/j.1365-294X.2002.01471.x

Thiele HU, 1977. Carabid beetles in their environments. A study on habitat selection by adaptation in physiology and behaviour. Springer, Berlin. 369 pp.

Thomas CFG, Green F, Marshall EJP, 1997. Distribution, dispersal and population size of the ground beetles, *Pterostichus melanarius* (Illiger) and *Harpalus rufipes* (Degeer) (Coleoptera, Carabidae), in field margin habitats. Biol Agric Hortic 15: 337-352. https://doi.org/10.1080/01448765.1997.9755208

Vickerman GP, Sunderland KD, 1977. Some effects of dimethoate on arthropods in winter wheat. J Appl Ecol 14: 767-777. https://doi.org/10.2307/2402808

Waldner T, Sint D, Juen A, Traugott M, 2013. The effect of predator identity on post-feeding prey DNA detection success in soil-dwelling macro-invertebrates. Soil Biol Biochem 63: 116-123. https://doi.org/10.1016/j.soilbio.2013.03.030

Wallinger C, Sint D, Baier F, Schmid C, Mayer R, Traugott M, 2015. Detection of seed DNA in regurgitates of granivorous carabid beetles. Bull Entomol Res 105: 728-735. https://doi.org/10.1017/S000748531500067X

Weber DC, Lundgren JG, 2009. Detection of predation using qPCR: effect of prey quantity, elapsed time, chaser diet, and sample preservation on detectable quantity of prey DNA. J Insect Sci 9: 1-12. https://doi.org/10.1673/031.009.4101

Zaidi RH, Jaal Z, Hawkes NJ, Hemingway J, Symondson WOC, 1999. Can multiple- copy sequences of prey DNA be detected amongst the gut contents of invertebrate predators? Mol Ecol 8: 2081-2087. https://doi.org/10.1046/j.1365-294x.1999.00823.x