

# Multiple lines of evidence for reproductive winter diapause in the invasive pest *Drosophila suzukii*: useful clues for control strategies

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**Abstract** Successful management of invasive pests, such as *Drosophila suzukii*, relies on a fine understanding of their biology. Genomic and physiological studies have suggested that the invasive success of *D. suzukii* is strongly associated with its ability to overwinter in a reproductive diapause state. Here, we coupled field surveys with comparative morphology and genetics to increase our understanding of *D. suzukii* overwintering behavior and provide useful indications for its management. The results of a 4-year-long field trapping in an Italian mountain region indicate that *D. suzukii* is continuously captured during winter months and that the number of captures is correlated with temperature. Capture patterns are also contrasting between sexes: while females are more abundantly caught during winter and summer, males are more abundant in spring and autumn. We found that overwintering could occur not only in natural environments, such as woods, but also in anthropic shelters. Comparative morphology and genetics further indicate that spermathecae may play an important

adaptive role during winter. Our results unveil complex winter biology in *D. suzukii* and highlight how the number of overwintering females is an earlier predictor of summer population size. We hence propose that in a given year infestation may be better forecasted by taking into account the captures of the previous winter. We recommend that control methods be diapause-aware. For instance, they should take place in late winter/early spring and close to natural environments, and not only in fruit ripening season and close to orchards.

**Keywords** Spotted wing drosophila · Diapause · Overwintering · Integrated pest management · Cyp4d20 cytochrome

## Key message

- We performed a 4-year-long field trapping of the alien pest of soft fruits *Drosophila suzukii* to enlarge our knowledge of its overwinter behavior.
- *Drosophila suzukii* activity extends throughout winter, when females outnumber males, and is highly affected by temperature.
- Spermathecae show signs of adaptation to overwintering.
- Knowledge of the overwintering reproductive diapause patterns can be useful in optimizing temporal and spatial dislocation of control measures.

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## Introduction

The effective management of an invasive pest relies on the fine understanding of its biology and ecology. This is a complex task normally achieved only by merging pieces of evidence gathered from different approaches, ranging from

field observation to morphological and genetic studies. Knowing the biology of the pest allows predicting its behavior and the way it is (or will be) adapting to the new environment, therefore helping to define appropriate management techniques.

The spotted wing drosophila, *D. suzukii* (Matsumura) (Diptera: Drosophilidae), is a well-established model organism in pest management. This species recently entered Western countries and has been responsible for widespread losses in the soft and thin-skinned fruit industry. A large body of knowledge is quickly accumulating that describes the behavior, physiology, and ecology of this pest, with the ultimate goal of foreseeing its spatio-temporal dynamics and enhancing the control strategies in the field (Cini et al. 2012; Asplen et al. 2015).

### Adaptation to temperate climates

*Drosophila suzukii* is native to Eastern Asia and has filled virtually all temperate regions of America and Europe within the past 8 years (Rota-Stabelli et al. 2013; Cini et al. 2014; Deprà et al. 2014). This suggests that *D. suzukii* was already capable of adapting to temperate climates characterized by alternating warm summers and cold winters. This is in contrast to most other cosmopolitan *Drosophila* species, such as *Drosophila melanogaster* or *Drosophila simulans*, which instead spread from a sub-equatorial ancestral range (Ometto et al. 2013). The short generation time coupled with high reproductive potential of *D. suzukii*, however, results in rapid population growth and considerable crop damage during the time of the year when the temperatures are favorable for *D. suzukii* development (Hamby et al. 2013; Wiman et al. 2014).

Several studies revealed that in Japan, which is a part of its native range, adults of *D. suzukii* are capable of overwintering in a reproductive diapause (Kanzawa 1939; Sasaki and Sato 1995; Mitsui et al. 2010). As observed in other insect species, this process is probably associated with an adjustment of the physiology in response to the adverse environmental conditions (Chapman 1998; Tauber et al. 1986; Denlinger 2002, 2008). In Japan, North America, and Europe, *D. suzukii* is indeed capable of surviving relatively harsh winter conditions by, for example, forming protective adult aggregates (Walsh et al. 2010; Zerulla et al. 2015), exploiting anthropic environments (Kimura 2004; Dalton et al. 2011; Jakobs et al. 2015), and developing into winter morphs (Asplen et al. 2015). In general, *D. suzukii* males are considered less capable than females to successfully undergo diapause and survive winter conditions (Dalton et al. 2011; Wiman et al. 2014), and is consistent with what is observed in other *Drosophila* species, in which overwintering occurs preferentially in females (Salminen and Hoikkala 2013).

Evidences of winter diapauses are clear when comparing patterns of molecular evolution between *D. suzukii* and other *Drosophila* species (Ometto et al. 2013). In particular, the slow evolving genome of *D. suzukii* is compatible with fewer generations per year compared to the closely related *Drosophila biarmipes*, likely due to the overwintering diapause that *D. suzukii* regularly undergoes in its ancestral Asian areal. In addition, the different patterns of molecular substitution in autosomes and the sexual chromosome suggests that the bottleneck associated with the diapause is stronger for males than for females (Ometto et al. 2013). However, many aspects of winter diapause in *D. suzukii* remain unclear, and a more detailed knowledge of this biological aspect promises to help in modeling early season risks and in defining more accurate management practices.

### Enhancing area-wide control strategies

Recent studies have focused on the screening of food attractants and the development of monitoring and control methods that can efficiently intercept *D. suzukii* population (Wu et al. 2007; Landolt et al. 2012; Cha et al. 2012, 2013, 2014; Kleiber et al. 2014; Grassi et al. 2015). It is not yet clear, however, which could be the most efficient period of the year and best spacing for trap deployment against *D. suzukii*. In most of the mass-trapping trials carried out so far, traps have been positioned along the perimeter and/or in the inner part of the orchards at the beginning of the fruit ripening stage, when the competition between food baits and mature fruits was very strong and may have reduced the trapping efficacy. Indeed, trapping with the currently available baits is poorly effective when applied to high *D. suzukii* population densities and only at the perimeter of treated orchards (Grassi et al. 2015). On the other hand, knowing the biology and the timing of *D. suzukii* diapause has the potential to identify the most suitable locations (e.g., close to the overwintering shelters) and periods (e.g., before, during, or after diapause) for trapping.

### Open questions and synopsis

Unresolved issues regarding winter diapause in *D. suzukii* include (1) the genotypic and phenotypic adaptations that allow *D. suzukii* winter diapause, (2) the sex-ratio of overwintering adults, (3) the yearly variations in overwintering capability, (4) the exact location of overwintering sites, and, more practically, (5) the exploitation of diapause to ameliorate population forecasting and management practices. In this study, we have integrated evidences from behavior, morphology, and genetics to increase our understanding of *D. suzukii* overwinter diapause. First, we report the results of a 5-year systematic survey of *D. suzukii* presence in the recently invaded region

of Trentino (Italy). Second, we focus on the long-term sperm-storage spermathecae (Pitnick et al. 1999), by comparing their morphology in *D. suzukii* and two other closely related species, *D. melanogaster* and *D. biarmipes*. Finally, we investigate the possible genetic basis of this adaptation by analyzing the expression of candidate genes for positive selection in *D. suzukii* (Ometto et al. 2013).

## Materials and methods

### Trapping

*Drosophila suzukii* populations were monitored weekly in 11 sites of Trentino (Italy), 6 from 2012 to 2015, 4 from 2013 to 2015, and 1 from 2012 to 2014. The sites, representative of different environments and located at different altitudes (see Table 1), were selected based on the presence or absence of potential winter shelters for overwintering *D. suzukii* individuals. In particular, we partitioned the sites into three categories: (1) those devoid of refuges and housing commercial orchards, where neither leaves nor fruits remain on the plants during the winter period; those offering winter shelters, which could be either, (2) artificial (composting plant and urban areas), or (3) natural (woody areas). In order to minimize captures of individuals hailing from surrounding areas, traps were placed at a distance of at least 15 m from environmental boundaries. Trapping was done using Droso-Traps (Biobest, Westerlo, Belgium) baited with ca. 200 ml of Droskidrink (produced by Azienda Agricola Prantil, Priò, Trento, Italy: 75 % apple cider vinegar and 25 % red wine) + 20 g/l of unrefined brown sugar and a drop of Triton™ X-100 (Sigma-Aldrich, St. Louis, Missouri, USA) to break surface tension (De Ros et al. 2015; Grassi et al. 2015). The contents of

traps were collected each week during the period of the study, and the bait solution was subsequently replaced with fresh one.

### Data analysis

The number of male and female *D. suzukii* from each trap was counted under magnification in the laboratory. Maximum and minimum daily and hourly temperatures were recorded for those sites ( $n = 6$ ) that were within 1 km distance from weather stations (owned by Fondazione Edmund Mach), to evaluate any possible correlation between captures and temperature and, in particular, to evaluate the dynamics of population drops (DPs) during winter. For this purpose, the period December–January was split into eight sample units (SUs) of 1 week each, for which we calculated the absolute maximum ( $T_{\max}$ ) and minimum ( $T_{\min}$ ) temperature, the number of days and hours below 0,  $-2$ , and  $-4$  °C, and the cumulated values and number of consecutive days and hours with  $T_{\min}$  below 0,  $-2$ , and  $-4$  °C. The correlation between each parameter and the DP for each site for each year ( $n = 15$ ) was tested by the Spearman's  $\rho$  (one tailed). The population drop rate (DR%) was calculated as  $(P_0/P_1) * 100$ , where  $P_0$  and  $P_1$  are mean captures before and after the DP, respectively. The SU corresponding with a DP was arbitrarily identified as the first SU in which the number of captures was less than  $(AV - SD)$ , where AV and SD are the average and standard deviation of the captures done during the three previous SUs. We expected a significant correlation between DR% and any parameter that might affect the *D. suzukii* population during winter.

We also tested (Spearman's  $\rho$ ) the impact of winter temperatures on population size by correlating the winter minimum temperatures and the number of *D. suzukii*

**Table 1** List of the sites where the *Drosophila suzukii* trap survey was carried out

Localities <sup>a</sup>	Elevation (m a.s.l.)	Environment <sup>b</sup>	Latitude	Longitude
Trento*	195	Public park <sup>B</sup>	46°3'15.89"N	11°7'25.17"E
Mezzocorona	213	Private garden <sup>B</sup>	46°12'40.52"N	11°07'28.23"E
Roverè*	234	Wood <sup>C</sup>	46°15'12.30"N	11°10'29.08"E
S. Michele a/A	249	Composting plant <sup>B</sup>	46°11'17.61"N	11°08'13.16"E
Pergolese*	250	Cherry orchard <sup>A</sup>	46°1'48.79"N	10°57'35.36"E
S. Michele a/A	272	Vineyard <sup>A</sup>	46°11'34.63"N	11°08'22.90"E
Zivignago	489	Cherry orchard <sup>A</sup>	46°04'15.74"N	11°14'28.75"E
Vattaro**	695	Wood <sup>C</sup>	45°59'24.71"N	11°13'50.31"E
Susà*	680	Cherry orchard <sup>A</sup>	46°03'16.95"N	11°13'30.16"E
Balbido	791	Cherry orchard <sup>A</sup>	46°0'50.23"N	10°48'34.65"E
Samone	800	Wood <sup>C</sup>	46°4'36.53"N	11°32'8.85"E

<sup>a</sup> Single asterisk indicates 2013–2015 monitoring activity, double asterisk indicates 2012–2014 monitoring activity

<sup>b</sup> Habitat typology: (A) orchard, (B) artificial, (C) wood

trapped after the occurrence of the winter DP (between first and eighth week of the year). Finally, we checked for a correlation between the winter and summer population sizes. In this way, we further defined a “population boost” whenever the number of captures increased to more than  $(AV + SD)$ . We considered for the analysis the 8 weeks following the boost of population. To better describe the trend, a best-fit curve was drawn for any correlation analysis.

The trends and temporal dynamics of the population sex-ratio were studied by analyzing the skew index along the years. In particular, for each week we calculated the sex skew index as the difference between female and male captures divided by total number of captures. This value varies between 1 (100 % females) and  $-1$  (100 % males) and, assuming captures are an unbiased sample of the population, can be used as a proxy for the sex-ratio of the population. A change point (CP) analysis was performed to estimate the beginning of a new trend along the skew-index series. This CP corresponded to the point at which the cumulative difference between the average value and each individual value reached the largest absolute value, and was estimated using the cumulative sum statistic method (Pettitt 1979). To test the null hypothesis that there was no CP, the Wilcoxon–Mann–Whitney test (one tailed) was used to compare the two data series, before and from the candidate CP (Siegel and Castellan 1988).

A possible (change of) preference in the overwintering habitats (orchards, artificial, and woods) was evaluated by an analysis of variance (ANOVA) on the number of flies caught in these sites during winter (first 8 weeks of the year).

## Insect rearing

We established *D. suzukii* and *D. melanogaster* colonies from wild-caught adults coming from multiple locations in Trentino (Italy). The *D. biarmipes* population was obtained from the San Diego stock center (line number 14023-0361.02). At the time of morphological analyses, all the colonies had been reared in the laboratory for 4–6 months. Rearing conditions were 21–23 °C and 70 % relative humidity, with a 16:8 light:dark photoperiod. All flies species were constantly provided with a water wick and artificial diet (Dalton et al. 2011) that served as both a food source and an oviposition medium.

## Morphology and volumetric analysis of spermathecae

Laboratory-reared *D. suzukii*, *D. biarmipes*, and *D. melanogaster* adult females were collected within 24 h after the emergence from the pupal stage. For each species, we sampled 30 females and divided them into three different

treatments: (i) 10 females were sampled within 1 day after eclosion and were considered virgins (Revadi et al. 2015); the remaining 20 females were kept with males and collected either, (ii) after 4 days (10 individuals), or (iii) after 10 days (10 individuals). All individuals were sacrificed and preserved in 70 % ethanol until being dissected in phosphate-buffered saline (PBS) solution. Spermathecae (two per individual) were separated from the rest of the female genitalia, left for 5 min in a cotton blue staining solution (0.05 % in water), further washed in PBS ( $2 \times 5$  min), and mounted on a glass slide with glycerin. Observations were made using a Leica LMD7000 microscope (Leica Microsystems GmbH). Basal diameter (excluding the basal cuticular ring) and median height of spermatheca were measured with the Leica application suite image analysis software. To avoid allometric effects resulting from the smaller body size of *D. melanogaster* and *D. biarmipes* compared to *D. suzukii*, spermatheca measures were adjusted by multiplying them by the ratio  $L_{\text{suz}}/L_{\text{dros}}$ , where  $L_{\text{suz}}$  was the average body length of *D. suzukii* females and  $L_{\text{dros}}$  was the average body length of *D. melanogaster* or *D. biarmipes* females (Dekker et al. 2015). We estimated the spermathecae volume by approximating their shape to a cylinder. Such an approximation leads to an underestimation of *D. suzukii* spermathecae, since they are enlarged at the median height, however, this proved to be conservative in our analyses. For each individual, we averaged the volume of its two spermathecae, since our preliminary data showed no intra-individual volumetric differences (data not shown). Volumes were analyzed for homoscedasticity (Levene test) and normality test (Shapiro–Wilk normality test), and their heterogeneity was tested using a two-way full factorial ANOVA, where the first factor was the species and second, the reproductive stage. The post hoc Tukey test was used for multiple comparisons.

## Gene expression analysis

We chose to study the expression of the gene *Cyp4d20*, which is expressed in spermathecae, because it has been shown to be a strong candidate for the action of positive selection in *D. suzukii* (Ometto et al. 2013). For each of the species tested (*D. melanogaster*, *D. biarmipes*, and *D. suzukii*), we collected the spermathecae from fifty 3-day-old mated females. We extracted total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA) and PureLink® RNA mini kit according to the manufacturer’s protocol. RNA samples were treated with amplification grade DNaseI (Invitrogen), in order to minimize genomic DNA contamination. RNA quality and quantity were assessed by standard agarose gel electrophoresis and spectrophotometric analysis using the Nanodrop 8000 (Thermo Fisher

Scientific, Wilmington, DE). First-strand cDNA was synthesized from 1.0 µg of total RNA using Superscript III (Invitrogen) and oligo-dT according to the manufacturer's protocol. Identical reactions omitting the reverse transcriptase were performed as controls for genomic DNA contamination. Three independently collected samples (biological replicates) were prepared and analyzed for each species separately. Quantitative PCR was performed to determine the relative mRNA expression level of *Cyp4d20* in spermathecae tissue of the three *Drosophila* species. Platinum SYBR green qPCR SuperMix-UDG (Invitrogen) was used at halved quantities that resulted in 10-µl reaction mixtures, containing 2 µl of cDNA and 0.2 µl of 10 µM each primer for relative quantification. Three technical replicates were performed for each sample. Primer sequences corresponded to conserved regions of exon–exon boundaries among *D. melanogaster*, *D. biarmipes*, and *D. suzukii* for the reference gene *Actin 5C* and the gene of interest *Cyp4d20*, designed for this study. The *Actin 5C* primer sequences used were Fwd 5'-CTTGCGGCATC-CACGAGACCAC-3' and Rev 5'-GGCGGTGATCTCCTTCTGCATACG-3', while the *Cyp4d20* primer sequences used were Fwd 5' GCAGATGGCTCTGCTGGACATCC-3' and Rev 5'-TGGTGGTGTGTCATCGCCCTCGAAC-3'. Negative controls included template-free qPCR reaction. Reactions were carried out using the Light Cycler 480 (Roche Diagnostics, Germany). The PCR conditions were 50 °C for 5 min and 95 °C for 5 min as initial steps, followed by 40 cycles of 95 °C for 30 s and 60 °C for 45 s. Dissociation curves were analyzed to verify the specificity of each amplification reaction. Relative quantification values were calculated using the  $\Delta\Delta C_t$  method normalized to the reference genes and related to the expression of calibrator (Pfaffl 2001), which in this study was *D. melanogaster*. In particular normalization was set as  $\Delta C_t = C_t(\text{sample}) - C_t(\text{reference})$ .  $\Delta\Delta C_t = \Delta C_t(\text{sample}) - \Delta C_t(\text{calibrator})$  and relative quantification =  $2^{-\Delta\Delta C_t}$ . One-way ANOVA and Tukey's multiple comparison tests were run to test for statistical significance of the observed differences. A *P* value of <0.05 was interpreted as statistically significant. Graphical representation was performed using GraphPad Prism 6.

## Results

### *Drosophila suzukii* is consistently captured during winter, and its presence best correlates with minimum temperatures

Our monitoring revealed consistent flying activity of *D. suzukii* during all 4 years across the 11 localities (Fig. 1a).

The numbers of catches were extremely low during the winters of 2012 and 2013 when compared with 2014 and 2015 (Fig. 1b). During the winters of 2013, 2014, and 2015 a huge drop ( $DR\% = 0.95 \pm 0.07\%$ ) of the *D. suzukii* population was observed at the beginning of the freezing period (49th–51st week of the year; Fig. 1c–e). Despite such drops, flights never ceased during the first 8 weeks of the year (Fig. 1c–e). This pattern is correlated with the low temperatures experienced by *D. suzukii* during this period, as shown by the significant correlation between *DR%* and temperatures (Table 2). More in detail, *DR%* is significantly correlated with both the temperatures recorded during the time frame in which the drop occurred (absolute and median minimum temperatures), and with the cumulated number of days and hours under 0 and  $-2$  °C (Table 2).

Starting from the end of February 2013 and May 2014 and 2015, we observed a period during which captures almost stopped in all monitored sites (Fig. 1c–e). This low-capture period (LCP) ended around the 26th–28th week of the year (Fig. 1c–e), when captures suddenly increased considerably, reaching thousands of individuals per week (Fig. 1a). The number of winter catches was significantly correlated with the average winter minimum temperature (exponential regression model,  $P = 0.002$ ; Fig. 2a). A significant correlation was also found between the total number of *D. suzukii* caught during winter and during the warmer season, i.e., along the 8 weeks following the end of the ensuing LCP (logarithmic relation,  $P < 0.05$ ; Fig. 2b).

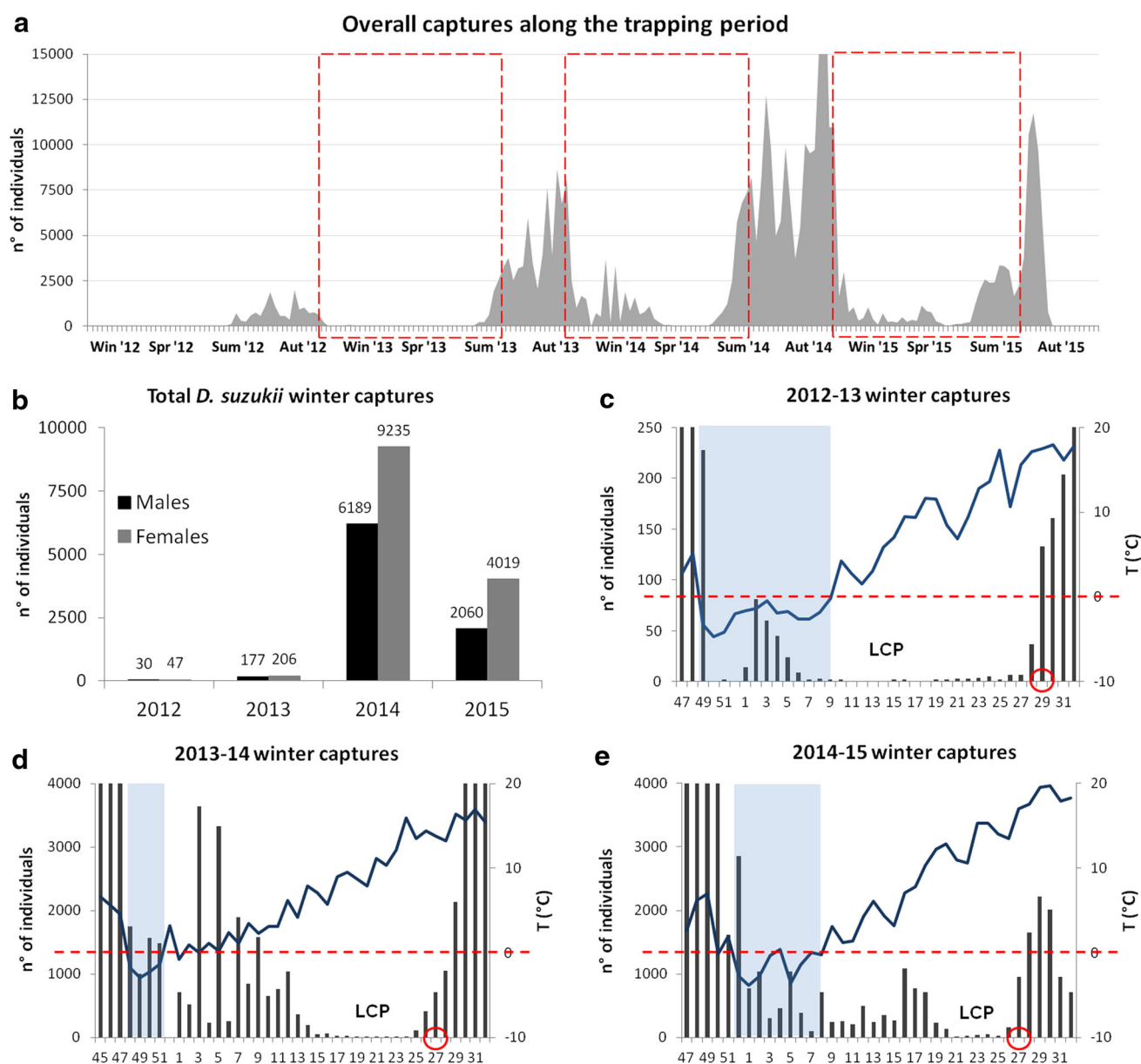
### Sex-ratio capturing pattern: consistently more females than males are captured in winter

The sex-ratio of *D. suzukii* captures varied according to a clear pattern, which we found to be conserved across the 4 years (Fig. 3). During the first (colder) months, traps contained mostly females (red areas in Fig. 3), while during spring males became more abundant (blue areas in Fig. 3). The sex-ratio switched toward more females between June and July. Finally, starting from the 40th week of the year (half of September), the bias returned in favor of males until the end of the year. All trend switches were significant according to Mann–Whitney test after Pettitt test (asterisks in Fig. 3).

### *Drosophila suzukii* overwinters in both woody and anthropic sites

Our winter monitoring indicates the ability of *D. suzukii* to overwinter in both wood and anthropic localities (Fig. 4). The majority of captures were from the woody areas, particularly in 2012 and 2013. In the following year, 2014, woody areas remained the main source of winter captures,





**Fig. 1** **a** *Drosophila suzukii* capture trend during the whole trapping period (winter 2012–autumn 2015). Red dashed-line areas correspond to **c–e**. **b** Total captures of *D. suzukii* males and females during winter in 2012–2015. **c–e** Relation between minimum weekly temperatures (blue lines) and numbers of individual trapped in three consecutive winters (periods span week 47–week 31 of the year). The blue areas

represent the extent of the freezing period, when minimum temperatures drop below zero (red dashed-line) and during which the drop of population occurred. The low-capture period (LCP) is shown and ends with the boost of population (red circles) in late spring. (Color figure online)

but an important fraction of captures came from artificial habitats, a trend that became more evident in 2015. Interestingly, orchards seem to be consistently avoided throughout all years. In fact, both sampling year [ANOVA,  $F_{(1,30)} = 19.47$ ,  $P < 0.001$ ] and environment [ANOVA,  $F_{(2,30)} = 6.76$ ,  $P < 0.01$ ] are strong determinants of the number of captures, suggesting a significant habitat preference in overwintering flies.

### Cytochrome is differentially expressed in *D. suzukii* spermathecae

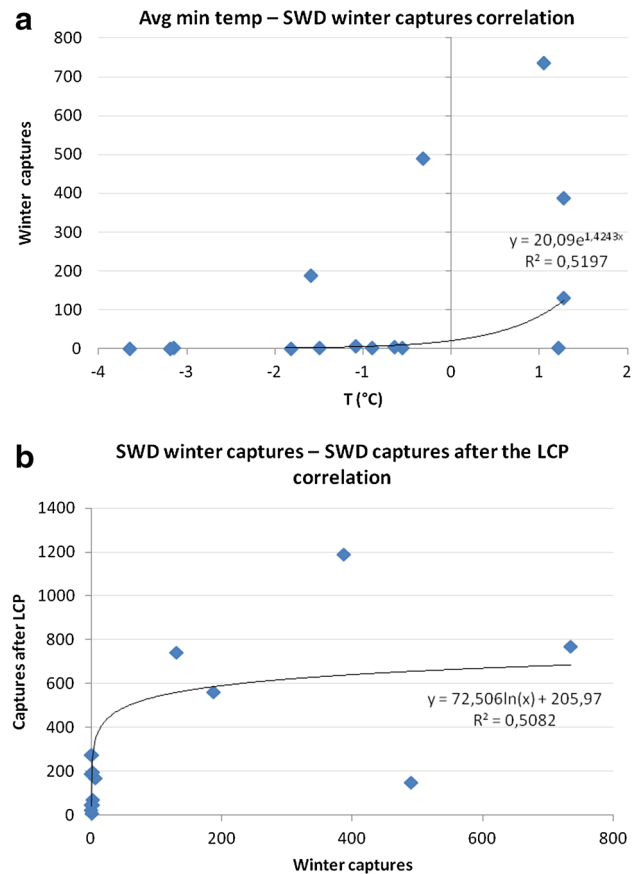
We previously identified several genes as putative target of positive selection in *D. suzukii* (Ometto et al. 2013). The strongest candidate was *Cyp4d20*, a cytochrome P450 that is expressed in various tissues but more specifically in adult spermathecae (Malta et al. 2014). Because of its possible

**Table 2** Analysis of correlation (Spearman's  $\rho$ ) between drop rate (DR%) of six localities ( $n = 15$ ) and each of the following meteorological parameters: minimum ( $T_{\min}$ ) and maximum ( $T_{\max}$ ) temperature (absolute and median), cumulative degrees ( $^{\circ}\text{C}$ ), consecutive days and number of days and hours under three reference temperatures (0,  $-2$ ,  $-4$   $^{\circ}\text{C}$ )

Parameters	Spearman's $\rho$	$P$
$T_{\min}$ absolute		
Med	-0.53	ns
Min	-0.72	<0.05
Max	-0.2	ns
$T_{\min}$ median		
Med	-0.62	ns
Min	-0.69	<0.05
Max	-0.18	ns
$T_{\max}$ absolute		
Med	-0.39	ns
Min	-0.56	<0.05
Max	-0.01	ns
$T_{\max}$ median		
Med	-0.35	ns
Min	-0.65	<0.05
Max	-0.16	ns
Cumulative		
<0	0.79	<0.01
<-2	0.77	<0.01
<-4	0.73	<0.05
Consecutive		
<0	0.37	ns
<-2	0.4	ns
<-4	0.65	<0.05
Number of days		
<0	0.77	<0.01
<-2	0.78	<0.01
<-4	0.63	ns
Number of hours		
<0	0.72	<0.05
<-2	0.61	<0.05
<-4	0.51	ns

For each parameter analyzed, absolute values refer to the period 1st December–31st January in 2 (2014–2015) or 3 years (2013–2015) according to the site (see Table 1), median values are calculated from the average of the eight sampling units (SUs, 7 days preceding the trap sampling)

role in overwintering, we performed quantitative PCR of this gene on the spermathecae of *D. suzukii* and two of its sister species. Results (Fig. 5a) showed that *Cyp4d20* has a fivefold higher expression in *D. suzukii* than in *D. biarmipes* and *D. melanogaster* [ANOVA,  $F_{2,24} = 19.59$ ,  $P < 0.0001$ ], suggesting its involvement in the sperm-



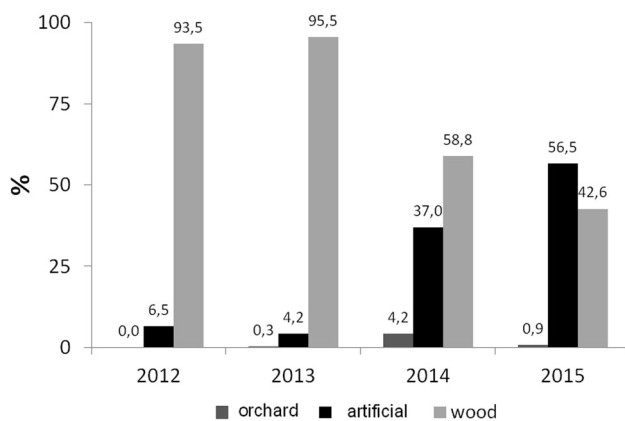
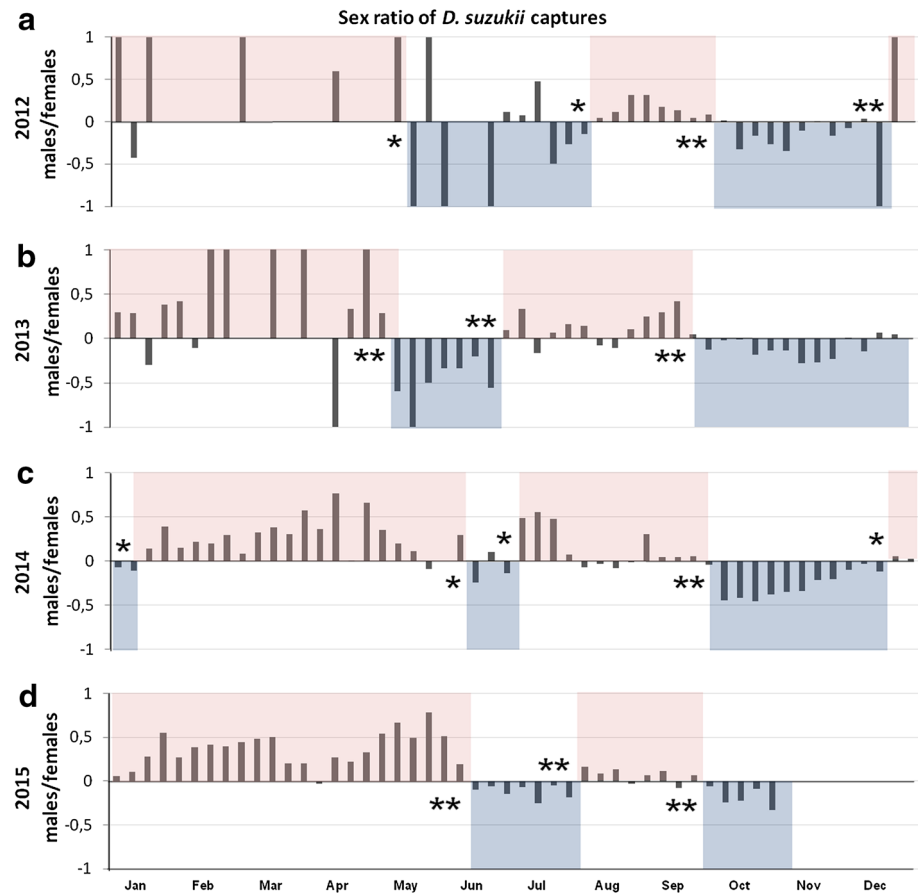
**Fig. 2** **a** Correlation between average minimum temperature and *Drosophila suzukii* captures in winter (first 8 weeks of the year). **b** Correlation between *Drosophila suzukii* winter captures and *D. suzukii* captures over the first 8 weeks after the end of the low-capture period (LCP). Captures were recorded from six sites that were within 1 km from FEM weather stations for either 2 or 3 years ( $n = 15$ ). Formula and  $R^2$  of the best-fit curves are shown.  $P < 0.05$  after Spearman  $\rho$  correlation analysis

storage process and ultimately in the ability of *D. suzukii* mated females to overwinter.

***Drosophila suzukii* spermathecae are larger, darker, ss and more enlargeable than in *D. melanogaster* and *D. biarmipes***

We further analyzed the morphology of spermathecae in *D. melanogaster*, *D. biarmipes*, and *D. suzukii*. In all three species, we observed a pair of spermathecae formed by a sperm duct and a chitinous capsule (Fig. 5b). The capsule has a typical bell shape, which is more elongated in *D. suzukii* and *D. biarmipes*, and shorter and apically flattened in *D. melanogaster*. In *D. suzukii*, the capsule is enlarged at the median height. Internally, a structure deriving from two consecutive invaginations of the sperm duct, possibly associated to the sperm pumping system, is visible in all three species. In *D. suzukii* a cuticular collar is present at

**Fig. 3** Sex-ratio of *Drosophila suzukii* captures, expressed in term of male–female skew, during the 4 years of trapping showing the prevalence of males (blue areas) or females (red areas). Statistical significance of the changing points was tested by Mann–Whitney test after Pettitt method: \* $P < 0.05$ , \*\* $P < 0.01$ . (Color figure online)



**Fig. 4** Percentage of *Drosophila suzukii* winter captures (2012–2014) grouped by environment typology: orchards, artificial areas, and woods

the base of the capsule; such a feature occurs with minor degree in *D. melanogaster* and is almost absent in *D. biarmipes*. Our analyses show that both species and reproductive stage are strong determinants of the spermathecae size [ANOVA,  $F_{(2,81)} = 92.8$ ,  $P < 0.001$ , and  $F_{(2,81)} = 5.7$ ,  $P < 0.01$ , respectively], as is their interaction [ANOVA,  $F_{(4,81)} = 3.06$ ,  $P = 0.02$ ]. In particular,

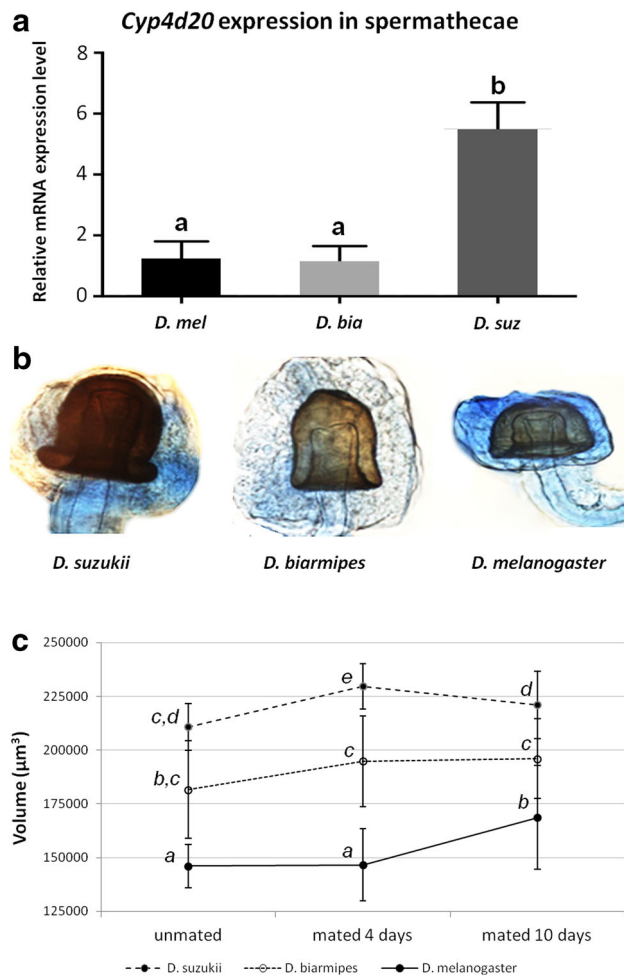
spermathecae are larger in size, darker in pigment, and enlarge more significantly upon mating in *D. suzukii* than in *D. melanogaster* and *D. biarmipes*. This also means that *D. melanogaster* spermathecae take longer to increase in size than those of the other two species which instead showed a similar temporal trend (Fig. 5c).

## Discussion

### Moving forward our understanding of the *D. suzukii* overwintering dynamics

While cold temperature is a limiting factor in the geographic range of several *Drosophila* spp. (Kimura 1988), it has been demonstrated that some of them are fairly cold-tolerant (Nyamukondiwa et al. 2011), being able to accumulate antifreeze substances, reduce the super cooling point and water content, regulate metabolic enzyme activity, or remove ice nucleation agents (Zachariassen 1985). Conversely, *D. suzukii* is considered to be a chill-intolerant species not able to overwinter in extremely cold climates (Kimura 2004; Dalton et al. 2011; Jakobs et al. 2015; Stephens et al. 2015). However, our data revealed





**Fig. 5** **a** Quantitative PCR analysis of *Cyp4d20* expression in spermathecae. Bars are shown as mean  $\pm$  standard error. Within each sub-figure, letters indicate statistical significance ( $P < 0.001$ ) after Tukey's multiple comparison test. **b** Comparative micrographs of the spermathecae of *Drosophila suzukii*, *D. biarmipes* and *D. melanogaster* virgin females. **c** Volume of spermathecae in three different reproductive stages: virgin females ( $n = 20$ ), females reared with males for 4 days ( $n = 20$ ), females reared with males for 10 days ( $n = 20$ ). Different letters indicate a statistically significant difference after two-way ANOVA followed by Tukey's test among fly species and mating status

continuous flight of *D. suzukii* adults throughout the winter even when the average minimum temperature is below  $0^\circ\text{C}$  (Fig. 1c–e), although in some sites we observed drops equal to 100 %. This not only suggests that some places are less suitable for overwintering, but also indicates that individuals can quickly get active, likely for feeding purposes whenever the climatic conditions are favorable. Most of the overwintering flies may in fact correspond to winter morphs (Asplen et al. 2015; Grassi personal communication), a larger and darker phenotype associated with the climatic conditions typical of cold environments and that has also been described in other *Drosophila* species

(Ayrinhac et al. 2004; Gibert et al. 2007). Winter morphs are more cold-tolerant than summer morphs (Zerulla et al. 2015; Stephens et al. 2015) and can enter into a state of partial quiescence to avoid freezing during the coldest hours of the day, but are otherwise ready to fly when it gets warmer.

Previous studies based on season-long monitoring of *D. suzukii*, conducted in raspberry plantings (Hamby et al. 2014) and mixed orchards (Harris et al. 2014), pointed to a sharp decline in the number of captures between October and November. Our data are partially consistent with this observation (Fig. 1a), indicating a drop of captures at the end of December (48th–49th week). The winter drop is associated to the beginning of the freezing period and is consistent with the relatively low cold tolerance observed in *D. suzukii* (Dalton et al. 2011; Zerulla et al. 2015; Jakobs et al. 2015; Stephens et al. 2015). Indeed, our analyses indicate that repeated exposure to temperatures below  $0^\circ\text{C}$  (even in non-consecutive days) is strongly correlated to the winter drop.

Our analyses further revealed the presence of an interesting LCP toward the end of spring (up to 26th week) followed by an increase in captures. One possible explanation for this pattern is the death of most of the overwintering adults (likely winter forms) before the development of the new generation (summer forms) in spring oviposition hosts (Briem et al. 2016). Alternative explanations would assume either a period during which flies remain inactive, or a sudden switch to alternative nutrient sources, such as pollen and nectar that would outcompete the attraction of the trap's bait. In partial support of the latter, we observed that the advance of the LCP in 2014 compared with 2015 (Fig. 1d, e) was accompanied by warmer temperatures during that season, which caused the early flowering and fruiting of several plant species (Italian Phenological Network, <http://iphen.entecra.it/cma/iphen/>).

A further interesting outcome of our results is that winter temperature does not only affect winter catches, but indirectly also the number of catches (i.e., the population size) in summer (Fig. 2). In particular, the exponential regression describing the correlation between winter minimum temperatures and number of winter catches suggests the presence of a temperature threshold (at around  $-0.5^\circ\text{C}$ ) below which captures are extremely rare (Fig. 2a). The correlation between the number of winter and summer catches is instead described by a logarithmic regression (Fig. 2b) which indicates that even if small-size winter populations will produce large summer populations, they will do it at a much lower magnitude than large-size winter populations. In other words, harsh winters can efficiently reduce the number of overwintering individuals,

which in turn will have a consequence for the population dynamics of the following generations.

Probably, the most surprising of our results is a sex-ratio variation in captures during the year. During winter, females are consistently more abundant compared to males, but this bias is repeatedly switched during the year (Fig. 3). Although we cannot exclude that our bait (Droskidrink, Grassi et al. 2015) is more attractive to females than to males in certain periods of the year, our data suggest that females are more cold-tolerant than males. This is in agreement with previous studies (Dalton et al. 2011; Zeru-ulla et al. 2015; Stephens et al. 2015) and with the genetic evidence of a reduced male effective population size (Ometto et al. 2013).

Our trapping data also indicate that there could be more than one preferential winter refuge environment for *D. suzukii*. While in winters of the first years (2012–2013) *D. suzukii* was mostly trapped in or nearby woody areas, in the following years captures were as abundant in wood as in anthropic sites (Fig. 3). As already suggested by other authors (Kanzawa 1939; Kimura 2004; Pelton et al. 2016), these environments can offer shelters (tree bark, leaves, artificial refuges) and heat sources (fermenting material and compost, home heating) to overwintering *D. suzukii* when natural climatic conditions would have otherwise decimated its population.

### Phenotypic and genotypic adaptations in spermathecae and their putative role in overwintering diapause

Our comparative analysis indicates that in *D. suzukii* spermathecae are larger and more expandable, and can therefore store more sperm, than in *D. melanogaster* and *D. biarmipes* (Fig. 5c). Furthermore, spermathecae of *D. suzukii* are clearly more melanised and/or sclerotized (Fig. 5b). We hypothesize that these are adaptations aimed at increasing protection of stored sperm from mutagenic ultraviolet-A radiation effects (Kvam and Dahle 2003) or to protect it from parasites and dehydration during the long-term storage (Sugumaran 2002; True 2003 and references therein). We also identified a putative genetic basis of long-term sperm-storage adaptation in the strong up-regulation of one of the cytochrome P450 (CYP) genes, *Cyp4d20* (Fig. 5a). In insects, CYP genes code for proteins with diverse functions, including detoxification of xenobiotics, metabolism and development of insects (Wilson 2001; Scott and Wen 2001; Li et al. 2007). After mating, female reproductive tract is filled with seminal fluid and sperm, both of which are potential allogeneic antigens. Therefore, the up-regulation of CYP genes in *D. suzukii* may be a mechanism to neutralize the possible toxic substances associated with sperms, thus permitting a long-term

storage of viable spermatozoa in spermathecae (Malta et al. 2014). This hypothesis is in agreement with what is observed in *Anopheles gambiae* (Shaw et al. 2014), where many genes, including metabolism and detoxifying cytochrome P450 enzymes, are up-regulated in spermathecae extracted from mated females compared to virgins.

Taken together, these results suggest that *D. suzukii* females may have adapted to the winter diapause and the concomitant paucity of males by maximizing the amount of sperm obtained from them as they exit diapause (compare with our field trapping of Fig. 1), and/or by storing sperm obtained from pre-winter matings in a specialized spermatheca, a quite common strategy among those insects that overwinter as adults (Hodek and Iperiti 1983; Neubaum and Wolfner 1999). Such behaviors would not be required in other *Drosophila*, such as *D. melanogaster*, which are adapted to conditions of relative constant temperature, where males should be similarly abundant all year long.

### Implications for *D. suzukii* management

Our results indicate that the number of winter catches correlate with the temperatures experienced during that particular winter. Even more importantly, these temperatures also correlate with the number of catches in the ensuing warmer season (Fig. 2b). This can be a precious indication for forecasting the population status of *D. suzukii* during late spring and summer, when the pest is causing direct damages to crops. In fact, the only currently available *D. suzukii* population dynamic model does not take into consideration survival and reproductive status during winter (Wiman et al. 2014). The present results suggest that information on the life history of the pest and the population structure after key bottleneck periods, such as winter diapause, is instead crucial to better predict serious outbreaks and increase the effectiveness of IPM methods. For example, a low number of individuals exiting the winter diapause will result in a delay of the population outbreak during the growing season, allowing reduced damage of the early crops (Wiman et al. 2014).

Based on our findings, we further advocate that population control methods based on behavior manipulation and applicable at a wide territorial scale, such as mass-trapping, attract-and-kill and push-and-pull, should be maximized close to winter shelter areas (reservoir during diapause) as well as in wild environments flanking orchards susceptible to *D. suzukii* attacks. In addition, trapping control methods carried out before the start of the flowering and fruiting season have the potential to be extremely effective because of the lack of competition between natural sources and bait traps. However, such strategies are likely more efficient in fruit growing areas characterized by a high fragmentation of the cultivated plots, as those present in Europe, where

small/medium-sized soft fruit orchards are often surrounded by crops not suitable to *D. suzukii* or interrupted by natural barriers (woods, hedges, and mountains). On the other hand, in areas characterized by large extensions of *D. suzukii* host plants, such as American agricultural areas, the implementation of an extensive off-season mass-trapping would be ineffective and economically unsustainable.

Our results also provide indication for the best periods of the year to launch insects for two types of potential *D. suzukii* biocontrol strategies: the sterile insect technique (SIT; Knipling 1959) and the Wolbachia-based incompatible insect technique (IIT; Laven 1967; Zabalou et al. 2004; Cattel et al. 2016). According to our observations, both techniques should be planned for late winter and early spring when there is paucity of males in the field (Fig. 3) and the consequent high ratio of released-males–resident-males would reduce the competition from resident males and increase the chances of effectively reducing the population. For the same reason, during late summer and autumn periods, when the ratio of resident males is high, SIT and IIT should be avoided.

In conclusion, our analysis provides a baseline to clarify some aspects of the peculiar *D. suzukii* reproductive diapause. We advocate that in the future this knowledge should be applied to demographical models, in order to better forecast *D. suzukii* growth rates in early season and improve IPM techniques.

### Author contribution statement

GA, OR-S, and VR-S conceived and designed research. AGr, AGo, GA, RK, LO, OR-S, VR-S, and VM conducted experiments and analyzed data. All authors contributed with the discussion of the results. GA, OR-S, VR-S, VM, LO, and RK wrote the paper.

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