For: Dr. Michael Traugott
Editor-in-Chief
Journal of Pest Science

Subject: manuscript submission - M. Valerio Rossi-Stacconi, Kaur Rupinder, Valerio Mazzoni, Lino Ometto, Alberto Grassi, Angela Gottardello, Omar Rota-Stabelli, Gianfranco Anfora - Multiple lines of evidence for reproductive winter diapause in the invasive pest *Drosophila suzuki*: useful clues for control strategies

Dear Dr.Traugott,

Please find attached our manuscript tackling the overwintering diapause in Drosophila suzukii.
D. suzukii is an invasive pest in all important affected production regions in Europe as well as North and South America. This pest has a wide host range and attacks several economically important commercial crops. Currently, *D. suzukii*'s management is not effective or sustainable, and results in a significant increase in production costs of affected crops. Overwintering diapause is a crucial aspect of this pest biology, partly responsible for its success in western countries; this behaviour should be well understood in order to successfully define control strategies.
Our author list include scientists from a broad expertise ranging from entomology, genetics, statistic, and whole-system IPM. This is indeed reflected by the multidisciplinary of our approach: we have coupled classical filed observation with comparative morphological and transcriptomic analyses of a key phenotypic trait involved with diapause, the spermatechae. Our results provide a more complete picture of the biology and seasonal field phenology of the pest in temperate climates. We ultimately propose that the knowledge of diapause that we have generates should be used to optimize IPM strategies such as mass-trapping and male release as well as population monitoring.
We believe that our work is not only of interest for biologist working on *D. suzukii* (interested on the biology behind diapause), but also those dealing directly with IPM and the control of the pets.
We kindly suggest possible reviewers who are recognized as leading scientists in the field of insect biocontrol:

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Looking forward to hearing from you, we send our best regards.

Sincerely,
Valerio Rossi, Omar Rota-Stabelli and Gianfranco Anfora

Journal of Pest Science

Special Issue: *Drosophila suzukii*

Research Article

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 to 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 four-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. In addition, during winter females were consistently trapped at higher numbers than males. We also found that overwintering not only occurs in anthropic shelters but also in natural environments such as woods. Comparative morphological and genetic studies indicate that *D. suzukii* spermathecae are larger in size, more pigmented, extend more after mating, and overexpress the spermathecae-related Cyp4d20 cytochrome: this suggests that females are able to collect and protect for UV more sperm than closely related species, a possible adaptation to the dormant reproductive state, when males are less likely to survive. We hence propose that early season population size can be better forecasted by taking into account the captures of the previous winter. We also recommend that control methods should be diapause-aware, therefore done in late winter/early spring and close to natural environments, and not only in fruit ripening season and close to orchards.

Keywords: diapause, overwintering, spotted wing drosophila, Integrated Pest Management, Cyp4d20 cytochrome

Key message:

- We studied 4 years long field trapping of the alien pest of soft fruits *D. suzukii*
- We enlarge our knowledge of its overwinter behavior
- *D. suzukii* is active through the winter, females outnumber males during winter, and temperature plays a key role
- Spermathecae show signs of morphological and transcriptional adaptation to overwintering
- We advocate that overwintering reproductive diapause should be used to define timing and spacing of control systems

Author Contribution Statement:

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

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 it will be) adapting to the new environment, therefore helping in the definition of appropriate management techniques.

Drosophila 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, the physiology and the ecology of this pest, with the ultimate goal of foreseeing its spatio-temporal dynamics and enhance its control in the field (Cini et al. 2012; Asplen et al. 2015).

Adaptation to temperate climates

D. suzukii is native of Eastern Asia and has filled virtually all temperate regions of Americas and Europe in just under 8 years (Rota Stabelli et al. 2013; Cini et al. 2014; Deprà et al. 2014). This suggests that *D. suzukii* was already well adapted to temperate climates characterized by alternations of warm summers and cold winters, 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* causes rapid population growth and considerable crop damage during the part of the year when the temperatures are favorable for *D. suzukii* development (Hamby et al. 2013; Wiman et al. 2015).

Several studies revealed that in Japan, which is 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), whilst altering their physiological processes in response to periods of 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, for example by forming protective adults aggregates (Walsh et al. 2010; Zerulla et al. 2015), probably 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), consistent with the observation that in insects overwintering occurs preferentially in females (Salminen and Hoikkala 2013).

The signature of repeated diapauses is also evident 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 pattern of molecular substitution in autosomes and in the sexual chromosome suggests that the bottleneck associated to 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 the definition of more accurate management practices.

Enhancing area-wide control strategies

The prevalent methods to control *D. suzukii* populations in the field rely on the use of pesticides, mainly adulticides (Cini et al. 2012). Yet, pesticide treatments have serious drawbacks, since the rapid generation turnover typical of *D. suzukii* requires frequent chemical interventions at the fruit ripening stage close to harvest, hence increasing the risk of residues in fresh market fruits. Therefore, more effective and sustainable solutions are urgently required.

Population controls by means of behavioral manipulation techniques with *D. suzukii* attractants, such as mass-trapping, attract-and-kill and push-and-pull, are potentially valid and efficient options also applicable at a wide territorial scale. In this regard, 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 deployed 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 only slightly effective when applied to high *D. suzukii* population densities and only at the perimeter of treated orchard (Grassi et al. 2015).

Clearly, knowing the correct timing and the optimal spatial distribution of control treatment is a key issue that deserves thorough exploration. In this respect, the occurrence of diapause may recommend the use of trapping in specific locations (e.g. close to the overwintering shelters) and periods of the year (e.g. immediately before, or after emergence from, the diapause).

Open questions and Synopsis

Unresolved issues regarding winter diapause in *D. suzukii* include 1) the genotypic and phenotypic adaptations that allow *D. suzukii* reproductive diapause; 2) the sex proportion of overwintering adults; 3) the yearly variations in overwintering capability, 4) the exact location of overwintering sites; and, on a more practical ground, 5) the exploitation of diapause to ameliorate population forecasting and management practices. In this article, we provide new pieces of evidence in support of winter reproductive diapause in *D. suzukii* and clarify some of its dynamics. We advocate that Integrate Pest Management (IPM) strategies, including mass-trapping, should take into account the population winter bottleneck in order to target the few-mostly female, individuals exiting this phase. This has the potential to considerably reduce the population size in spring and delay the exponential population growth during the rest of the year.

Materials and Methods

Trapping

D. suzukii populations were monitored weekly in eleven sites of Trentino (Italy), six of which from 2012 to 2015 four from 2013 to 2015, and one from 2012 to 2014. The sites, representative of different environments and located at different altitudes (see Tab. 1), were selected based on the presence or absence of potential winter shelters to overwintering *D. suzukii* individuals. In particular, we partitioned the sites in three categories: 1) those devoid of refuges and housing commercial orchards, where neither leaves nor fruits remain on the plants during the winter period; sites offering winter shelters were further divided in two categories, depending on whether they were 2) artificial (composting plant and urban areas) or 3) natural (woody areas). 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/liter 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 sample 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 from weather stations (owned by Fondazione Edmund Mach), to evaluate any possible correlation between captures and temperature trends and, in particular, in association to significant drops of population (DP) during winter. To this purpose the period December–January was split in 8 sample units (SU) of 1 week each and for which we calculated the absolute maximum (Tmax) and minimum (Tmin) temperature, the number of days and hours below 0 °C, -2 °C and -4 °C, and the cumulated values and number of consecutive days and hours with Tmin below 0 °C, -2 °C and -4 °C. The correlation between each parameter and the drop of population 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 (P0/P1)*100 where P0 and P1 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 SU's. 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* trapped after the DP had occurred during winter months (between week 1 and 8). Finally, to assess whether there is any correlation between the winter and summer population sizes we also analyzed these two parameters. In this way we defined a boost of population as the SU in which the number of captures was more than (AV+SD) as previously defined. We considered for the analysis the 8 weeks following it. 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 females and males captures divided by total number of captures. This value varies between 1 (100% females) and -1 (100% males) and can be used as a proxy for the sex-ratio. A change point analysis was performed to estimate the beginning of a new trend along the skew-index series. This change point (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 (Cusum) 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) of 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* rearing from wild-caught adults collected from multiple locations in Trentino (Italy), during the summer of 2011-2014. The *D. biarmipes* population derived from the San Diego stock center (LINE Number 14023-0361.02). Rearing conditions were 21-23 °C and 70% relative humidity, with a 16:8 Light:Dark photoperiod. Flies were 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 hours after the emergence from the pupal stage. For each species, we sampled 30 females and divided them into three different treatments: i) 10 virgin females were sampled one day after eclosion (virgin); 20 females were kept in rearing vials 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 ethanol 70% 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 minutes in a Cotton Blue staining solution (0.05% in water), further washed in PBS (2x5min), and mounted on a glass slide with glycerin. Observations were made on 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_{suz}/L_{dros}, where L_{suz} was the average body length of *D. suzukii* females and L_{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 we preliminary found no volumetric intra-individual differences (data not shown). Volumes were analyzed for homoschedasticity (Levene test) and normality (Shapiro-Wilk normality text), and their heterogeneity tested using a two-way full factorial ANOVA where the first factor was the species and the second the reproductive stage. The post-hoc Tukey test was used for multiple comparisons.

Gene expression analysis

For each of the species tested, (*D. melanogaster, D. biarmipes* and *D. suzukii*) a total of 50 3 days-old mated females were dissected to collect spermathecae. Total RNA was extracted 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 (-RT) 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 (GOI) *Cyp4d20,* designed for this study. The *Actin 5C* primer sequences used were Fwd5'-CTTGCGGCATCCACGAGACCAC-3' and Rev 5'-GGCGGTGATCTCCTTCTGCATACG-3', while the *Cyp4d20* primer sequences used were Fwd5' GCAGATGGCTCTGCTGGACATCC-3' and Rev 5’-TGGTGGTGTCATCGCCCTCGAAC-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 30s and 60°C for 45s. Dissociation curves were analysed to verify the specificity of each amplification reaction. Light Cycler 480 SV1.5.0 software (Roche) was used to extract Ct values and LinReg software was used to calculate reaction efficiencies (Ruijter et al. 2009). Relative quantification values were calculated using the ΔΔCt 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 ΔCt = Ct (sample) – Ct (reference). ΔΔCt = ΔCt (sample) - ΔCt (calibrator) and Relative quantification = $2^{-\Delta\Delta Ct}$. One-way ANOVA and Tukey’s multiple comparison test was 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 GraphPadPrism 6.

Results

D. suzukii is consistently captured during winter months, and its presence best correlates with minimum temperatures

Our monitoring revealed consistent flying activity of *D. suzukii* during all four years across the 11 localities (Fig. 1a). The numbers of catches were extremely low during the winters 2012 and 2013 if compared with 2014 and 2015 (Fig. 1b). During the winters 2013, 2014 and 2015 a huge drop (DR% = 0.95±0.07 %) of the *D. suzukii* population was observed in correspondence of the beginning of the freezing period (49^{th}-51^{th} week of the year; Fig. 1c-e). Despite such drops, however, the flights never really ceased during the first eight 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 the minimum temperatures recorded during the drop (Table 2). In particular, DR% was significantly affected by the absolute and median minimum temperatures (per week, or sample unit SU) recorded during the time frame in which the drop occurred ($P < 0.05$ in both cases), but also by the cumulated values and number of days and hours under 0°C, -2°C ($P < 0.01$ and $P < 0.05$ for days and hours respectively). On the contrary, maximum temperatures correlated only for their minimum values and the number of consecutive cold days under -4°C ($P < 0.05$ in all cases).

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 greatly (boost of population), reaching thousands of individuals per week in a short time (Fig 1a). The number of winter catches was significantly correlated with the average winter minimum temperature following an exponential regression model (P = 0.002; Figure 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 LCP (logarithmic relation, $P < 0.05$; Fig. 2b).

Consistently more females than males in winter trapping

In all monitored sites, the sex ratio of *D. suzukii* population varied according to a clear pattern conserved across years (Fig. 3). For instance, during the first (colder) months traps contained mostly females, while in spring males were more abundant, and this bias lasted one to three months, depending on the year. During summer a second trend reversal occurred and 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).

D. suzukii overwinter in both wooden and anthropic sites

Our winter monitoring indicates the ability of *D. suzukii* for overwintering in (or close to) both wood and anthropic localities (Fig. 4). The bulk of captures was consistently obtained from the woody areas, particularly in 2012 and 2013. In the following year, 2014, wooden areas remained the main source of winter captures, but an important fraction of captured flies came from artificial habitats, a trend that became much 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 for the number of captures.

Larger, darker, more enlargeable spermatechae in D. suzukii

In all three species, *D. melanogaster*, *D. biarmipes* and *D. suzukii*, we observed a pair of spermathecae formed by a sperm duct and a chitinous capsule (Fig. 5a). The capsule has a typical bell shape, more elongated in *D. suzukii* (where it is also enlarged at the median height) 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 the base of the capsule; such a feature occurs with minor degree in *D. melanogaster*, and it is almost absent in *D. biarmipes*. Our analyses based on 20 replicates per species show that 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 *D. melanogaster* and *D. biarmipes* (Fig. 5b).

A differentially expressed cytochrome in D. suzukii spermathecae

We previously identified several genes as putative target of positive selection in *D. suzukii* (Ometto et al. 2013). Interestingly, one of the most promising candidates was *Cyp4d20*, a Cytochrome P450 that is expressed in various tissues but more specifically in adult spermathecae (Malata et al. 2014). Quantitative PCR (Fig. 6) showed that *Cyp4d20* has a five-fold higher expression in mated *D. suzukii* female spermathecae than in *D. biarmipes* and *D. melanogaster* (Anova, $F_{2,24} = 19.59$, $P < 0.0001$), suggesting its involvement in the sperm-storage process and ultimately in the ability of *D. suzukii* mated females to overwinter.

Discussion:

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

Our four-year long field trapping trial confirms the overwintering reproductive diapause of *D. suzukii* and sheds new lights into its dynamics.

It has already been demonstrated that some *Drosophila* species possess more pronounced cold tolerance than others (Nyamukondiwa et al. 2011), for instance by activating freeze-tolerance strategies such as accumulation of antifreeze substances, reduction of the super cooling point and water content, regulation of metabolic enzyme activity or removal of ice nucleation agents (Zachariassen 1985). Cold temperature is a limiting factor in the geographic range of several *Drosophila* spp. (Kimura 1988), and *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 continuous flight of *D. suzukii* adults throughout the winter even when the average minimum temperature was below 0 °C (Fig. 1c-e), (although in some sites we observed drops equal to 100%); this not only indicates that some places are less suitable to host overwintering, but also indicates that individuals can quickly get active, likely for feeding purposes whenever the climatic conditions are favorable. Most of these overwintering flies may in fact correspond to winter morphs (Asplen et al. 2015; A. 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 in a state of partial quiescence to avoid freezing during the coldest hours of the day, but remain ready to fly.

The number of captures between January and July follows a specific pattern, with a sudden drop of the capture rate at the end of December (week 48 - 49) and an increase of captures at the end of June (week 26 - 28) (Fig. 1c-e). The drop is associated to the beginning of the freezing period and it is consistent with the relatively low cold tolerance observed in *D. suzukii* (Dalton et al. 2011; Zerulla et al. 2015; Jackobs et al. 2015; Stephen et al. 2015). Supporting this hypothesis, our analyses showed that repeated exposure to temperatures below 0 °C (even in non consecutive days) is strongly correlated to the severity of the drop in the number of captures. The ensuing low capture period (LCP) could then be due to the death of most of the overwintering adults before the development of the new generation of the year. An alternative explanation for this pattern would assume a period during which flies remained inactive or moved first to more suitable winter habitats and then to alternate nutrient sources, such as pollen and nectar from the early flowering plants, that outcompete the attraction of the trap's bait. For instance, we recorded warmer temperatures during season 2014 than 2015, which caused the anticipation of flowering and fruiting of several plant species (IPHEN - Italian Phenological Network, http://iphen.entecra.it/cma/iphen/) and a corresponding anticipation of the beginning of the LCP (Fig. 1d-e). The early start of the plant phenological phases is also consistent with the later end of the LCP in 2013 (28th week of the year) compared with 2014 and 2015 (26th week of the year). We also found clear indication that winter temperature does not only affect winter catches, but indirectly also influences 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 °C) below which captures are extremely rare (Fig 2a). How the number of winter catches is related to that of the summer catches is instead described by a logarithmic relation (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 the following generations. Another interesting outcome is that during winter active females are consistently more abundant compared to males (Fig. 3). Although we cannot exclude that our bait (Droskidrink, Grassi et al. 2015) is more attractive to females than to males in certain period 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; Zerulla et al. 2015; Stephen et al. 2015) and with the genetic evidence of a reduced male effective population size (Ometto et al. 2013).

Our trapping finally indicates that there is more than one preferential winter refuge environment for *D. suzukii*. While in winters of the first years (2012 - 2013) *D. suzukii* could be trapped almost only in or nearby wooden areas, in the following years captures were as abundant in wood as in anthropic sites (Fig. 3). The massive presence of overwintering adults in both site typologies agrees with the limited adult plasticity of cold tolerance observed by Jakobs et al. (2015). As already suggested by other authors (Kanzawa 1939; Kimura 2004), these environments can offer shelters (tree bark, leaves, artificial refuges) and heat sources (fermenting material and compost, home heating) to overwintering *D. suzukii*.

Phenotypic and genotypic adaptions in spermathecae and their putative role in overwintering diapause

Our comparative analysis indicates that spermathecae are larger and more expandable in *D. suzukii* than in other species, suggesting that in this species females are capable of storing more sperm than other Drosophila (Fig 4). Furthermore, spermathecae of *D. suzukii* are clearly more melanised and/or sclerotized, likely to increase protection of stored sperm from mutagenic UV radiation. We identified a putative genetic basis of such difference in the strong up-regulation of one of the Cytochrome P450 (CYP) genes, *Cyp4d20* (Fig. 5). 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 may be a mechanism to detoxify the possible toxic substances associated with sperms, thus permitting a long-term storage of viable spermatozoa in spermathecae (Malata et al. 2014). This hypothesis is in agreement with what 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 virgin females' ones.

Taken together, these results suggest that females have adapted to the winter diapause and the concomitant paucity of males in late winter/early spring by maximizing the chances of getting a lot of sperm from the rare male encounters when they exit diapause (compare with our field trapping of Fig. 1). On the other hand, it cannot be excluded that overwintering females are already mated and store sperm in the spermatheca, a quite common strategy among those insects that overwinter as adults (Hodek and Iperti 1983; Neubaum and Wolfner 1999). Such behaviors would not be required in other Drosophila, such as *D. melanogaster*, which are adapted to condition of relative constant temperature, when males are similarly abundant all year long.

Implications for D. suzukii *pest management*

Our results indicate that the number of catches during winter changes in different years and that these differences correlate with the winter temperatures. Even more importantly, these temperatures also correlate with the catches of the following part of the 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. Indeed, the only currently available *D. suzukii* population dynamic model does not take into consideration survival and reproductive status during winter (Wiman et al. 2014). 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. A low number of individuals exiting the winter diapause thus 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 results, 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 fruit growing areas 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 have 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). According to our observations both techniques should be planned for late winter and early spring because at that time of the year there is paucity of males in the field (Fig 3). Such timing promises to be more effective because the ratio of released-males to resident-males would be higher, therefore reducing the competition from resident-males and increasing chances of effectively reducing population. For the same reason, late summer and autumn, when the ratio of resident males is high, should be avoided.

In conclusion, our analysis provided a baseline to clarify some aspects of the peculiar *D. suzukii* reproductive diapause. In the future, this knowledge should be translated into existing and new population development models in order to better forecast *D. suzukii* growth rates in early season and improve IPM techniques.

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Figure's Captions

Fig. 1 a) *Drosophila suzukii* capture trend during the whole trapping period (winter 2012 – autumn 2015). Red dashed-lines areas correspond to figures 1c-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 to week 31 of the year). The blue areas represent the extent of the freezing period , when minimum temperatures drop below zero (dotted red line) and during which the drop of population occurred. The low-capture period (LCP) is shown and ends with the boost of population in late spring.

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 6 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 showed. P < 0.05 after rho Spearman correlation analysis.

Fig. 3 Sex ratio of *Drosophila suzukii* captures, expressed in term of male-female skew, during the four 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.

Fig. 4 Percentage of *Drosophila suzukii* captures (2012-2014) grouped by environment typology

Fig. 5 a) Comparative micrographs of the spermathecae of *Drosophila suzukii, D. biarmipes* and *D. melanogaster* virgin females. b) Volume of spermathecae in 3 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.

Fig. 6 Quantitative PCR analysis of *Cyp4d20* expression in spermathecae. Bars are shown as means ± Standard Error. Letters indicate statistical significance (P < 0.001) after Tukey's multiple comparison test.

Figure 1

Click here to download Figure Fig1.tif

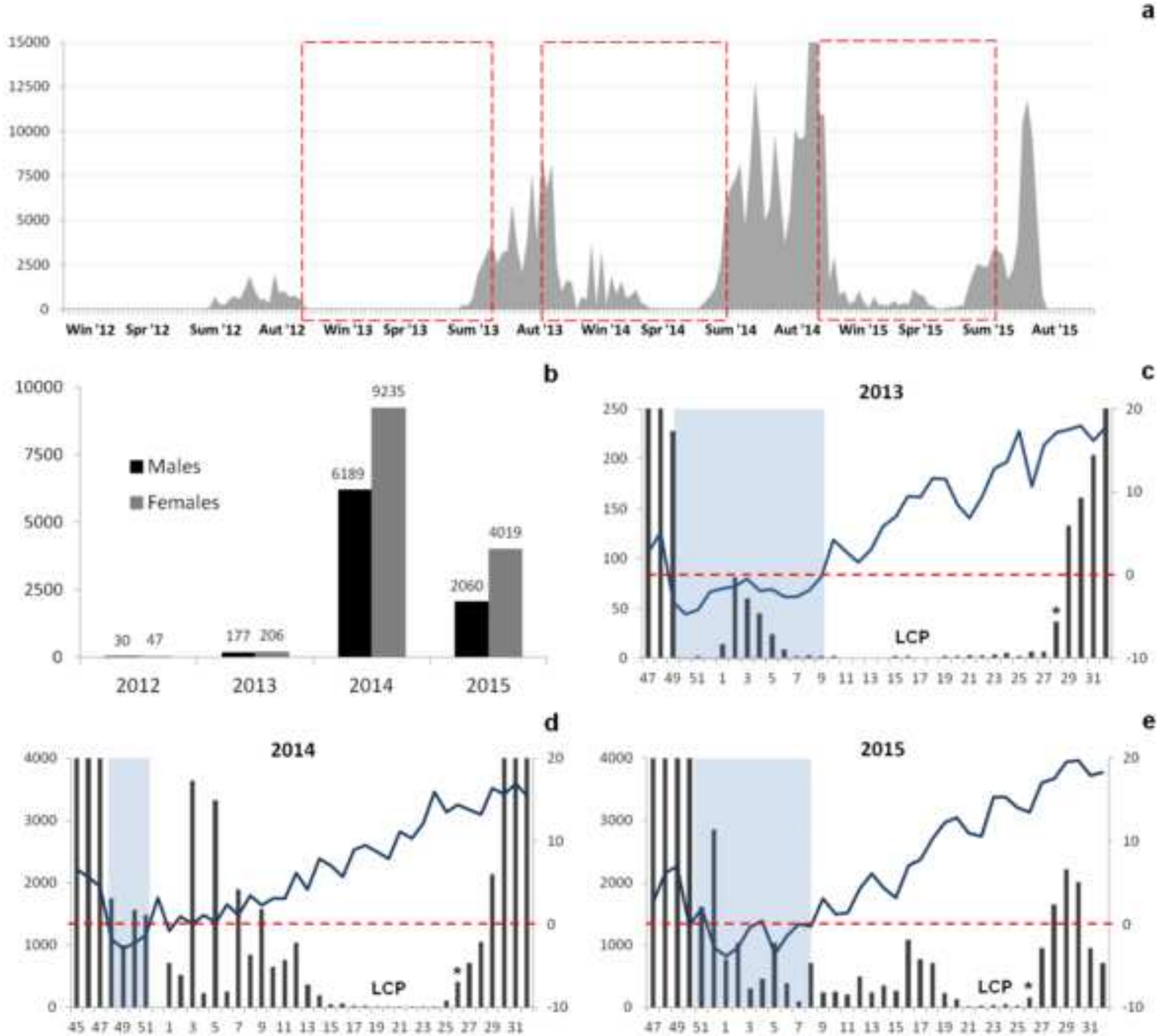

Figure 2

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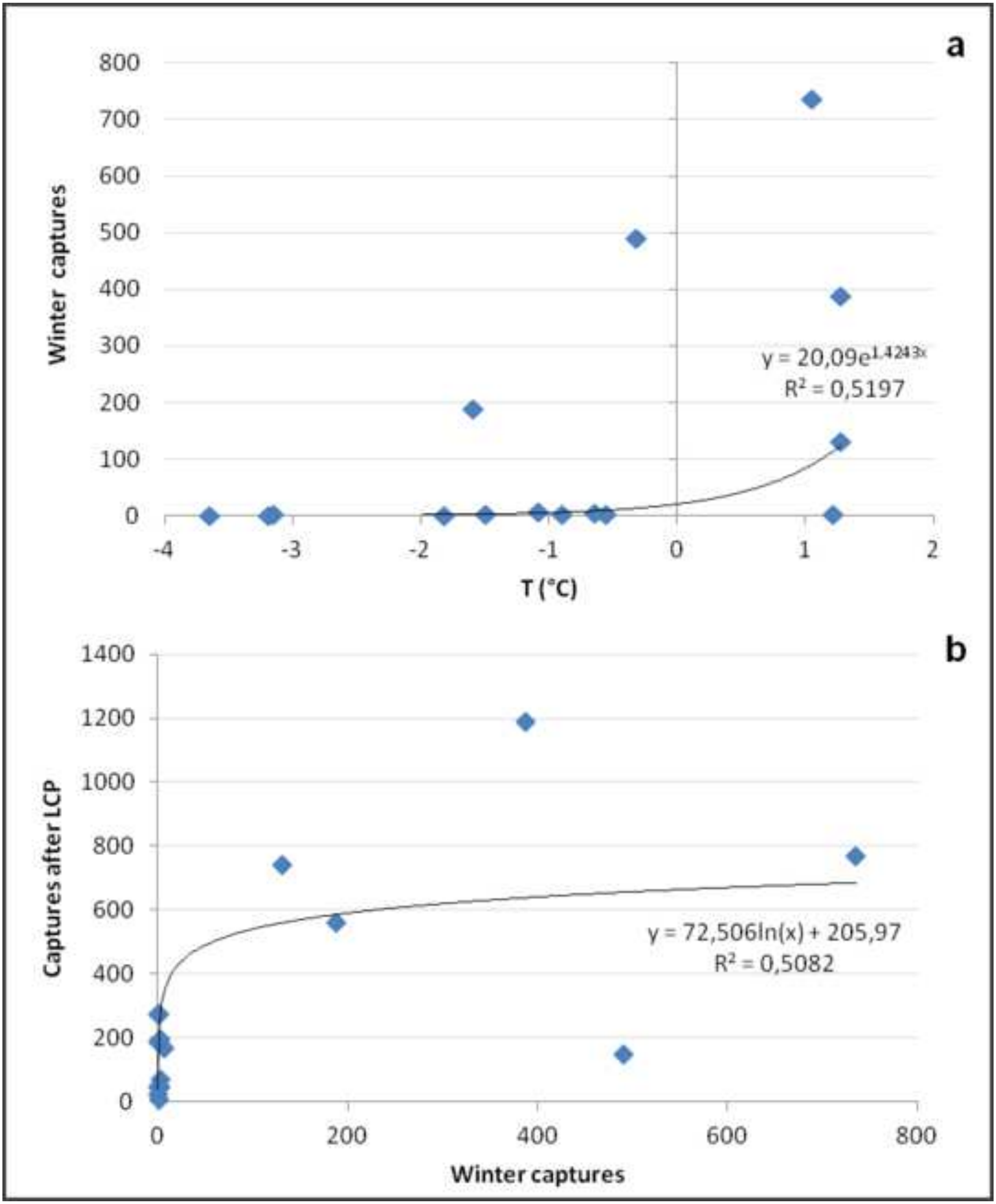

Figure 3

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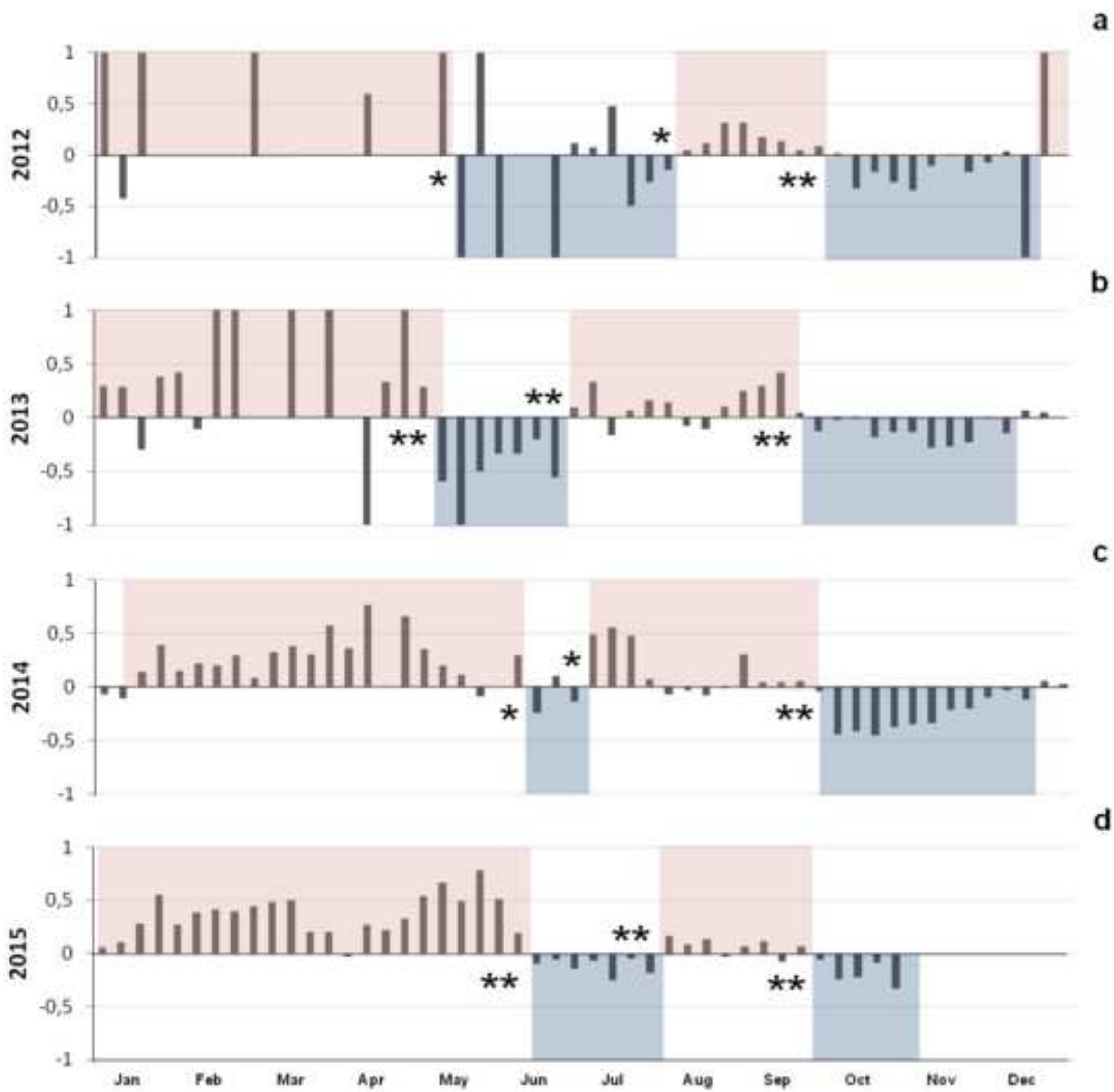

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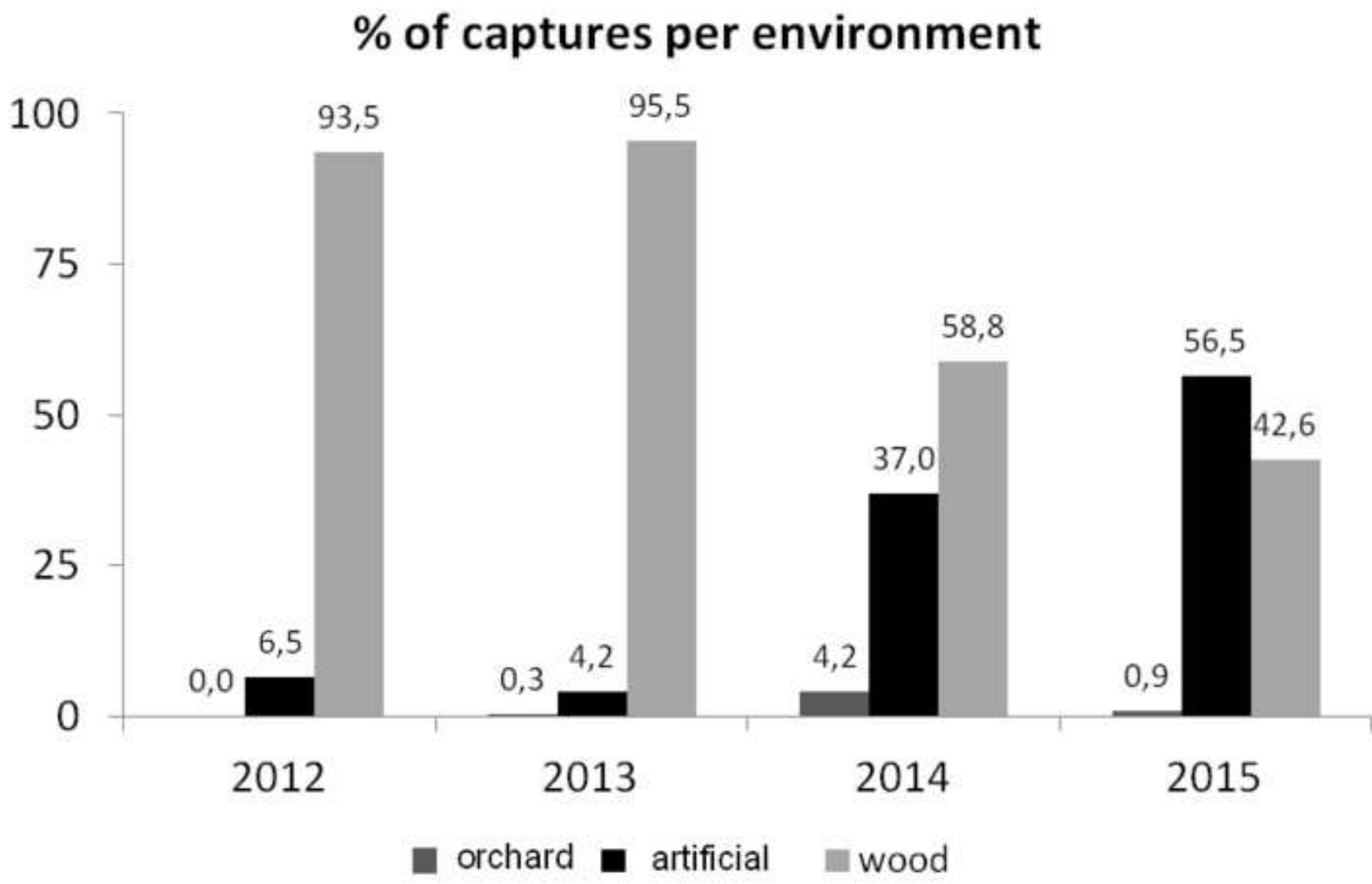


Figure 5

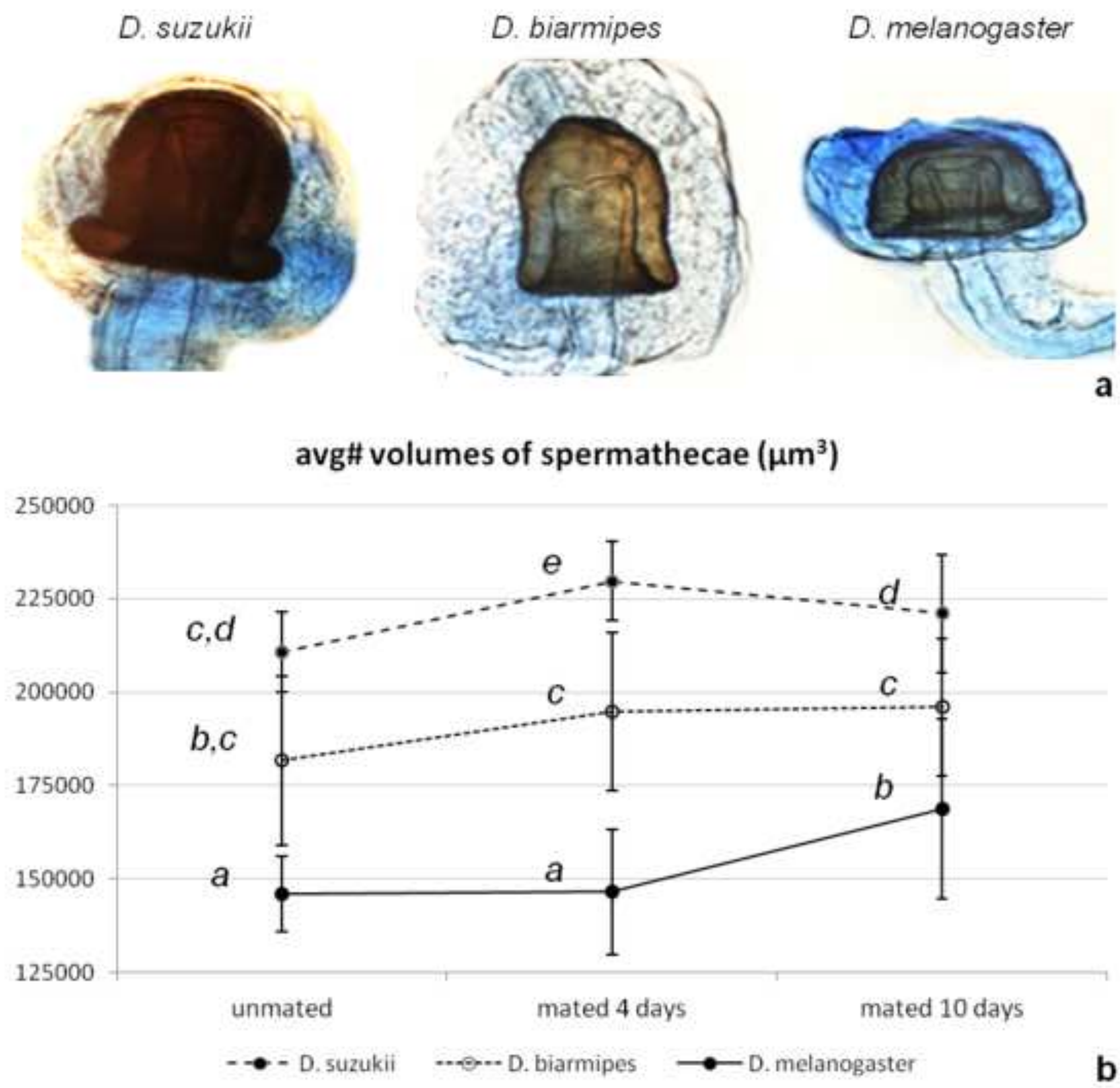
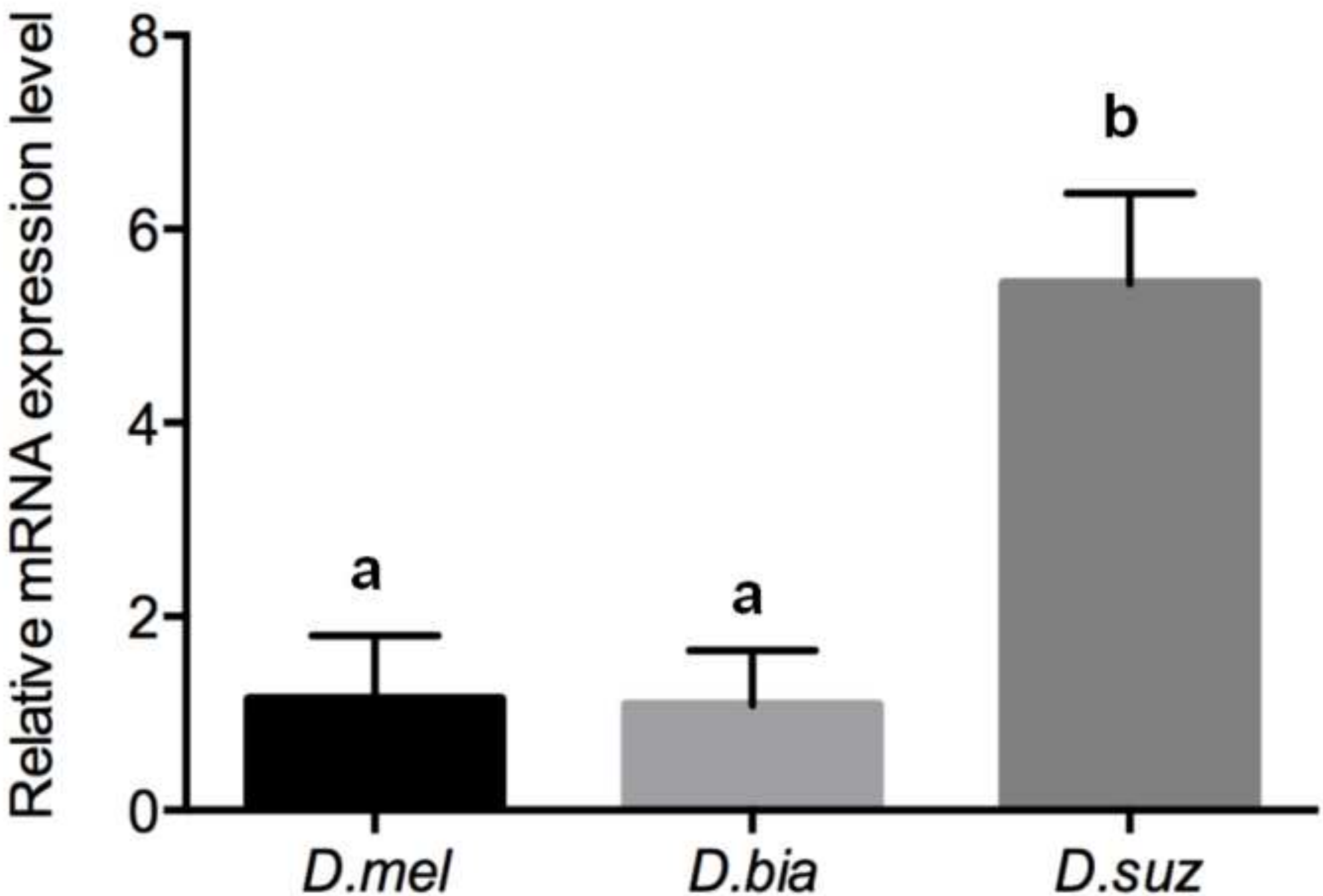

D. suzukii
D. biarmipes
D. melanogaster
a
avg# volumes of spermathecae (µm³)
250000
225000
200000
175000
150000
125000
e
c,d
d
c
c
b,c
b
a
a
unmated
mated 4 days
mated 10 days
D. suzukii
D. biarmipes
D. melanogaster
b

Figure 6



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

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

[a] (*) indicates 2013-2015 monitoring activity, (**) indicates 2012-2014 monitoring activity.
[b] Habitat typology: A) Orchard; B) Artificial; C) Wood.

Tab. 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}) an maximum (T_{max}) temperature (absolute and median); cumulative degrees (°C), consecutive days and number of days and hours under three reference temperatures (0, -2, -4°C). For each parameter are analysed absolute values refer to the period 1st December – 31st January in two (2014-2015) or three years (2013-2015) according to the site (see Table 1); Median values are calculated from the average of the 8 Sampling Units (SU, 7 days preceding the trap sampling).

Parameter		**Spearman's rho**	**P**	**Parameter**		**Spearman's rho**	**P**
	Med	-0,53	ns		< 0	0,79	< **0.01**
T_{min} Absolute	Min	-0,72	< **0.05**	Cumulative	< -2	0,77	< **0.01**
	Max	-0,2	ns		< -4	0,73	< **0.05**
	Med	-0,62	ns		< 0	0,37	ns
T_{min} Median	Min	-0,69	< **0.05**	Consecutive	< -2	0,4	ns
	Max	-0,18	ns		< -4	0,65	< **0.05**
	Med	-0,39	ns		< 0	0,77	< **0.01**
T_{max} Absolute	Min	-0,56	< **0.05**	Nr of Days	< -2	0,78	< **0.01**
	Max	-0,01	ns		< -4	0,63	ns
	Med	-0,35	ns		< 0	0,72	< **0.05**
T_{max} Median	Min	-0,65	< **0.05**	Nr of Hours	< -2	0,61	< **0.05**
	Max	-0,16	ns		< -4	0,51	ns