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Overwintering of two pupal parasitoids of *Drosophila* under natural conditions

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ABSTRACT

The overwintering capacity of biocontrol agents is of fundamental relevance for biological control of pests in temperate regions. In this study we tested the cold tolerance of the indigenous *Drosophila* pupal parasitoids *Pachycrepoideus vindemmiae* and *Trichopria drosophilae* at constant low temperature in the laboratory and exposed different preimaginal parasitoid stages in the field during winter. We evaluated whether semi-natural habitats promote overwintering via more favorable microclimatic conditions as well as higher host availability compared to orchards. Further, we studied the parasitoids' phenology in a semi-field experiment during autumn. We found that *P. vindemmiae* larvae and pupae were most cold tolerant under laboratory and field conditions, while all preimaginal stages of *T. drosophilae* displayed similar cold tolerance. Semi-natural habitats buffered temperature extremes, yet overwintering survival was not enhanced compared to orchards. Suitable overwintering hosts were present in all habitats at times when parasitoids were still active parasitizing. These results demonstrate that *P. vindemmiae* overwinters most likely as larva or pupa and that *T. drosophilae* can overwinter in a preimaginal life stage. Further, we provide evidence that both parasitoids can overwinter in a wide range of habitats and that the availability of hosts for overwintering is unlikely a limiting factor for the parasitoids during fall.

1. Introduction

The spotted wing drosophila, *Drosophila suzukii* (Diptera: Drosophilidae), is an invasive pest infesting soft skinned fruits throughout Europe and other world regions (Asplen et al., 2015; Cini et al., 2012; Walsh et al., 2011). With its serrated ovipositor, the pest oviposits in healthy, ripening fruits causing severe economic loss in the small fruit industry (Goodhue et al., 2011; Walsh et al., 2011). Since *D. suzukii* is highly polyphagous with a wide range of cultivated and wild host plants (Kenis et al., 2016; Lee et al., 2015; Poyet et al., 2015), its management requires a landscape-scale approach, including semi-natural habitats, with biological control as an important management tool (Haye et al., 2016).

Conservation and augmentation biological control can contribute to the management of *D. suzukii* (Gabarra et al., 2015; Rossi-Stacconi et al., 2019). We here focus on two pupal parasitoids, *Pachycrepoideus vindemmiae* (Rondani) (Hymenoptera: Pteromalidae) and *Trichopria drosophilae* (Perkins) (Hymenoptera: Diapriidae), that utilize *D. suzukii* as a host in the invaded areas (Kremmer et al., 2017; Miller et al., 2015; Rossi-Stacconi et al., 2015). *Pachycrepoideus vindemmiae* is a generalist idiobiont ectoparasitoid with a global distribution and a broad host range including several families of the order Diptera (Noyes, 2019; Rossi-Stacconi et al., 2013). *Trichopria drosophilae* is a generalist idiobiont endoparasitoid of the genus *Drosophila* and distributed across Eurasia and America (Carton et al., 1986; Lee et al., 2019; Wang et al., 2016). The parasitism behavior of *P. vindemmiae* and *T. drosophilae* is well investigated under both laboratory and field conditions (Amiresmaeili et al., 2018; Kaçar et al., 2017; Rossi-Stacconi et al., 2015; Wolf et al., 2021). In contrast, fundamental aspects of their biology, such as their overwintering ecology, have so far received little attention.

Cold periods are important factors shaping the life history of insects with consequences for their fitness and their realized geographical distribution (Bale et al., 2002; Sinclair, 2015). From a physiological point of view, overwintering success depends on the cold tolerance of the overwintering developmental stage (Bale et al., 2002; Clark and Worland, 2008; Sinclair et al., 2003). A previous laboratory study on the cold tolerance of different life stages of *T. drosophilae* revealed that the parasitoid most likely overwinters in a preimaginal life stage (Amiresmaeili et al., 2020). Parasitoid eggs, larvae and pupae can tolerate 0°C

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for one month with survival rates of 35% to 45% but also withstand sub-zero temperatures for the same period albeit with strongly reduced survival rates (Amiresmaeili et al., 2020). *Trichopria drosophilae* adults survived exposure to 0°C for 7 to 21 d depending on their nutrition status and survived sub-zero temperatures for about a week (Amiresmaeili et al., 2020). The cold tolerance of *P. vindemmiae* and its overwintering stage has thus far not been addressed.

The overwintering ability of arthropods is also influenced by the overwintering (micro-) habitat (Sinclair, 2015). Semi-natural habitats could provide particularly suitable microclimatic conditions, since forest and hedge vegetation, as well as ground cover such as leaf litter and forest mulch, buffer temperature extremes (Suggitt et al., 2011; Tougeron et al., 2016; Wallingford et al., 2018). For example, Pfiffner and Luka (2000) have reported higher abundance of overwintering arthropods and higher species richness in soil samples from semi-natural habitats compared to adjacent arable fields. Further, semi-natural habitats could provide overwintering hosts, since forest habitats are a hotspot of *Drosophila* spp. (Basden, 1955; Burla and Bächli, 1991; Trivellone et al., 2020). Whether semi-natural habitats are an important source of overwintering hosts for parasitoids strongly depends on the seasonal synchrony of parasitoid-host interactions (Tougeron et al., 2020; Wetherington et al., 2017). Additionally, the host species could influence parasitoid cold tolerance through its body size and/or nutritional value (Harvey, 2005; Ismail et al., 2012).

We aimed to assess parasitoid overwintering under controlled laboratory conditions and to validate the findings under more realistic conditions in the field, where temperatures fluctuate. We hypothesized that semi-natural habitats promote overwintering of *P. vindemmiae* and *T. drosophilae* due to (i) more favorable microclimatic conditions and (ii) higher host availability in autumn. To test the first hypothesis, we first addressed gaps in knowledge of the cold tolerance of *P. vindemmiae* in laboratory experiments and subsequently exposed preimaginal stages of both parasitoid species to microclimatic conditions in soil in orchards and three semi-natural habitats; forest, forest edge and hedge, during the winter period of two consecutive years. To address the second hypothesis, we assessed parasitism by both parasitoid species during the autumn of two consecutive years. We simultaneously studied *Drosophila* spp. in the different habitats in autumn using sentinel traps and investigated whether host species influenced cold tolerance of the two parasitoid species.

2. Materials and methods

2.1. Insects

All insect cultures were maintained at standard rearing conditions (i.e., 22 ± 1°C, 70 ± 5% RH, 16L:8D). *Drosophila melanogaster*, *Drosophila subobscura* (Diptera: Drosophilidae) and *D. suzukii* cultures were obtained and reared on an artificial diet as described in Amiresmaeili et al. (2020, see supplementary information).

The pupal parasitoids *P. vindemmiae* and *T. drosophilae* cultures originated from individuals captured in Zurich-Affoltern (Switzerland) in 2017 (Trivellone et al., 2020). *Trichopria drosophilae* cultures were kept in vented plastic flight cages (22 × 33 × 18 cm), while a vented 1.3 l plastic vial (11 cm diameter) was used for *P. vindemmiae*. Twice a week parasitoids were provided with fresh *D. melanogaster* pupae on paper towel for oviposition as well as water and honey. Host pupae were obtained by lining the walls of plastic vials containing last instar larvae of *Drosophila* spp. with moistened paper towel strips and the pupae attached to the strips were removed after 24 to 48 h.

2.2. Laboratory cold tolerance

2.2.1. Stage-specific cold tolerance of P. vindemmiae

Drosophila subobscura was chosen as host for the overwintering experiments since this species can overwinter as immatures (Sørensen et al., 2015). To assess the cold tolerance of the preimaginal stages, *D. subobscura* pupae on paper towel were exposed to *P. vindemmiae* in a flight cage at a ratio of about 2.5:1 (pupae:parasitoid) for 30 h. For each sample, 30 potentially parasitized pupae were placed in a plastic box (5 × 3 × 1.5 cm) equipped with a small piece of wet cotton. To obtain different preimaginal stages of the parasitoid, potentially parasitized pupae were either used for the experiment immediately (parasitoid eggs) or stored for 8 d (larvae) or 18 d (pupae) at 22°C. After 7 d acclimatisation at 10°C (12L:12D), followed by 7 d at 5°C (darkness), parasitized pupae were exposed to 0°C for 28 d (darkness). Subsequently, the pupae were transferred to 10°C (light) for 6 h and then kept at 22°C (16L:8D) until eclosion of parasitoids. Three weeks after the first parasitoid eclosion, all individuals were counted and sexed. Five replicates per preimaginal stage were subjected to the cold treatment or kept at 22°C as control. To assess the parasitoid developmental stage, an additional 30 potentially parasitized pupae per stage were exposed to the cold treatment and were dissected immediately. The survival rate for each sample was defined as the number of eclosed parasitoids divided by the sample size, excluding host pupae with fly eclosion.

The cold hardiness of *P. vindemmiae* adults was determined using 2–4 d old, unfed individuals. To address the long-term cold tolerance, five replicates of 30 males and 30 females each were subjected to the same acclimatisation and cold exposure as described above for the preimaginal stages. To assess the short-term cold tolerance, single individuals were placed into 1.5 ml microcentrifuge tubes sealed with wet cotton. After 6 h acclimatisation at 10°C, the tubes were transferred to either 0°C or −5°C for 1, 3, 5 or 7 d. At the end of the cold exposure, adult survival was assessed immediately after another 6 h acclimatisation at 10°C and again after 24 h at standard rearing conditions due to potential chill coma of the insects (Andersen et al., 2015). For each temperature and time period, 7 to 8 replicates, each consisting of 16 individuals, were conducted. As a control, 30 adults were only subjected to acclimatisation. Adult sex was determined at the end of the experiment and the survival rate per sex was calculated as the number of adults alive divided by the total number of adults within one replicate.

2.2.2. Host specific cold tolerance of P. vindemmiae and T. drosophilae

Pupae of *P. vindemmiae* and *T. drosophilae* were acclimatised and cold-exposed as described above. However, pupae of *D. subobscura*, *D. melanogaster* and *D. suzukii* were used as a host and exposed to 0°C for 14 d. The experiment was conducted as a complete 3 x 2 factorial design with host species as the first factor and parasitoid species as the second. Each replicate consisted of 30 potentially parasitized pupae. Five replicates for each combination of factors were subjected to the cold treatment, while another 5 were kept at 22°C as a control. The survival rate for each replicate was defined as the number of eclosed parasitoids divided by the initial number of parasitized host pupae, i.e., the total number of pupae minus pupae with fly eclosion.

2.3. Field sites

Over a period of two years, 14 fruit growing farms located around Zurich-Affoltern and the northwest of the canton of Zurich (Switzerland) were selected for field experiments and observational studies (Farm 1 to 14, Table S1). At each farm, 4 sites were selected. One site was located in the farm's orchard (A) and 3 in the surrounding semi-natural habitats; hedge (B), forest edge (C), and forest (D). All the sites located in semi-natural habitats were within maximally 650 m from the farms' orchards. In both forest and hedge habitats, natural fruit sources such as *Rubus fruticosus* (blackberry), *Prunus spinosa* (blackthorn) and *Sambucus nigra* (elder) were available. The fruit crops grown in the orchards differed both within and between farms (Table S2).

2.4. Field overwintering of P. vindemmiae and T. drosophilae

The overwintering field experiments were conducted at 4 farms in

winter 2018/2019 (Season I) and 8 farms in winter 2019/2020 (Season II, Table S1), all of which contained an orchard, hedge, forest edge and forest site. At each site, seven samples of *D. subobscura* pupae were exposed to the field conditions. Six of the samples consisted of 100–120 pupae that had been parasitized by either *P. vindemmiae* or *T. drosophilae* and contained either eggs, larvae or pupae (see below). The seventh sample, consisting of 100 unparasitized pupae, served as a control. All samples were put in mesh bags and buried together with a temperature logger (HOBO 64K Pendant®, Onset Computer Corporation, Massachusetts, United States) approximately 2 cm deep in soil within a galvanized wire mesh cage (30 × 30 × 12 cm, 13 mm mesh size). On-site available soil cover (e.g., leaf litter, sticks, moss) was placed on top of the soil within the cage to mimic the natural cover at each site.

Host pupae used for the experiments were parasitized 15–17 d, 7–9 d or 1–2 d prior to the assays to obtain parasitoid pupae, larvae and eggs, respectively. Parasitism took place for 24–48 h within parasitoid flight cages exposing host pupae to parasitoids at a ratio of 8:1 in Season I and 1:3 in Season II. For each sample of parasitoid larvae and pupae, 100 of the host pupae exposed to parasitoids were selected for the experiment, whereby visibly not parasitized host pupae (empty pupae due to fly eclosion, fly pharates, or red eyes of fly pupa visible) were excluded. Since the parasitism state of host pupae was not assessable for parasitoid eggs, egg samples contained a total of 120 host pupae. Parasitized and unparasitized *D. subobscura* pupae were acclimatised prior to field exposure for 4 d at 10°C (12L:12D), 6 d at 4°C (darkness) and 3 d at 0°C (darkness). The egg samples were excluded from the 0°C treatment in Season II, since our laboratory assays demonstrated the vulnerability of *P. vindemmiae* eggs to elongated exposure at 0°C and field temperature in Season I rarely dropped this low. To prevent desiccation of samples during the acclimatisation process, they were kept in boxes containing a saturated NaCl solution.

Controls (N = 1–6) for each parasitoid preimaginal stage without cold acclimatisation were kept at standard laboratory conditions. Additionally, 16 controls were subjected to cold acclimatisation prior to storage at standard laboratory conditions (Table S3). To control for developmental progress of parasitoids during the field exposure, one additional sample per parasitoid stage (N = 50–100 host pupae) was buried at the hedge site of Farm 11 and subsets of those parasitized pupae were recouped and dissected regularly.

The samples were placed in the field in late December in 2018 and mid-December in 2019 and were recollected in early March after 76 and 83 d of field exposure, respectively. In the laboratory, sample mesh bags were opened and stored in vented 0.25 l plastic vials. After 7 d at 10°C (12L:12D), followed by 7 d at 15°C (14L:10D) all samples were transferred to standard rearing conditions. Parasitoid eclosion was checked once per week until no further eclosion was recorded over two consecutive weeks for all samples. The eclosion rate for each sample and control was defined as the sum of eclosed parasitoids divided by the sample size.

Based on the hourly measurements of field temperature, the average temperature and the average daily temperature range (i.e., the difference between the daily maximum and minimum temperature) over the entire experimental period were calculated for each site. Temperature data from two sites were missing due to logger malfunctioning (Table S1).

2.5. Parasitoid phenology

In a semi-field experiment, 3 flight cages (16 × 11 × 11 cm) for each parasitoid species and 3 empty control cages were installed 20 cm above ground under a shelter at Agroscope Reckenholz (Zurich, Switzerland). Flight cages with parasitoids each contained either a colony of 20 2–4 d old *P. vindemmiae* or *T. drosophilae* adults (sex ratio 50:50). Parasitoids were provided with water and honey on cotton pads. Every week, 5-10 adult parasitoids per flight cage were added, using parasitoids that had been previously exposed to field conditions in a 0.25 l plastic vial for one week. From mid-August (calendar week, CW 34) to early December (CW 49) 2019 two samples of 25 *D. subobscura* pupae were weekly exposed in the lower half of petri-dishes (4.5 cm diameter) in each experimental cage for 6 d. Subsequently, the pupae were transferred into vented 0.25 l plastic vials. One sample was left outside in a Styrofoam box filled with leaf litter and the other one was transferred to the standard rearing conditions in the laboratory. Laboratory samples were checked weekly for parasitoid and fly eclosion until no further eclosion occurred for two consecutive weeks. Host pupae without any eclosion were dissected under a stereomicroscope. The number of parasitized host pupae was defined as the sum of eclosed parasitoids and the number of dead parasitoids found during the host pupae dissection.

The samples left outside were checked weekly for parasitoid and fly eclosion from CW 36 through CW 50 in 2019 and seven times between CW 9 and CW 33 in 2020. The average degree days (DD) until adult eclosion in autumn 2019 and spring 2020 were calculated for each parasitoid species. Therefore, the daily average temperatures and a developmental threshold of 10°C for *T. drosophilae* and 11°C for *P. vindemmiae* (Wang et al., 2018) were used. DD for each sample with parasitoid eclosion were calculated for the time period between parasitism and the observation of alive, eclosed parasitoids during eclosion checks. When only dead parasitoids were observed during an eclosion check, the DD were estimated as the average DD between the previous and the current check. Eight samples with only 1–2 dead adults were excluded since those potentially remained from the parental generation.

Temperature and relative humidity next to flight cages and within the Styrofoam box were recorded throughout the experimental period using data loggers (MicroLite II Temperature/RH Logger Fourier Technologies, United States; HOBO 64K Pendant® Temperature Data Logger). Missing data points were filled in with data from the automatic meteorological station at the institute site, which did not differ substantially from temperatures recorded by data loggers (Fig. S1). For all weekly sample exposures, the weekly average temperature, the average daily maximum and minimum temperature, and the average daily relative humidity were calculated.

2.6. Host availability

To investigate the availability of potential overwintering hosts, sentinel traps were deployed within each 6 orchards, hedges, forest edges, and forests from mid-August (CW 34) through mid-November (CW 45) 2019 (Table S1). The traps consisted of 0.8 l plastic vials with 11 holes (3 × 4 mm) and a plastic lid. To detect frugivorous *Drosophila* spp. that were reproductively active, a banana-bait (40–60 g smashed banana mixed with 2–4 ml Nipagin solution, i.e., 40:1 mixture of distilled water and 10% Nipagin solution in 95% ethanol) was provided in 80 ml dressing cups (festag, Switzerland) with two water-soaked cotton pads to prevent desiccation. Each trap was hung 10–60 cm above ground and within the shade or half-shade wherever possible. Once per week the entire trap was exchanged. Therefore, the holes were sealed with tape, trapping all insects inside. In the laboratory, the trapped drosophilids were anesthetized with carbon dioxide, transferred into 70% ethanol, and counted. Subsequently, the bait was transferred into a 0.8 l plastic vial with vented lid and stored at standard rearing conditions for 3 weeks. Thereafter, the vials were placed in a drying oven at 47 ± 2°C for about 3 h. Eclosed drosophilids were counted and stored in 70% ethanol.

For each site, a random subsample of 20 drosophilids (or less if fewer individuals eclosed) from the time of the first and last eclosion was identified to species level by a specialist (Irene Bühlmann, Biotopia Ökobüro, Zug, Switzerland). For the 5 sites with their last eclosion in November, an additional subsample from October was selected for species identification.

2.7. Statistical analysis

All statistical analyses were conducted with R version 4.0.3 (R Core Team, 2018) and all raw data were stored in a repository (Häner et al., 2021). The two parasitoid species were always analysed separately.

2.7.1. Laboratory cold tolerance

Because no *P. vindemmiae* adults survived long-term cold exposure, the data was not statistically analysed. The short-term cold tolerance of adults and the preimaginal stage-specific cold tolerance of *P. vindemmiae*, as well as the host-specific cold tolerance of both parasitoid species were analysed by means of generalized linear models (GLMs) with binomial distribution and logit link function. In case of overdispersed data, a quasi-binomial distribution with logit link function was specified to adjust model estimates and p-values through the inclusion a dispersion parameter into the models. The model to analyse the short-term cold tolerance of *P. vindemmiae* adults included the continuous explanatory variables duration of cold exposure and temperature (0°C or −5°C), the categorical variable sex, and all two-way interactions. The response variable was the observed number of parasitoids alive divided by the sum of alive and dead parasitoids. The explanatory variables for the stage-specific cold tolerance of *P. vindemmiae* and the host-specific cold tolerance for *P. vindemmiae* and *T. drosophilae* included the experimental treatment (control or cold), parasitoid developmental stage or host species, and their respective interaction. The response variable was the observed number of eclosed parasitoids divided by the sum of eclosed parasitoids and host pupae with unknown fate (i.e., no parasitoid or fly eclosion). An analysis of deviance (using function Anova in car package, Fox and Weisberg, 2019) was conducted for all models followed by a Tukey post-hoc test.

2.7.2. Field overwintering

An analysis of covariance followed by a Tukey post-hoc test was conducted to assess the effect of habitat on the average temperature and the average daily temperature range while accounting for the covariate altitude. The average daily temperature range was log transformed to reduce the skewness of the original data.

Survival of the overwintering parasitoids was analysed by means of GLMs with binomial distribution and logit link function. In case of overdispersed data, a quasi-binomial distribution with logit link function was specified to adjust model estimates and p-values. The response variable was the observed number of eclosed parasitoids divided by the sum of eclosed parasitoids and host pupae with unknown fate (i.e., no detectable parasitoid or fly remains). The models included habitat, developmental stage, experimental season as well as all two-way interactions as explanatory variables. In the case of significant interaction terms, a separate analysis was conducted, while non-significant interaction terms were excluded from the final model. An analysis of deviance was conducted for all models followed by a Tukey post-hoc test.

2.7.3. Parasitoid phenology

Parasitism in semi-field cages and subsequent adult eclosion from laboratory samples of both parasitoid species were analysed using a GLM with binomial distribution and logit link function. In case of overdispersed data, a quasi-binomial distribution with logit link function was specified to adjust model estimates and p-values. The response variable to model parasitism in semi-field cages consisted of the number of parasitized host pupae divided by the sum of parasitized and not parasitized host pupae. The response variable to model subsequent adult eclosion consisted of the number of parasitized host pupae without eclosion divided by the sum of parasitized host pupae without eclosion and parasitoid eclosions. The weekly average temperature was used as explanatory variable in all models. The Spearman's correlation between calendar week and the weekly average temperature was calculated.

2.7.4. Host availability

The host availability in the different habitats was modelled using a two-part hurdle modelling approach (pscl package, Zeileis et al., 2008). Thereby, the probability of drosophilid offspring observation (irrespective of their number) was modelled with a zero hurdle model with binomial distribution and logit link function (model part one), while a zero truncated negative binomial model with log link function (count model, model part two) was used to model the number of eclosed drosophilids. The full model was identical for both model parts. The number of eclosed drosophilids was used as response variable and the explanatory variables included habitat, weekly average temperature measured at the closest weather station in Lägern (47.48193/8.39722, Switzerland), altitude of the sites and the number of trapped drosophilids. All continuous explanatory variables were scaled to assure equal contribution to the analysis using the z-score standardization (i.e., mean set to 0 and variance to 1). Samples for which the number of eclosed drosophilids (11 cases) and the number of trapped drosophilids (4 additional cases) could not be determined were excluded from the analysis. A stepwise model selection using the R package lmtest (Zeileis and Hothorn, 2002) was performed separately for both model parts. Tukey test was used for pairwise comparison. The Spearmans's correlation between calendar week and the weekly average temperature was calculated.

Based on the identified eclosed *Drosophila* spp. from 53 samples, the Shannon species diversity index according to Chao et al. (2013) was calculated with the R package SpadeR (Chao et al., 2016) for each habitat type using raw species indices. Obtained Shannon entropies were back transformed to effective number of species by taking their exponential (i.e., Hill number, ${}^{q}D$, with $q = 1$, here referred to as Shannon Diversity). Further, the community composition between habitat types and months was compared with a non-metric multidimensional scaling (NMDS, R package vegan, Oksanen et al., 2020) analysis using species indices.

3. Results

3.1. Laboratory cold tolerance

3.1.1. Stage specific cold tolerance of P. vindemmiae

All dissected host pupae still contained the assigned preimaginal stage of parasitoids after cold exposure. The cold treatment, the parasitoid developmental stage and their interaction significantly affected the eclosion rate of *P. vindemmiae* (GLM with quasi-binomial distribution, cold: $X^2 = 18.46$, $df = 1$, $p < 0.001$; stage: $X^2 = 24.38$, $df = 2$, $p < 0.001$; interaction: $X^2 = 22.47$, $df = 2$, $p < 0.001$). One month of cold exposure to 0°C strongly reduced the survival of the egg stage compared to the larval (Tukey, $p < 0.001$) and pupal stages ($p < 0.001$, Table 1).

In the control group all adults survived the acclimation period. No *P. vindemmiae* adults survived the long-term (28 d) cold exposure to 0°C.

Table 1
Cold tolerance of *P. vindemmiae* preimaginal stages. The eclosion of *P. vindemmiae* adults from eggs, larvae, and pupae without cold treatment (control) and subjected to 28 d at 0°C (cold) is displayed. The survival rate for each replicate was defined as the number of eclosed parasitoids divided by the initial number of parasitized host pupae, i.e., the total number of pupae minus pupae with fly eclosion. Averages were calculated over 5 replicates with each 24–30 parasitized *D. subobscura* pupae. Letters indicate significant differences between developmental stages within a treatment (Tukey post-hoc analysis).

Treatment	Developmental stage	Average eclosion (SD) [%]
control	egg	50.67 (14.79)[a]
control	larva	63.59 (18.80)[a]
control	pupa	58.00 (16.93)[a]
cold	egg	0.67 (1.49)[A]
cold	larva	47.33 (22.29)[B]
cold	pupa	51.33 (13.25)[B]

In the short-term cold tolerance experiment, temperature, duration and sex significantly affected adult survival (GLM with binomial distribution, temperature: $X^2 = 9.28$, $df = 1$, $p = 0.002$, duration: $X^2 = 394.05$, $df = 1$, $p < 0.001$, sex: $X^2 = 8.83$, $df = 1$, $p = 0.003$). Further, significant interactions were found for temperature × duration ($X^2 = 16.33$, $df = 1$, $p < 0.001$) and sex × duration ($X^2 = 5.48$, $df = 1$, $p = 0.019$). The survival rate declined over duration of cold exposure for both temperatures tested, but more quickly at −5°C (Fig. 1). At 0°C males displayed with an average survival of 41.57 ± 33.69% ($\bar{x} \pm SD$) higher cold tolerance than females (29.95 ± 30.33%). No such difference was apparent at −5°C (males: 28.49 ± 32.99%, females: 25.79 ± 32.84%).

3.1.2. Host specific cold tolerance of P. vindemmiae and T. drosophilae

The host species had a significant effect on the eclosion rate of *P. vindemmiae* (GLM with quasi-binomial distribution, $X^2 = 39.4$, $df = 2$, $p < 0.001$, Table 2), whereas the two weeks of cold exposure had no effect ($X^2 = 2.7$, $df = 1$, $p = 0.1$). In the control, the eclosion rate of *P. vindemmiae* from *D. subobscura* hosts was reduced compared to *D. melanogaster* and *D. suzukii* (Tukey, *all* $p < 0.001$). After cold exposure, the eclosion rate was higher from *D. melanogaster* compared to *D. subobscura* and *D. suzukii* (Tukey, $p < 0.001$ and $p = 0.049$, Table 2). The eclosion rate of *T. drosophilae* was significantly reduced after two weeks of cold exposure (GLM with quasi-binomial distribution, $X^2 = 145.7$, $df = 1$, $p < 0.001$) and differed between host species ($X^2 = 10.31$, $df = 2$, $p = 0.006$). Parasitoid eclosion from *D. suzukii* was significantly lower in comparison to *D. melanogaster* and *D. subobscura* after cold exposure (Tukey, $p = 0.036$ and $p = 0.003$, respectively, Table 2), while no difference was observed between host species in the control treatment. Considering the eclosion rate in the respective control group, the relative survival in *D. suzukii* hosts was reduced by the cold treatment in both parasitoid species compared to the other two hosts (Table 2).

3.2. Field overwintering

Over both winter seasons, habitat significantly affected the average daily temperature range (Ancova, $F_{3,41} = 26.5$, $p < 0.001$) but not the average temperature ($F_{3,41} = 1.92$, $p = 0.141$) after controlling for altitude of the experimental sites. The average daily temperature range was larger in orchards compared to hedges (Tukey, $p = 0.001$), forest edges and forests as well as in hedges compared to forests (Tukey, *all* $p < 0.001$, Fig. 2 B). Also, temperatures measured in the two winter seasons differed. The average temperature across habitats was higher in Season II (4.72 ± 0.7, $\bar{x} \pm SD$) compared to Season I (3.44 ± 0.63).

The habitat type had no effect on eclosion of *P. vindemmiae*. The experimental season (GLM with quasi-binomial distribution, $X^2 = 262.59$, $df = 1$, $p < 0.001$) and the parasitoid developmental stage ($X^2 = 405.16$, $df = 2$, $p < 0.001$) significantly affected the eclosion rate of *P. vindemmiae*. No *P. vindemmiae* eggs survived winter field exposure, whereas in Season I 23.03 ± 7.27% ($\bar{x} \pm SD$) and in Season II 4.66 ± 3.62% of larvae and pupae developed into adults. Due to a significant interaction of the experimental season and the development stage ($X^2 = 15.65$, $df = 2$, $p < 0.001$), a separate analysis of the data for each experimental season was conducted. Significantly more pupae than larvae survived winter field exposure in Season I (Tukey, $p = 0.023$), whereas the opposite was observed in Season II ($p = 0.005$). Over both experimental seasons, no difference in survival was observed between larvae and pupae (Tukey, $p = 0.531$, Fig. 3).

The habitat type did not affect *T. drosophilae* eclosion. While the eclosion rate between the two experimental seasons did not differ for *T. drosophilae*, the parasitoid developmental stage significantly affected adult eclosion (GLM with binomial distribution, $X^2 = 69.29$, $df = 2$, $p < 0.001$). Eclosion was higher for larvae compared to eggs and pupae (Tukey, $p < 0.001$ and $p < 0.001$, respectively) and higher for eggs than pupae ($p = 0.016$, Fig. 3).

Overall, 58% of non-parasitized control flies survived the winter. Of the field samples used to control for developmental progress of the parasitoids during field exposure, a high proportion of the dissected host pupae was either not parasitized or the parasitism state could not be identified (Fig. S2). From those dissected host pupae where parasitism was detected, most *T. drosophilae* were still found to be in the intended

Table 2
Host-specific parasitoid cold tolerance. The eclosion of *T. drosophilae* and *P. vindemmiae* from pupae of *D. melanogaster*, *D. subobscura*, and *D. suzukii* without cold treatment (control), and after 14 d of exposure to 0°C (cold) is displayed. Survival rates after correction for control mortality are shown below. The survival rate for each replicate was defined as the number of eclosed parasitoids divided by the initial number of parasitized host pupae, i.e., the total number of pupae minus pupae with fly eclosion. Averages were calculated over 5 replicates with each 30 parasitized host pupae. Letters indicate significant differences between host species within a treatment and parasitoid species (Tukey post-hoc analysis).

	Average eclosion (SD) [%]		
Treatment	Host species	*T. drosophilae*	*P. vindemmiae*
control	*D. melanogaster*	76.00 (15.71)^{a}	83.33 (5.27)^{a}
control	*D. subobscura*	70.00 (19.58)^{a}	50.67 (8.30)^{b}
control	*D. suzukii*	60.67 (10.90)^{a}	76.67 (6.24)^{a}
cold	*D. melanogaster*	15.33 (5.06)^{A}	78.00 (8.69)^{A}
cold	*D. subobscura*	20.67 (7.23)^{A}	51.33 (15.02)^{B}
cold	*D. suzukii*	3.33 (2.36)^{B}	62.67 (16.06)^{B}
Average cold survival corrected by control [%]			
D. melanogaster		20.18	93.60
D. subobscura		29.52	101.32
D. suzukii		5.49	81.74

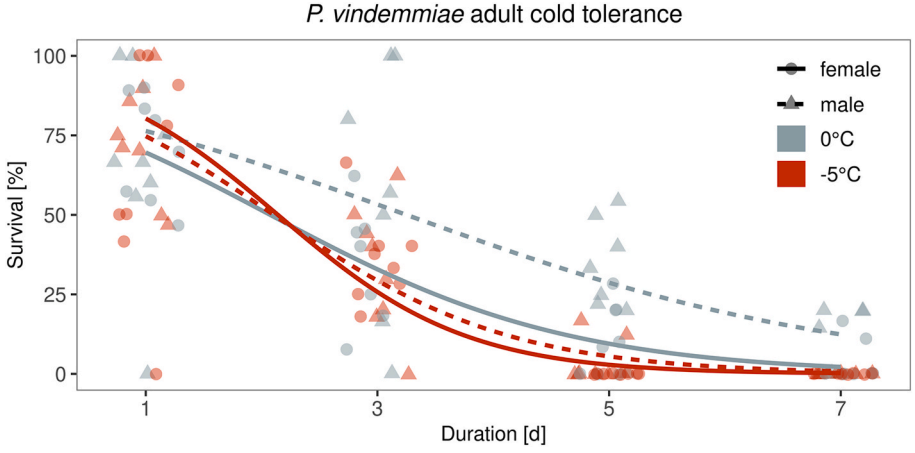

Fig. 1. | Cold tolerance of *P. vindemmiae* adults. The survival rate of *P. vindemmiae* females (dots) and males (triangles) subjected to 0°C (**grey**) and −5°C (**red**) for 1, 3, 5 and 7 days are displayed. Each dot or triangle represents the survival rate per sex for one sample with a total of 16 adults (mixed sex). The survival rate was defined as the number of alive adults of a sex divided by the total number of adults of a sex contained in a sample. The logistic regression curves display female (solid line) and male (dashed line) survival rates in response to duration of cold exposure as predicted by the GLM with binomial distribution.

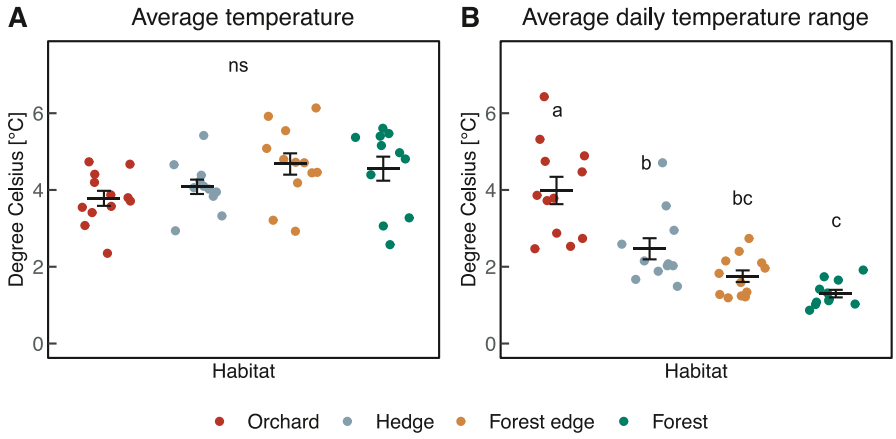

Fig. 2. | Winter field temperatures at 2 cm soil depth in two consecutive winter seasons. The average temperature (**A**) and the average daily temperature range (**B**) measured at a soil depth of 2 cm are displayed. Each dot represents one site ($N = 12$) and the colors indicate the habitat type. The black bars indicate the average and standard error over both experimental seasons and across the different sites. Letters indicate significant differences between habitats (Tukey post-hoc analysis).

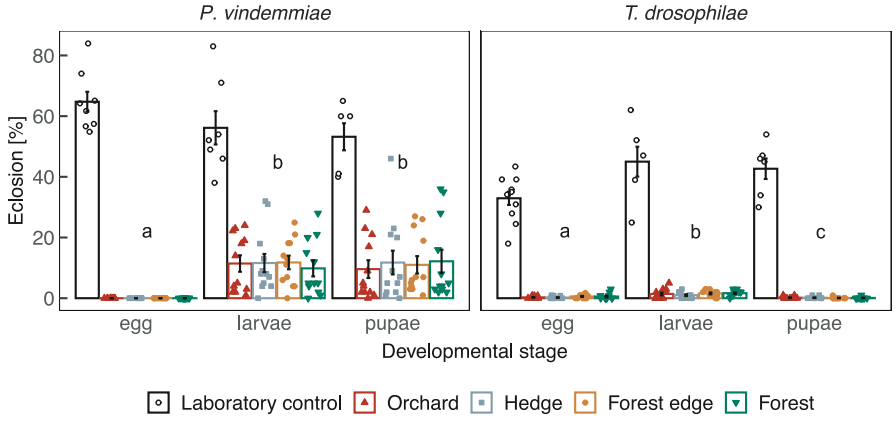

Fig. 3. | Parasitoid eclosion after winter field exposure. The eclosion from the egg, larval, and pupal stage of *P. vindemmiae* (left) and *T. drosophilae* (right) after winter field exposure in the habitats forest, forest edge, hedge, and orchard over both experimental seasons are displayed. Each dot represents one site, while the bars indicate the average and the standard error across 12 sites. Grey dots display the eclosion from the control samples without field exposure and acclimatisation, while the corresponding bars display the average and standard error over 8–11 replicates. $N = 100$–120 for experimental samples and $N = 50$–120 for the control. Letters indicate significant differences between developmental stages (Tukey post-hoc analysis).

preimaginal stage at the time of dissection. For *P. vindemmiae* larger deviations were detected in Season I, when larval and pupal stages were predominantly observed as pupae and pharates.

3.3. Parasitoid phenology

The weekly average temperature declined over the experimental period (Spearman correlation, $r = -0.93$, $df = 14$, $p < 0.001$, Figure S3 D), while the relative humidity remained high (82.81 ± 5.17%, $\bar{x} \pm SD$). The parasitism rate was positively associated with weekly average temperature for both *P. vindemmiae* (GLM with quasi-binomial distribution, $\beta = 0.36$, $SE = 0.04$, $p < 0.001$) and *T. drosophilae* (GLM with quasi-binomial distribution, $\beta = 0.25$, $SE = 0.03$, $p < 0.001$, Fig. 4, Fig. S3 A and B). Parasitism by both parasitoid species ceased completely after the weekly average temperature dropped below 10°C to an average of 4.37 ± 0.66°C ($\bar{x} \pm SD$) during CW 45 to CW 49 (Fig. S3 A and B). The weekly average temperature had no effect on *P. vindemmiae* and *T. drosophilae* eclosion from samples transferred to the laboratory (Fig. 4). While on average 94.18 ± 6.62% ($\bar{x} \pm SD$) of *T. drosophilae* eclosed, the average eclosion rate of *P. vindemmiae* was much lower and fluctuated strongly between calendar weeks and replicates (31.42 ± 31.48%). Fly eclosion in both parasitoid treatments was low at the beginning and then increased to levels recorded in the no-parasitoid control cages in CW 45 (Figure S3 C).

Offspring eclosion under field conditions in autumn occurred exclusively from host pupae parasitized in CW 34 and CW 35 (August) after 232 ± 8 and 272 ± 30 ($\bar{x} \pm SD$) DD for *P. vindemmiae* and *T. drosophilae*, respectively. Eclosion in the following spring occurred in May for *P. vindemmiae* after 313 ± 82 DD and between February and May for *T. drosophilae* after 299 ± 56 DD (Table S4). While *P. vindemmiae* eclosed from host pupae parasitized in August until mid-October (CW 34–41), *T. drosophilae* only occasionally eclosed from host pupae parasitized after mid-September (CW 37, Fig. S4).

3.4. Host availability

The number of eclosed *Drosophila* spp. individuals from banana-baits differed between habitats. An earlier cessation occurred in semi-natural habitats compared to orchards where there were more than twice as many individuals (Fig. 5). The weekly average temperature declined over the experimental period (Spearman correlation, $r = -0.94$, $df = 10$, $p < 0.001$).

The probability of offspring production (zero hurdle model) was positively associated with weekly average temperature and the number of drosophilids trapped (Table 3). The number of eclosed drosophilids from banana-baits (count model, Table 3) was positively associated with weekly average temperature and significantly affected by the habitat (Hurdle with negative binomial distribution, $X^2 = 21.92$, $df = 3$, $p < 0.001$). Fewer eclosions were observed in forests (Tukey, $p < 0.001$) and forest edges ($p < 0.001$) compared to orchards (Fig. 5).

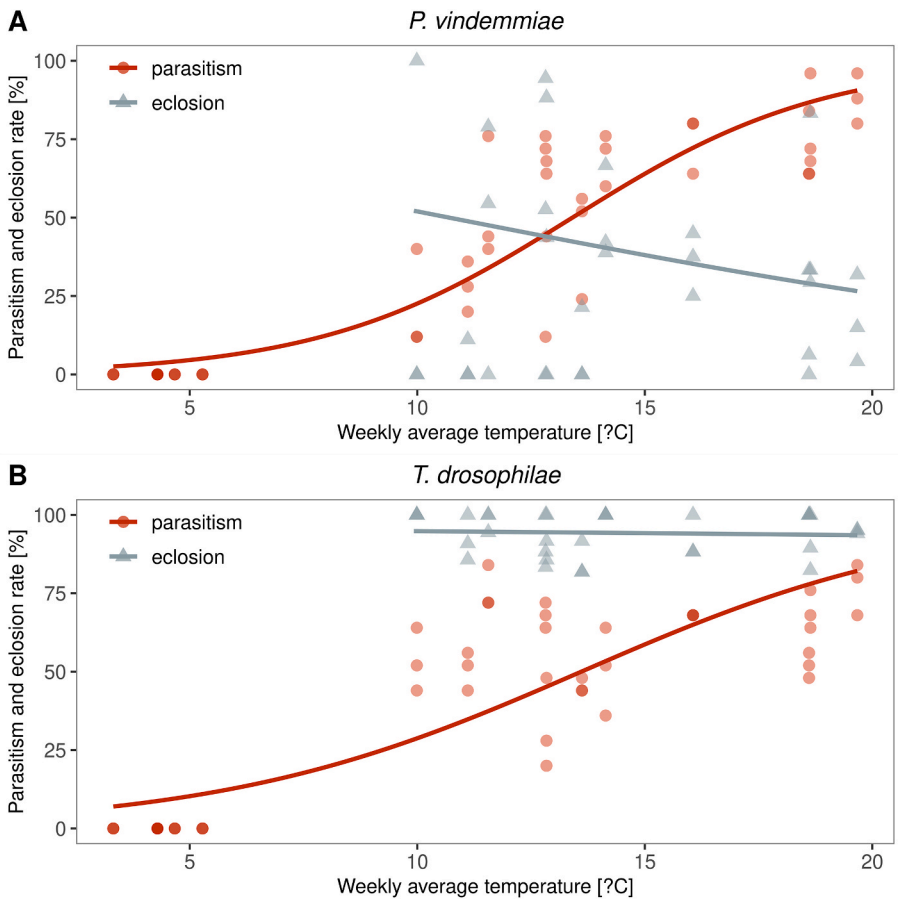

Fig. 4. | Parasitism in field cages and subsequent parasitoid eclosion. The parasitism rate in field cages (red dots) in response to the weekly average temperatures from mid-August (CW 34) through early December (CW 49) in 2019 and the subsequent parasitoid eclosion rate in the laboratory (grey triangles) are displayed for *P. vindemmiae* (A) and *T. drosophilae* (B). Each dot or triangle represents the parasitism or eclosion rate of a sample ($N = 25$, 3 replicates per CW, 48 samples in total). The parasitism rate is defined as the number of parasitized *D. subobscura* pupae (i.e., eclosed parasitoids plus the number of host pupae containing dead parasitoids) divided by the sample size. The eclosion rate is defined as the number of eclosed parasitoids divided by the number of parasitized host pupae. The logistic regression curves display parasitism (red) and eclosion rates (grey) in response to weekly average temperature as predicted by the GLMs with binomial (*T. drosophilae* eclosion) or quasi-binomial distribution (*P. vindemmiae* and *T. drosophilae* parasitism, *P. vindemmiae* eclosion).

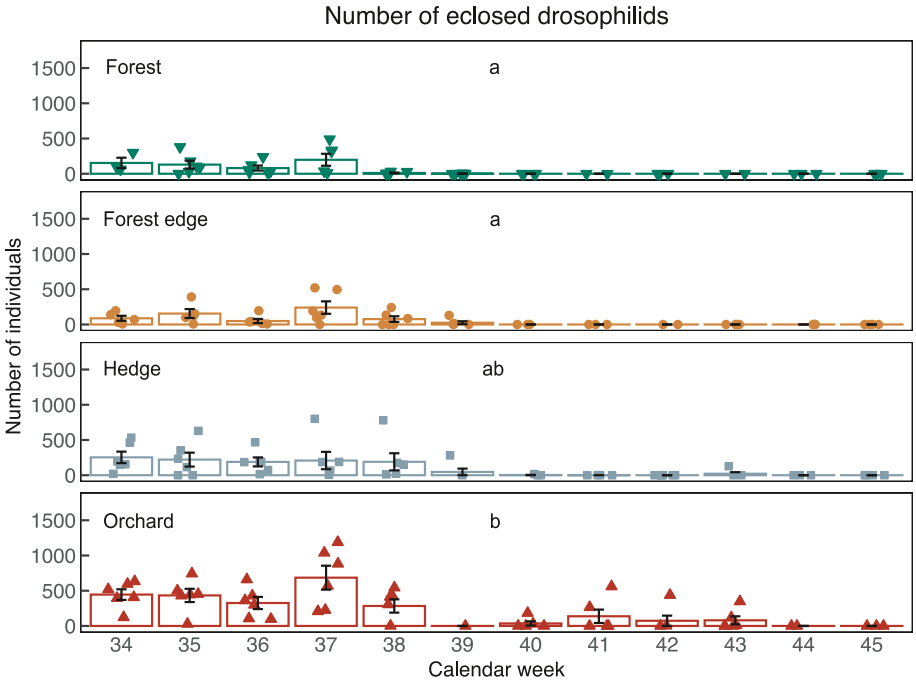

Fig. 5. | Host availability in semi-natural habitats and orchards. The number of drosophilids that eclosed from banana-baits placed in orchards and the three semi-natural habitats forest, forest edge, and hedge from mid-August (CW 34) to early November (CW 45) in 2019 are displayed. Each dot represents one site, while the bars indicate the average and the standard error across 10 sites. Letters indicate significant differences between habitats (Tukey post-hoc analysis).

Table 3
Influence of habitat and temperature on *Drosophila* spp. eclosion. The model coefficients of the two-part hurdle model are displayed. The count model (negative binomial distribution with log link) assessed the effect of habitat and weekly average temperature on the number of eclosed drosophilids from banana-baits exposed in orchards (i.e., intercept), hedges, forest edges and, forests in autumn 2019. The zero hurdle model (binomial distribution with logit link) assessed the effect of weekly average temperature and the number of trapped drosophilids on the offspring production (irrespective of their number). Continuous variables (weekly average temperature and number of trapped drosophilids) were scaled to assure equal contribution to the analysis using the z-score standardization. Significant p-values are indicated in bold.

Count model coefficients (truncated negative binomial with log link)

Variable	Estimate	Std. Error	z value	Pr(>|z|)
(Intercept)	5.67	0.21	27.15	**< 0.001**
Hedge	−0.57	0.27	−2.07	**0.038**
Forest edge	−1.1	0.28	−3.91	**< 0.001**
Forest	−1.24	0.29	−4.26	**< 0.001**
Weekly average temperature	0.46	0.17	2.68	**0.007**
Log(theta)	−0.19	0.13	−1.4	0.163

Zero hurdel model coefficients (binomial with logit link)

Variable	**Estimate**	**Std. Error**	**z value**	**Pr(>|z|)**
(Intercept)	9.3	2.51	3.7	**< 0.001**
Weekly average temperature	2.06	0.44	4.69	**< 0.001**
Number of trapped drosophilids	32.46	7.71	4.21	**< 0.001**
Log-likelihood = −811.1, *df* = 9				

In total 897 specimens belonging to seven *Drosophila* spp. were identified to species level in the subsamples of eclosed drosophilids. *Drosophila simulans* (Diptera: Drosophilidae) was most abundant with a total of 335 identified individuals, while *D. suzukii* was rarest with only 20 individuals (Fig. 6). The observed species diversity was higher in semi-natural habitats than in orchards (Shannon diversity, forest: $^1D = 6.72$, forest edge: $^1D = 5.28$, hedge: $^1D = 5.59$, orchard: $^1D = 4.53$), yet no difference was found within the community composition between the habitats and sampling months (Fig. S5).

4. Discussion

Our study together with laboratory data from Amiresmaeili et al. (2020) demonstrates that preimaginal life stages of both *P. vindemmiae* and *T. drosophilae* possess the highest cold tolerance and are able to overwinter under field conditions in Switzerland. Parasitism by both parasitoid species ceased when field temperatures permanently dropped below 10°C in late October. Overwintering survival did not differ between semi-natural habitats and orchards despite buffered temperature extremes in semi-natural habitats. While the number and temporal availability of potential overwintering hosts was higher in orchards, *Drosophila* spp. diversity was higher in semi-natural habitats.

4.1. Cold tolerance of parasitoid life stages

In the laboratory, *P. vindemmiae* adults tolerated temperatures of 0°C or lower only for a few days. Eggs did not survive extended periods of

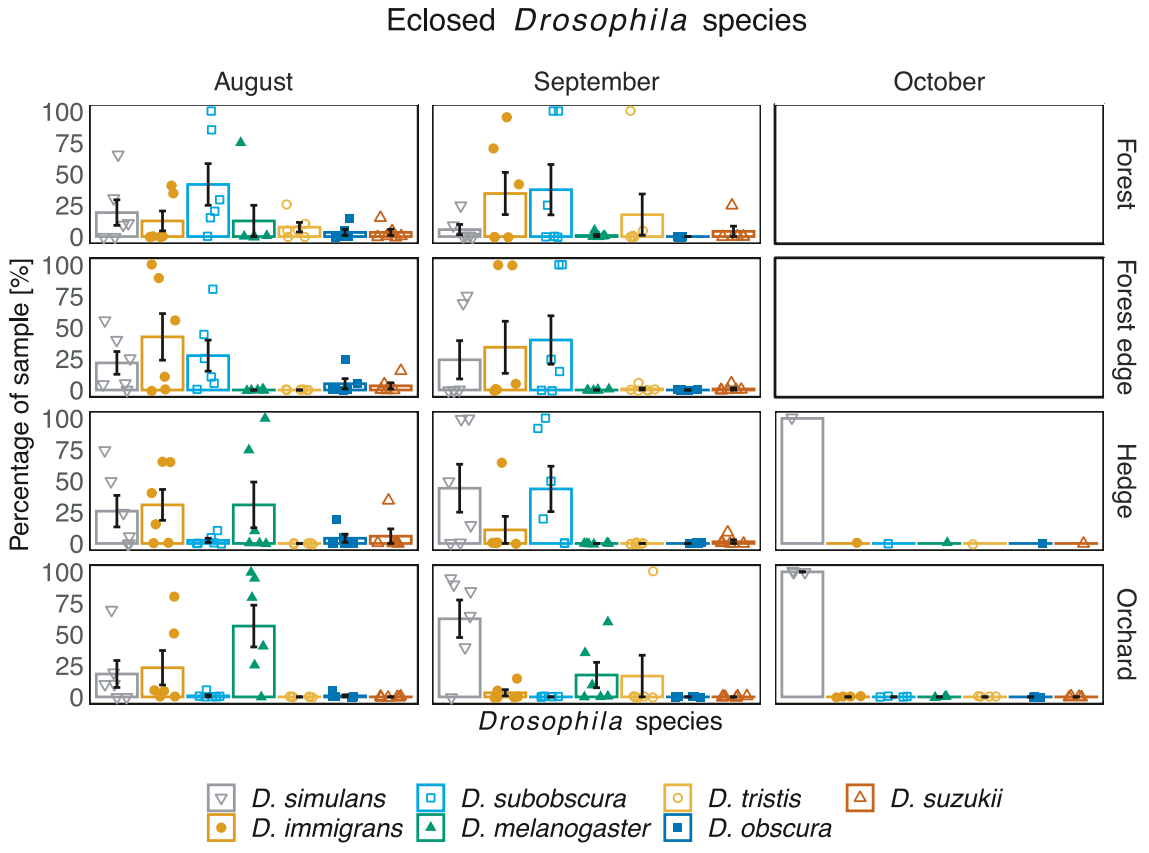

Fig. 6. | Eclosed *Drosophila* species. The percentage of eclosed drosophilids in a random subsample ($N = 1$–20) from banana-baits placed in orchards and the three semi-natural habitats forest, forest edge, and hedge in August (CW 34 and 35), September (CW 36 to 39) and October (CW 40 to 43) are 2019 is displayed. Each habitat is represented with 6 samples in the months of August and September, while in October the habitats are represented by 4 (orchard), 1 (hedge) or no samples (forest and forest edge). Each dot represents a random subsample from one experimental site, while the bars indicate the average and the standard error across the subsamples.

cold under both laboratory condition and winter field exposure. In contrast, around 50% of the larvae and pupae survived 1 month at 0°C in the laboratory and 11% survived winter field exposure. Based on this evidence, we conclude that *P. vindemmiae* most likely overwinters as larva or pupa.

In the case of *T. drosophilae*, all preimaginal stages survived winter field exposure, confirming the results from an earlier laboratory study (Amiresmaeili et al., 2020). Pupae of *T. drosophilae* were the most vulnerable stage followed by eggs and larvae. These results, however, need to be taken with caution since numbers analysed were very low (Fig. 3). The parasitoid may also overwinter as an adult, since some honey-fed adults were shown to tolerate constant temperatures of 0°C for about 20 d and −5°C for about 10 d (Amiresmaeili et al., 2020). Winter field temperatures in the soil microhabitat in our study rarely dropped below 0°C and never lower than −3°C. Over a period of eight years (from 2010 through 2018) the meteorological station at the institute recorded 4 periods of 7 to 22 d with continuous frost in the upper soil layer.

Interestingly, the overwintering survival and cold tolerance of *T. drosophilae* was considerably lower than expected based on previous results for the same parasitoid colony (Amiresmaeili et al., 2020). Differences in temperature steps and length of cold acclimatisation between the two studies likely caused the different results. In our study, cold acclimatisation prior to field exposure negatively affected parasitoid survival. The mortality in controls subjected to acclimatisation was two-fold higher compared to those without acclimatisation (Fig. S6). Additionally, negative, non-lethal effects caused by cold acclimatisation have been reported by Colinet and Boivin (2011), which might further have affected survival of field exposed parasitoids. Also, we cannot exclude that *T. drosophilae* lost some of its cold tolerance due to adaption to laboratory rearing conditions, as it was, for example, observed in the fly *Pseudosarcophaga affinis* (Diptera: Sarcophagidae, House, 1967). In our study, only 40% of non-acclimatised laboratory control samples of *T. drosophilae* eclosed from *D. subobscura* (Fig. 3), whereas 60–80% eclosion were reported by Rossi-Stacconi et al. (2017) and Wang et al. (2018) using *D. melanogaster* and *D. suzukii* as hosts. This suggests a suboptimal quality of the *D. subobscura* host. Suboptimal host quality is not expected to affect *P. vindemmiae* survival as strongly as *T. drosophilae*, since the species displays a notable physiological flexibility and ability to adapt to different host sizes (Rossi-Stacconi et al., 2013). It was even able to successfully parasitize partially decomposed host pupae from the overwintering field samples after their transfer to the laboratory (personal observation).

Many insect species are known to pass the winter in a diapausing stage, and diapause may increase insect cold tolerance (Denlinger, 2008). Starting our experiments, we assumed no diapause as this phenomenon has not been observed in *P. vindemmiae* (Pickens et al., 1975; Aluja et al., 1998). Similarly, for *Trichopria* species no evidence for diapause was found, but literature is scarce (but see O'Neill, 1973). Based on our results, we assume that the insects used in our study either received adequate cues for entering a diapause or that no such phenomenon exists in both examined species. Specifically, a number of *P. vindemmiae* individuals survived the winter in the field. The low survival of *T. drosophilae* after winter field exposure matched the eclosion rate from eggs laid during September in the parasitoid phenology experiment (Figure S3 B), where both the ovipositing female and the developing offspring were exposed to the full set of cues for any potential diapause.

Overall, the observed cold tolerance in the field was similar to the results obtained in the laboratory assays with constant low temperatures, which agrees with Wang et al. (2018). Over prolonged periods of cold, *P. vindemmiae* pupae seem to be the most cold hardy life stage. Compared to *T. drosophilae*, *P. vindemmiae* appears much better adapted to cope with typical winter conditions in Switzerland. This may explain why *P. vindemmiae* is more abundant and widespread than *T. drosophilae*, which is abundant in the south but less frequently observed north of the Alps (Knoll et al., 2017; Trivellone et al., 2020). Likewise, the distribution of *P. vindemmiae* includes northern countries such as Denmark, Sweden and Canada (Noyes, 2019), while, to the authors' best knowledge, the most northern record of *T. drosophilae* was made in Germany (Herz et al., 2021).

4.2. Habitat effect on parasitoid overwintering survival

In general, temperature influences the overwintering success of insects through its effect on the metabolic rate and thus the energy reserve depletion (Sinclair, 2015; Williams et al., 2015). Warmer winter temperatures can negatively affect fitness and survival of insects due to increased energy consumption. For instance, larvae of *Eurosta solidaginis* (Diptera: Tephritidae) overwintering under the snow experienced warmer conditions that resulted in lower survival and fecundity in comparison to less sheltered counterparts (Irwin and Lee Jr, 2003). Likewise, simulated winter warming of 4°C increased energy reservoir depletion in the butterfly *Erynnis propertius* (Lepidoptera: Hesperiidae, Williams et al., 2012). Low temperatures, however, can induce chill injuries negatively affecting insect survival (Koštál et al., 2004). For example, survival of *T. drosophilae* preimaginal life stages was much lower after one month exposure to −5°C than to 0°C, while both temperatures are well above their supercooling point (Amiresmaeili et al., 2020). In our study, the average temperature and the parasitoid eclosion after winter field exposure did not differ among habitats, although semi-natural habitats buffered temperature extremes compared to orchards (Fig. 2 B). While our samples were exposed in soil, larger temperature fluctuations in less protected microhabitats such as leaf litter or bare ground could be expected (personal observation), which then could lead to differences in parasitoid overwintering survival. Also, a weak effect of habitat could have been masked by a variety of other factors independent from temperature, such as desiccation, suffocation, entomopathogenic fungi or predatory nematodes.

4.3. Parasitoid phenology

For *Drosophila* spp. to serve as overwintering hosts, their temporal availability must be in accordance with the parasitoids' phenology. Suitable overwintering hosts must be found prior to cessation of parasitism by the parasitoids, and parasitoids must reach their overwintering developmental stage before temperature drops below the developmental threshold.

In this study, parasitism by both parasitoid species was recorded until late October (Fig. S3 A, B). Although an effect of photoperiod cannot be completely excluded, the strong correlation between weekly average temperature and calendar week suggests temperature as a leading factor mediating host parasitism. No more parasitism by both parasitoid species was observed after field temperature dropped below 10°C to about 5°C in early November (Fig. 4, Fig. S3 A, B, D). Based on a preliminary assay in autumn 2018 (Fig. S7) and Colombari et al. (2020), we assume a parasitism threshold of 7–8°C for *T. drosophilae*. Similarly, parasitism by *P. vindemmiae* was exclusively observed for average temperatures above 8°C in the preliminary assay in 2018.

The observed parasitism and eclosion rates in the semi-field experiment differed from those reported by Rossi-Stacconi et al. (2017) and Wang et al. (2018) at similar temperatures under laboratory conditions. Although those authors used a different ratio of parasitoids to host pupae and a different *Drosophila* host species, differences may predominantly result from their use of constant thermal regimes. These fail to capture the effect of daily and seasonally fluctuating temperatures on parasitism and parasitoid eclosion rate (Delava et al., 2016). For example, a drop in the minimum temperature in our semi-field experiment below the survival threshold resulted in an increased mortality even if the average temperature remained well above this threshold (e.g., *P. vindemmiae* eggs, Fig. S3, Fig. S7). Likewise, when the maximum temperature is sufficiently high, parasitism may occur even at an average temperature

below the 7–8°C determined as the parasitism threshold. Furthermore, fluctuating thermal regimes may allow insects to repair heat or cold-induced damage (Colinet et al., 2016; Koštál et al., 2016). For example, the eclosion rate of the aphid parasitoid *Aphidius colemani* (Hymenoptera: Braconidae) increased by approximately 50% when exposed to 4°C with a daily heat peak (2 h at 20°C) in comparison to constant 4°C (Colinet et al., 2006).

In accordance with the estimated developmental threshold of about 10°C for both parasitoids (Wang et al., 2018), adult eclosion under field conditions during autumn 2019 was exclusively observed from host pupae parasitized in August (Fig. S4). Of these, adults eclosed in October shortly before the temperature dropped below 10°C. Further, *P. vindemmiae* eclosion in 2020 was restricted to May irrespective of the parasitism time, whereas eclosion of *T. drosophilae* was spread over several months. Hence, it appears that the conditions needed for eclosion after overwintering are different for the two parasitoid species.

4.4. Host availability in different habitats

To overwinter in preimaginal life stages, both parasitoid species depend upon finding suitable host pupae in autumn. We expected to find more reproductively active *Drosophila* spp. in semi-natural habitats compared to orchards due to their buffered microclimatic conditions (Suggitt et al., 2011; Tougeron et al., 2016) and a larger diversity of *Drosophila* spp. as reported previously (Basden, 1955; Burla and Bächli, 1991; Trivellone et al., 2020).

While our expectation was fulfilled regarding species diversity, we observed larger numbers of drosophilids in orchards (Fig. 5). The latter was most likely caused by the higher resource availability, such as fresh and rotting fruits as food, and composting heaps and human infrastructure as shelter (Boulétreau-Merle et al., 2003; Hughan and Dreves, 2014; Schou et al., 2015). It must be noted that the number of eclosed drosophilids as well as the observed frequency and species diversity are biased by trap bait attractiveness, intra- and interspecific competition over limited bait resources, and parasitism in the field. Therefore, our results should be regarded as indices of abundance reflecting the relative occurrence of various *Drosophila* species in their natural habitats. Nevertheless, the observed distribution of *Drosophila* spp. among habitats in this study is similar to faunistical records of drosophilids in Switzerland (Burla and Bächli, 1991; Trivellone et al., 2020) and southern Italy (Antonacci et al., 2017). Among the seven *Drosophila* spp. identified from subsamples of drosophilids eclosed from field-exposed bait traps, *D. simulans*, *D. immigrans*, *D. subobscura* and *D. melanogaster* made up the largest proportion. Although *D. suzukii* made up on average 21% of the drosophilids trapped at the time of collection, eclosion was only recorded in low numbers, since the pest enters a reproductive diapause in autumn (Rossi-Stacconi et al., 2016).

Not all *Drosophila* spp. are equally suitable as overwintering hosts. In our study, fewer adults of both parasitoid species eclosed from cold exposed *D. suzukii* pupae than from the smaller *D. melanogaster* and *D. subobscura*. Similarly, *Aphidius ervi* (Hymenoptera: Aphidiinae) that eclosed from larger aphid hosts after cold treatment displayed a reduced fitness compared to those eclosed from smaller ones (Ismail et al., 2012). Since the host is the only nutrient source for developing solitary pupal parasitoids, the host species limits the parasitoids' body size and the amount of energy reserves (Harvey, 2005; Kishani Farahani et al., 2016). Amongst other factors, these are known to affect insect cold tolerance (Chown and Gaston, 2010; Ismail et al., 2012; Sinclair, 2015). The absolute energy demand hypothesis predicts an increased energy demand by larger body size and thus lower fitness under stress (Blanckenhorn, 2005), explaining the observed differences in host-specific cold tolerance.

We have no information on the host suitability of some of the other commonly found *Drosophila* spp., but based on our records we can assume that there are sufficient suitable hosts available. Since the *Drosophila* community composition in all four habitats were similar besides some additional species recorded in semi-natural habitats (Fig. 6, Fig. S5), the availability of hosts for overwintering is unlikely a limiting factor for the parasitoids during fall. However, *P. vindemmiae* could profit from a higher host availability in orchards during summer and autumn, since the parasitoid is more abundant in agricultural habitats (Knoll et al., 2017; Kremmer et al., 2017; Trivellone et al., 2020) with recordings until early-October in Switzerland (Knoll et al., 2017). In contrast, *T. drosophilae* is less likely to benefit from a higher host abundance in orchards as it appears to be more common in semi-natural habitats and was almost exclusively recorded in August (Knoll et al., 2017; Trivellone et al., 2020). However, this may be different in regions with different climatic conditions, since the phenology of parasitoids and their hosts depends on the local climate in which they live. For example, trapping success of both *T. drosophilae* and *P. vindemmiae* was highest in November in cultivated habitats in south-eastern France (Kremmer et al., 2017).

5. Conclusion

Our results show that the microclimatic conditions within soil in orchards and the three semi-natural habitats tested were equally suitable for parasitoid overwintering, and that the availability of suitable overwintering hosts did not differ among the habitats. Future studies should focus on the effect of different microhabitats (e.g., bare soil, leaf litter, snow cover) on parasitoid overwintering success within different habitat types. Further, we demonstrate that *P. vindemmiae* is most likely overwintering in the larval or pupal stage while *T. drosophilae* can overwinter in all preimaginal life stages. In addition, it is possible that *T. drosophilae* can also overwinter as adults. This, however, needs to be confirmed in future studies.

Since both pupal parasitoid species are known to attack *D. suzukii* under laboratory conditions (Knoll et al., 2017; Miller et al., 2015; Rossi-Stacconi et al., 2013), and *T. drosophilae* was successfully used to reduce *D. suzukii* infestation in cherry orchards through early season releases in semi-natural habitats (Rossi-Stacconi et al., 2019), knowing their overwintering ecology could improve the biological control of *D. suzukii*. First, in the context of conservation biocontrol, knowledge of the requirements for successful overwintering of parasitoids will allow the protection and promotion of overwintering habitats (Gontijo, 2019). Second, the phenology of parasitoids and their hosts determines the ideal release period for augmentative releases. Third, knowledge about the critical temperatures for the overwintering life stage could permit the estimation of the overwintering success based on the winter temperatures in a specific year and thus the level of biocontrol provided in the following fruiting season.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

All raw data are stored in a repository, as cited in the MS.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jtherbio.2022.103231.

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