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Cold tolerance of the mealybug parasitoid *Anagyrus vladimiri*

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Abstract We investigated the lower thermal limits of *Anagyrus vladimiri* Triapitsyn (Hymenoptera: Encyrtidae), a natural enemy of mealybugs. Parasitoids were cooled to measure supercooling points and the lower lethal temperature LLT_{50}. To investigate survival after long-term cold exposure, parasitoid adults and eggs, larvae, and pupae within their host mummy were gradually acclimated. Adults were then exposed for three days to 7 °C, 5 °C, 3 °C, and 1 °C, and immatures for varying durations to 5, 1, and − 4 °C. Parasitoids were investigated for survival and reproduction. To assess the impact of fluctuating temperature, parasitoid pupae were subjected to daily warming to 10 °C from baseline temperatures of 5 °C, 1 °C, and − 4 °C during four-day-cold exposure. Finally, eggs, pupae and adults were exposed to winter conditions in Switzerland in a semi-field setup. The LLT_{50} was − 17.24 °C for adults and 0.94 °C for pupae. Both values were above the supercooling points. No adult survived three days at 3 °C and lower. Likewise, no emergence occurred from eggs or larvae exposed for four days and longer to 1 °C or seven days to 5 °C. Pupae were cold-hardier surviving seven days at 5 °C and three days at 1 °C. Parasitoids surviving cold exposure were still able to reproduce. Daily warming decreased emergence of cold exposed pupae at a baseline temperature of 1 °C but not at 5 °C and − 4 °C. No eggs, pupae and adults survived winter conditions in the semi-field experiment. We thus consider *A. vladimiri* a chill-susceptible species with very limited cold-tolerance of the investigated population and low chances of survival during winter in Northern Switzerland.

Keywords Cold hardiness · Overwintering · *Anagyrus pseudococci* · Parasitoid · Biological control · Encyrtidae

Introduction

Cold tolerance is an important factor for the success and environmental safety of biological control agents. It determines the capacity to store insect biocontrol agents at low temperature as well as their survival during cold periods in the greenhouse or field. The latter may be desired for native and classical biological control agents. In greenhouses, however, exotic agents are often used in augmentative biological control, where winter survival and establishment are not desired and considered to increase the risk of lasting

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non-target effects (Hatherly et al. 2005; Boivin et al. 2006).

Mealybugs (Hemiptera: Pseudococcidae) are important agricultural pests in the greenhouse and in the field, causing substantial damage by weakening the plants, transmitting viruses and excreting honeydew, which often leads to sooty molds (Charles 1982; Ben-Dov et al. 1997; Sether et al. 1998; Tsai et al. 2010; Daane et al. 2012). The fact that mealybugs have a protecting wax cover and often hide inside tiny crevices or under bark makes them difficult to control with insecticides (Walton and Pringle 2004; Daane et al. 2012; Mani and Shivaraju 2016; Sharon et al. 2016) and thus are interesting targets for biological control. *Anagyrus vladimiri* Triapitsyn (Hymenoptera: Encyrtidae) is commercialized and widely used in the field and in greenhouses as a biological control agent against several mealybug species such as *Planococcus ficus* Signoret and *Planococcus citri* (Risso) since more than 25 years (Franco et al. 2008; Lucchi and Benelli 2018; EPPO 2021; Mouratidis et al. 2021). It is considered as one of the most common and effective parasitoids of mealybugs in vineyards (Mansour et al. 2023), either being released on large acreages (Lucchi and Benelli 2018) or providing natural regulation (Daane et al. 2012). It has further recently gained increased attention as a potential biocontrol agent against the invasive Comstock mealybug *Pseudococcus comstocki* (Kuwana), which has invaded several European countries including Switzerland, where it was found for the first time in 2016 and is causing damages in pome and stone fruit orchards (Terrettaz et al. 2020; Ricciardi et al. 2021; EPPO 2023).

Anagyrus vladimiri is part of the *Anagyrus pseudococci* species complex that has just recently been untangled (Andreason et al. 2019). In the literature, the species has previously been referred to as *Anagyrus* sp. near *pseudococci* and sometimes cited as *Anagyrus pseudococci* (Guerrieri and Pellizzari 2009; Ricciardi et al. 2021). *Anagyrus vladimiri* is of old-world origin and has been confirmed to be present in Europe (Spain, Italy), Israel, Russia, Turkmenistan, and Tunisia (Andreason et al. 2019). The species has been released in California (USA) to control *Planococcus* spp., and although it has been stated initially as non-persistent (Bartlett 1978), records of this species have been made there later on (Andreason et al. 2019). Daane et al. (2004) have examined the influence of temperature on *A. vladimiri* (cited as *A. pseudococci*, Andreason et al. 2019). They found that the species can successfully develop and hatch when exposed to winter temperatures in Berkeley, California, USA where average low winter temperatures are around 5–8 °C warmer than in Northern Switzerland. The minimum developmental temperature was 11.6 °C and the wasps could complete development between 14 and 34 °C, while no oviposition could be observed at 14 °C and below (Daane et al. 2004). In the related *Anagyrus ananatis* Gahan (Hymenoptera: Encyrtidae) it was found that survival of larvae and pupae during cool storage was affected by temperature, developmental stage and acclimation status (Pandey and Johnson 2005). While immatures stored at 14.8 °C for eight weeks had emergence rates comparable to the control, storage at 10.1 °C resulted in high mortality, especially in non-acclimated individuals (Pandey and Johnson 2005). A few other studies have investigated thermal requirements of *Anagyrus* species for successful development (Pandey 2002; Chong and Oetting 2006) and survival of adults during long-term storage at 20–27 °C (Sagarra et al. 2000). However, cold tolerance of insects is highly variable among life-stages and species and thus generalizations should not be made (Binazzi et al. 2015; Amiresmaeili et al. 2020). With the overall increased demand for biological control agents in greenhouses and the advancement of *P. comstocki* into non-Mediterranean countries such as Switzerland, an investigation into the biology of *A. vladimiri* in colder environments becomes pertinent. The present study was therefore conducted to investigate the lower thermal limits of *A. vladimiri* and to compare cold-hardiness among the different developmental stages. We chose to evaluate the three measures that are commonly used to assess cold tolerance in insects at the population level, supercooling points, lower lethal temperature, and lower lethal time (Hanson et al. 2013), complemented by an overwintering experiment under Swiss outdoor conditions.

Material and methods

Insects and rearing conditions

Planococcus citri originated from collections of infested greenhouse plants from the botanical garden

in Zurich, Switzerland. Identity was confirmed by molecular sequence analysis (D. Zwahlen, Agroscope). The mealybugs were reared on sprouting field bean *Vicia faba* and potatoes *Solanum tuberosum*. Mealybug egg sacks were added weekly with a brush onto fresh plants.

Anagyrus vladimiri adults were provided by Bioplanet (Cesena, Italy) and were originally collected in Northern Italy and then reared continuously on *P. citri*. They were supplied with small honey droplets and moist cotton pads. Sprouting potatoes with mealybugs (third instar and older) were offered as hosts. After 24 h of parasitization, the potatoes were transferred into 1.3 l ventilated vials until emergence of the next parasitoid generation, 23 ± 2 days later. All rearing took place in mesh cages of 47.5 × 47.5 × 47.5 cm (BugDorm-4E4545, Mega View science Co. Ltd., Taiwan) in climate-controlled chambers at 22 ± 0.3 °C, 70% RH, and a L:D 16:8 photoperiod.

The following parasitoid developmental stages were used in the experiments: (1) Adult females and males, 3–7 days after emergence and fed with honey, (2) pupae, 15–19 days after oviposition, (3) larvae, seven days after oviposition, and (4) eggs, within 24 h after oviposition. Larvae and eggs were only used in a subset of the experiments. Parasitized mealybugs were identified by a black scar on their body surface for containing eggs and larvae, whereas at the parasitoid pupal stage mummified mealybugs were collected. Only mealybug mummies with intact pupal cases and no visible deformations were selected for experiments. To confirm the desired parasitoid stage inside the mealybug several individual mealybugs from the same oviposition date were dissected.

Measurement of super-cooling points (SCPs)

The super-cooling point is determined to be the lowest temperature before the exothermic reaction of crystallization (Vigier and Vassoille 1987). Super-cooling points of adult and pupal *A. vladimiri* were measured using 0.255 mm diameter (30-gauge) type-T copper-constantan thermocouples (Omega, Hypodermic W/SMPW connector, Stamford CT, USA). The body of the parasitoid was glued ventrally with high vacuum grease (Dow Corning Corporation, USA) to the tip of the thermocouple and enclosed within a 2 ml microcentrifuge tube. The tubes with the insect and the thermocouple were placed inside a Styrofoam cube (pre-cooled to 5 °C) closed with a rubber stopper and deposited into a − 80 °C freezer (SGMDF-700VX, Panasonic). The standardized Styrofoam cube with 20 cm length has a cooling rate of around 1 °C min^{-1} (Carrillo et al. 2005). The thermocouples were connected to a laptop via an analog–digital converter (USB-Personal Daq/55, Measurement Computing, IOtech, USA). The current temperature was measured four times per second and was visualized with the program DASYLab (Version 11, 2010, National Instruments, Ireland).

SCP measurements of female and male adults were conducted with 20–21 individuals each. For SCP measurements of pupae (n = 40), the pupae were dissected from mealybug mummies and the pupal body was attached ventrally to the thermocouple without the pupal case. According to previous studies, SCP values may differ significantly upon daytime (Amiresmaeili et al. 2020), therefore, all SCPs measurements were started at 2 pm.

Short-term exposure to measure lethal temperature (LT)

Lethal temperatures were measured using the thermocouples described above. The thermocouples were enclosed within a 2 ml microcentrifuge tube containing five individuals (adults or pupae, mixed sex). The tubes with the insects and the thermocouple were placed inside a pre-cooled (5 °C) Styrofoam cube and added to the − 80 °C freezer. Samples with adults were cooled at a rate of − 1 °C min^{-1} to − 8 ± 1 °C, − 11 ± 1 °C, − 16.5 ± 1.5 °C, − 20 ± 1 °C and − 23.5 ± 1.5 °C, whereas samples with pupae were cooled to 4 ± 1 °C, 0 ± 1 °C, − 4 ± 1 °C, − 8.5 ± 1.5 °C, − 16.5 ± 1.5 °C and − 20.5 ± 1.5 °C (temperature categories; all means ± SE). When the designated temperature was reached, the Styrofoam cube with the parasitoids was removed from the − 80 °C freezer and the lowest temperature measured by the thermocouples was marked. The Styrofoam cube containing the insects was subsequently kept at room temperature until the thermocouples inside the tubes showed a temperature above 18 °C, then the insects were placed back to the 22 °C rearing chamber. Control samples were placed into the pre-cooled Styrofoam cube for 1 min, subsequently rewarmed to room temperature, and then put at 22 °C.

After rewarming, adult parasitoids were transferred into a 250 ml vial to examine the survival after 1 h and 24 h. Since this type of storage resulted in high mortality, adults in later experiments were provided with a humid cotton pad and honey for humidity and nutrition. The pupae were transferred into squared plastic vials (2.0 × 2.0 × 2.5 cm) and checked daily for hatching individuals. For each temperature category, a total of 80 individual adults (40 females, 40 males, n = 13–16) and 50–55 (n = 10–11) pupae were examined. The pupae, out of which no parasitoids had hatched, were dissected under the stereomicroscope and the developmental stage recorded.

Acclimation for long-term exposure

Before the long-term cold exposure, *A. vladimiri* were acclimatized stepwise to cold conditions. Different cooling devices were used to obtain the different temperatures: (1) 10 °C: a thermostatic chamber (Aqualytic TC 256, Tintometer GmbH), (2) 7 °C: a versatile climate chamber (MLR-352H, Panasonic), (3) 5 °C: a walk-in cold room, and (4) 3 °C and colder: a cooling box (CoolFreeze CFX28, Dometic).

From rearing at 22 ± 0.3 °C and a L:D 16:8 photoperiod, adult parasitoids were placed to 10 ± 0.1 °C and a L:D 14:10 photoperiod and then to 7 ± 0.2 °C and a L:D 12:12 photoperiod and finally to 5 ± 0.1 °C and a L:D 10:14 photoperiod for two days each. Pupae, larvae and eggs were acclimated at 10 ± 0.1 °C and a L:D 14:10 photoperiod and then 7 ± 0.2 °C and a L:D 12:12 photoperiod for four days each. Acclimatized insects were subsequently exposed to the experimental cold treatments. Subsequently, *A. vladimiri* were re-acclimated using the same steps in reverse order and with shorter duration, i.e., one day for the adults and two days per step for pupae, larvae and eggs.

Adult parasitoids were kept in groups of five inside cylindrical plastic vials (2 cm diameter × 6 cm height) during the acclimation, the experiment, and the re-acclimation. The vials for the adults were provided with honey, a piece of humid cotton pad, and a strip of paper towel for hiding. The pupal samples were kept in groups of five inside a squared plastic vial (2.0 × 2.0 × 2.5 cm) and embedded in cotton. For larval and egg samples, 20 parasitized mealybugs were placed on a potato inside a 250 ml vial. Control individuals always underwent the acclimation and re-acclimation steps as the experimental individuals, without the cold exposure step in between. Temperatures during acclimation were confirmed using a temperature logger (HOBO 64 K Pendant®, Onset Computer Corporation, Massachusetts, USA). RH was confirmed to be over 70% inside all experimental cooling devices.

Long-term exposure at constant temperature

Acclimated adults were exposed to one of the following temperatures for three days: 1 ± 0.3 °C, 3 ± 0.3 °C, 5 ± 0.1 °C, and 7 ± 0.2 °C (all in darkness). Survival was recorded 1 h and 24 h after re-acclimation. For each temperature treatment and the acclimation control in total 50 females and 50 males (n = 10) were tested. The pupae were exposed to the following temperatures for three and seven days: 5 ± 0.1 °C, 1 ± 0.5 °C, −4 ± 0.2 °C. An additional treatment consisted of pupae exposed to 5 °C for 11 days. In addition to the acclimation control, batches of pupae were kept at constant 22 °C to demonstrate the hatching capability. For each cold treatment 100 pupae were tested, 50 pupae were tested for the hatching- and 50 pupae for the acclimation control. After re-acclimation, the pupae were surveyed for hatching individuals. Pupae, out of which no adult parasitoids had hatched for at least 37 days, were dissected and the stage of the insect body inside the pupal case was reported. Larvae and eggs were exposed to the following temperatures for four and seven days: 5 ± 0.1 °C, 1 ± 0.5 °C, or −4 ± 0.2 °C as well as for 11 and 14 days to 5 ± 0.1 °C or 1 ± 0.5 °C. For each treatment a total of 100 larvae and 100 eggs were used.

Long-term exposure at fluctuating temperature

To assess whether warming during periods of cold would enhance survival of pupae, we conducted an experiment with fluctuating temperatures. Samples with pupae were prepared and acclimated as described above. Based on meteorological information, test samples experienced a 6 h daily warming from the baseline temperature to 10 °C. As baseline temperatures 5 °C, 1 °C and −4 °C were chosen and samples were exposed to the respective treatment for four days. Control samples were continuously kept at the baseline temperatures. Additionally, an acclimation control was conducted where insect survival was

assessed after the acclimation process. For each treatment, ten boxes with each five pupae were used.

Reproduction after cold exposure

Females surviving cold treatments (treated at adult stage) and females that hatched from cold-treated pupae in the short and/or long-term exposure experiments were investigated for their fertility. Treatments for adults included short exposure, i.e., individuals were cooled to the respective temperature at 1 °C min^{-1} and subsequently rewarmed, to temperatures of − 10.4 °C to − 7 °C (n = 21), and long-term exposure of three days to 7 °C (n = 20), or three days to 4 °C (n = 20). Control individuals (n = 21) were taken directly from the rearing. The females were added individually to an 800 ml ventilated plastic vial together with two untreated adult males from the rearing. The vial contained honey, a wet cotton pad, and two field bean plants on which ten living mealybugs (third nymphal stage and young adults) were placed. Offspring presence and sex were recorded after eight weeks. Since in Hymenoptera diploid offspring develop into females while haploid offspring develop into males, the presence of female offspring was used as indicator for sexual reproduction. For females emerging from cold-treated pupae, individuals were tested that had either experienced short exposure to temperatures of − 1 °C to 4.7 °C (n = 30), or long-term exposure of three days to 5 °C (n = 39), seven days to 5 °C (n = 21) or 11 days to 5 °C (n = 4). Control individuals (n = 27) were taken from the rearing as pupae and emerged in squared plastic vials.

Semi-field experiments

Semi-field experiments were conducted in Zurich, Switzerland during winters 2020/2021 and 2022/2023. The temperature during the experimental periods was measured hourly with temperature loggers (HOBO 64 K Pendant®, Onset Computer Corporation, Massachusetts, USA) placed within the boxes.

Cylindrical plastic vials (2 cm diameter × 6 cm height) provided with honey, a piece of humid cotton pad, and a strip of paper towel for hiding, containing five adults were placed inside a plastic box (50 × 30 × 20 cm) located on a table at 1 m height under a shelter. Ten replications were performed with males and females assigned randomly to the vials, resulting in a total of 50 examined adult females and 50 adult males. After acclimation, the adults were exposed to outside temperatures for 14 days from 16 to 30 January 2021. Afterwards, insects were re-acclimatized to 22 °C (see above) and survival was recorded after 1 h. As a control, ten vials with females and ten with males were acclimated, re-acclimated and then kept for 14 days at 22 °C. Another 20 newly emerged adult females and males were kept at 22 °C 70% RH, and a L:D 16:8 photoperiod to assess lifespan under optimal conditions. They were kept individually in 250 ml vials supplied with honey and water ad libitum and checked every other day for survival.

Similar to the adult parasitoids, 50 pupae, separated into groups of five each within a plastic box as described above were exposed to field conditions for 14 days from 21 December 2020 to 3 January 2021. Subsequently, the pupae were brought to the laboratory, re-acclimated and examined daily for their hatching ability. Each 50 pupae were kept at 22 °C or received the acclimation treatment without outdoor exposure. Pupae, out of which no parasitoid hatched, were dissected and the stage inside was reported.

To obtain parasitoid eggs, two field bean plants carrying 30 living mealybugs (third nymphal stage and young adults) each were offered to parasitoids in the rearing cage during 24 h. After parasitization, the plants were transferred separately into 800 ml ventilated plastic vials. One plant was kept in the laboratory as hatching control, whereas the other was placed inside a Styrofoam cube filled with leaf litter under the shelter outdoors. Between the lid, which was held in place by six toothpicks, and the lower part of the Styrofoam cube an 0.5 cm gap allowed gas and temperature exchange. The experiment was repeated ten times between 12 October and 10 May (Fig. 5). Samples were placed outdoors during mid-day when temperatures were at least 10 °C. No samples were exposed in weeks when this condition could not be met. Emergence from controls was assessed four weeks after parasitization, whereas emergence from field samples was checked weekly from 12 April onwards until 24 May 2023, when all samples were transferred to the laboratory and kept for another four weeks with regular checks for emergence.

Data processing and statistical analysis

The data analysis was conducted using R version 4.2.1 (R Core Team 2023). Initial models contained all possible two-way interactions and were subsequently simplified.

Super-cooling points (SCPs)

Residuals of mean SCPs were confirmed to be normally distributed by Shapiro–Wilk test and homoscedasticity was confirmed by Levene's test. The mean SCPs were analyzed by one-way ANOVA and the differences of SCP means between adult female, adult male and pupa were analyzed using Tukey post-hoc test.

Lower Lethal temperatures (LLT)

The temperatures, at which 50% of adults and pupae survive (LLT_{50}), were calculated by logistic regression. For the calculation of the LLT_{50}, the minimum temperature of each thermocouple measurement was used. For further analysis of the LLT experiments, the temperature categories instead of the minimum temperatures were used. Since groups of each five adults and pupae were evaluated in the assays, numbers of survived and dead males and females and of individuals hatching from pupae from each vial were compared among treatments to avoid pseudoreplications. The influence of temperature category and sex as explanatory variables on adult survival after 1 h were modelled as binomial response variables using generalized linear models (GLM) with a logit link function followed by Dunn's post-hoc tests that employed a Bonferroni-Holm correction to adjust for multiple comparisons. Due to overdispersion a model from the binomial family was chosen that contained an additional estimate of a dispersion coefficient to analyze the influence of temperature category on hatching ability of pupae.

Long-term exposure

The survival of adults and the hatching capability of pupae, larvae and eggs after long-term exposure to cold temperatures were analyzed using generalized linear models with binomial distributions and a logit link function followed by Dunn's post-hoc tests that employed a Bonferroni-Holm correction to adjust for multiple comparisons. Since groups of each five adults and pupae and groups of 20 larvae and eggs were evaluated in the assays, numbers of survived males and females and of individuals hatching from pupae, larvae and eggs from each vial were compared among treatments to avoid pseudoreplications. For adults, the influence of the factors temperature and sex on survival after 1 h and 24 h was examined. To evaluate the hatching ability of pupae, larvae and eggs after long-term cold exposure, the combination of temperature and time was aggregated into a single factor "temperature–time". This allowed the inclusion of the hatching control and acclimation control (which do not possess a time and temperature component) into the analysis and can be justified by the accumulation of cold damage that results from prolonged exposure to cold.

Constant vs. fluctuating temperatures

The hatching capability of pupae after four days exposure to either constant or fluctuating temperatures at different baseline cold temperature were analyzed using generalized linear models with binomial distributions and a logit link function followed by Dunn's post-hoc tests that employed a Bonferroni-Holm correction to adjust for multiple comparisons. Since groups of each five were evaluated in the assays, numbers of individuals hatching from pupae from each vial were compared among treatments to avoid pseudoreplications. The influence of the factors baseline temperature and temperature fluctuation on hatching ability was evaluated.

Reproduction after cold exposure

The ability to reproduce and to reproduce sexually (i.e., to produce female offspring) after exposure to cold either in the adult or pupal stage was analyzed using generalized linear models with binomial distributions and a logit link function.

Semi-field experiment

No statistical analyses were conducted due to non-emergence or low numbers of samples.

Results

Super-cooling points (SCPs)

The super-cooling points differed significantly among females, males, and pupae (ANOVA: $F_{2,\ 78} = 76.02$, $p < 0.001$). Pupal SCP (-25.83 ± 0.31 °C, mean ± SE) was significantly lower than male SCP (-21.68 ± 0.62) and female SCP (-18.94 ± 0.39 °C). Male SCP was significantly lower than female SCP (Tukey post-hoc test: all $p < 0.001$; Table S1).

Short-term exposure: Lethal temperatures (LT)

Short-term exposure to low temperatures had a significant effect on adult survival recorded 1 h after rewarming to 22 °C ($\chi^2 = 385.71$, df = 5, $p < 0.001$) (Fig. 1a, b). The effect differed between sexes ($\chi^2 = 4.36$, df = 1, $p = 0.037$) and the interaction between sex and temperature was not significant. Females in the control survived equally well to those shortly exposed to − 8 °C and − 11 °C (Fig. 1a; Table S2). When exposed to − 16.5 °C and below, survival was significantly reduced (Dunn test, all: $p \leq 0.013$). Also in males there was no significant difference in survival between the control and those exposed to − 8 °C and − 11 °C (Fig. 1b). Survival at − 16.5 °C was significantly lower than at the higher temperatures, but significantly higher than at − 23.5 °C (Dunn test, all: $p \leq 0.023$; Table S3). The LLT_{50} for adult *A. vladimiri* (both sexes combined) 1 h after the cold exposure was calculated to be − 17.24 °C. After 24 h the survival had decreased approximately by 50% across all treatments, including the control. Therefore no further analyses were conducted for survival after 24 h.

Short-term exposure to low temperatures also had a significant effect on hatching ability of pupae ($\chi^2 = 59.36$, df = 6, $p < 0.001$; Fig. 1c, Table S4). No significant difference was detected in the hatching rate of the control compared to 4 °C and 0 °C. At all lower temperature categories hatching was significantly reduced, except for the comparison with 0 °C (Dunn test, all: $p \leq 0.003$). The temperature, at which 50% pupae survived (LLT_{50}) was 0.94 °C. From the non-emerged pupae 73% were in the pupal stage, 15% undeveloped and 7% adults inside the pupal case, for 5% the state could not be determined.

Long-term exposure at constant temperature

Long-term exposure to low temperatures had a significant effect on adult survival recorded 1 h after rewarming ($\chi^2 = 519.99$, df = 4, $p < 0.001$), but not sex or the interaction between temperature and sex (Fig. 2a). No difference in survival was observed between the acclimation control, and long-term exposure at 7 °C and 5 °C, whereas no survival was observed at 3 °C and 1 °C (Dunn test, all: $p \leq 0.001$; Table S5). Survival 24 h after rewarming did not change for the acclimation control, yet it was reduced by about 3% in individuals exposed to 7 °C and 12% in individuals exposed to 5 °C (data not shown).

In the case of pupae, the aggregated factors temperature and exposure time had a significant effect on hatching ($\chi^2 = 501.82$, df = 8, $p < 0.001$, Fig. 2b).

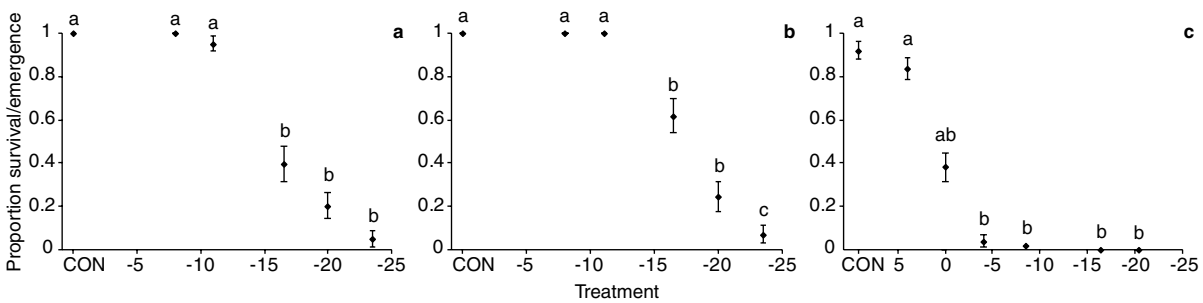

Fig. 1 Survival (mean proportion ± SE) of *Anagyrus vladimiri* after short-term exposure to cold: (**a**) females, (**b**) males, (**c**) pupae. For adults, survival was recorded 1 h after rewarming to 22 °C. Survival of pupae was measured as hatching success from pupae after exposure. Different letters above bars indicate significant differences (GLM with binomial distribution followed by Dunn post-hoc test, $p \leq 0.05$). CON: individuals were placed into Styrofoam cube cooled to 5 °C and rewarmed subsequently

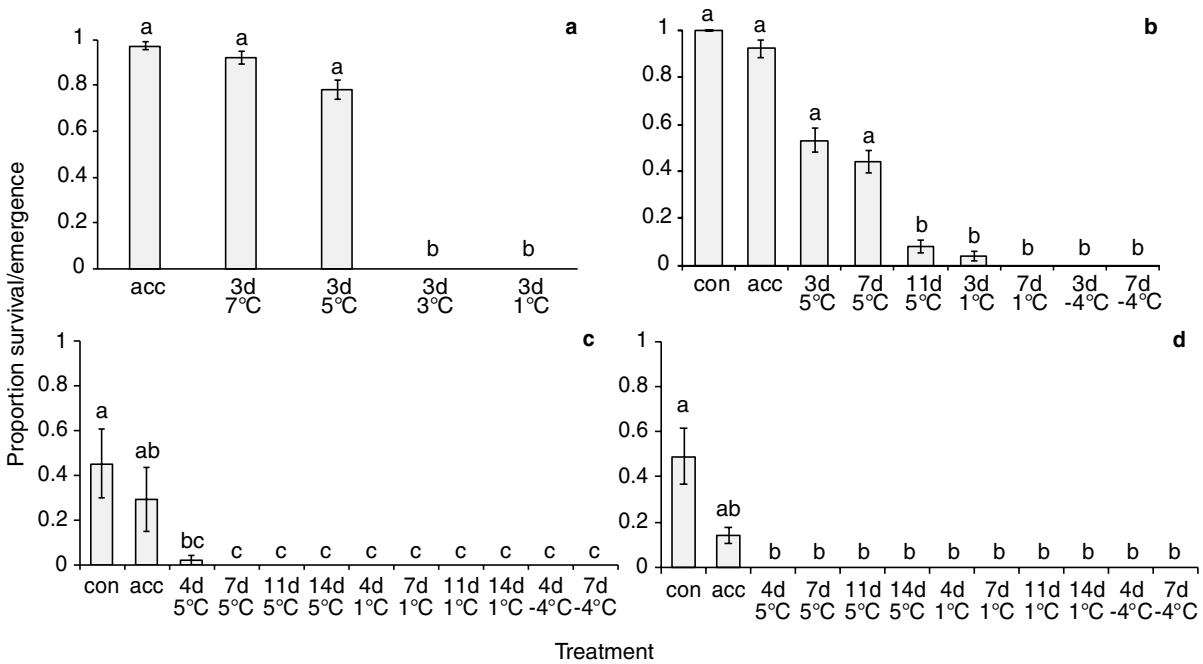

Fig. 2 Survival (mean proportion ± SE) of *Anagyrus vladimiri* after long-term exposure to cold: (**a**) adults, (**b**) pupae, (**c**) larvae, (**d**) eggs. For adults, survival was recorded 1 h after rewarming to 22 °C. Survival of immatures was measured as hatching success from pupae after cold exposure. Different letters above bars indicate significant differences (GLM with binomial distribution followed by Dunn post-hoc test, $p \leq 0.05$). con: Hatching control; acc: individuals exposed to acclimation only

Hatching was not significantly different between the hatching control, acclimation control, three days 5 °C and seven days 5 °C but it was significantly reduced in all other cold exposure treatments (Dunn test, all: $p \leq 0.003$; Table S6). From the non-emerged pupae, 70% were in the pupal stage, 13% undeveloped and 7% adults inside the pupal case.

Likewise, after long-term exposure of larvae, hatching was significantly affected by the factor temperature–time ($\chi^2 = 264.37$, df = 11, $p < 0.001$; Fig. 2c). While the acclimation process itself had no significant effect on hatching, hatching in all cold treated samples was significantly lowered to 0%, except for the four days 5 °C treatment, which was not significantly different from the acclimation control (Table S7).

Also, eggs were significantly affected by temperature–time after long-term exposure to cold ($\chi^2 = 270.06$, df = 11, $p < 0.001$; Fig. 2d). Hatching in all cold treated samples was reduced to 0%, which was significantly different from the control, but not from the acclimation control (Table S8).

Long-term exposure at fluctuating temperature

The factor temperature ($\chi^2 = 128.59$, df = 2, $p < 0.001$) but not the factor fluctuation significantly affected hatching of pupae. The interaction of the two factors however was significant ($\chi^2 = 13.54$, df = 2, $p = 0.001$). At constant temperatures hatching after exposure to 1 °C was only marginally reduced compared to 5 °C (Dunn test, $p = 0.054$), but hatching after exposure to − 4 °C was significantly lower compared to 5 °C ($p < 0.001$) and 1 °C ($p = 0.029$; Table S9). At fluctuating temperatures hatching after exposure to 1 °C and − 4 °C was significantly reduced compared to the 5 °C treatment (both $p < 0.001$) but no difference was observed between 1 °C and − 4 °C (Fig. 3; Table S10). At 5 °C no significant difference between the constant and the fluctuating temperature treatment was observed, whereas hatching after exposure to 1 °C with daily fluctuation was significantly reduced compared to constant 1 °C ($p = 0.01$).

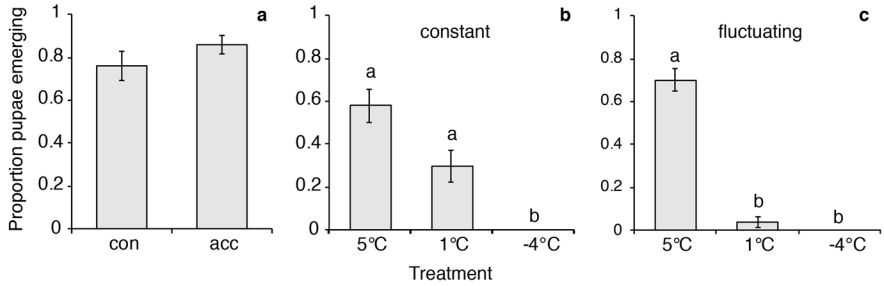

Fig. 3 Hatching of *Anagyrus vladimiri* (mean proportion ± SE) from pupae: (**a**) in the hatching control and acclimation control, (**b**) after exposure to constant cold temperatures, (**c**) after exposure to fluctuating cold temperatures. Different letter above bars indicate significant differences (GLM with binomial distribution followed by Dunn post-hoc test, $p \leq 0.05$), hatching control and acclimation control were not included in the statistical comparisons. con: Hatching control; acc: individuals exposed to acclimation only

Reproduction after cold exposure

Sublethal exposure to cold did not have a significant influence on adult reproduction ($\chi^2 = 3.37$, df = 3, $p = 0.339$; Fig. 4a), neither did it influence sexual reproduction (i.e., indicated by the presence of female offspring) ($\chi^2 = 1.28$, df = 3, $p = 0.734$). Likewise, no influence of sublethal cold treatment during the pupal stage on reproduction ($\chi^2 = 6.85$, df = 4, $p = 0.144$; Fig. 4b) or sexual reproduction ($\chi^2 = 0.93$, df = 4, $p = 0.920$) was detected.

Semi-field experiment

The mean (± SE) daily temperature during the adult exposure was 2.72 ± 0.73 °C with a minimum temperature of − 3.55 °C measured at 6:00 am on 27 December 2020. No adult survived the semi-field exposure, whereas 75% of adults survived in the acclimation control. At 22 °C, adult females survived for 85.38 ± 5.93 days and adult males for 46.70 ± 4.00 days. The mean daily temperature during the pupal exposure was 3.69 ± 0.82 °C with a minimum temperature of − 4.27 °C measured at 7:00 am on 21 January 2021. No pupae hatched after semi-field exposure, whereas 95% hatched from the hatching control and 48% from the acclimation control. From the non-emerged pupae 66% were in the pupal stage, 9% undeveloped and 25% adults inside the pupal case.

Temperatures experienced by the different egg samples during semi-field exposure are presented in Fig. 5b. All control samples showed adult emergence of between four and 18 individuals. In contrast, only from samples exposed after 26 April 2023 adult emergence was observed (Fig. 5a).

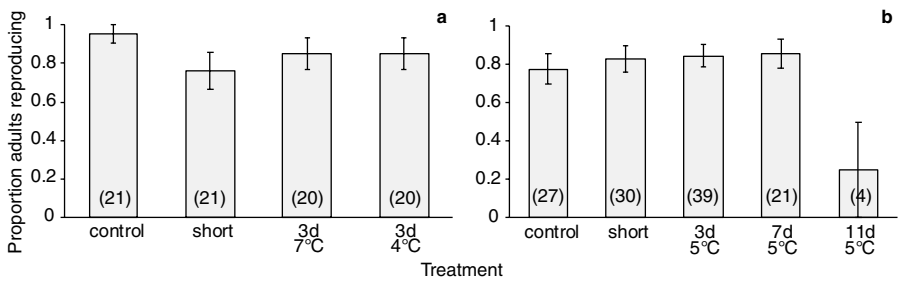

Fig. 4 Proportion of successfully reproducing females of *Anagyrus vladimiri* (mean ± SE) after exposure for different durations (given in days) to cold temperatures during the (**a**) adult, (**b**) pupal stage. Sample sizes are given in brackets within the bars. No significant differences were detected among treatments (GLM with binomial distribution; $p < 0.05$). Control: Hatching control, short: adults shortly cooled to temperatures between − 7.0 °C and − 10.4 °C, pupae shortly cooled to temperatures between 4.7 °C and − 1.0 °C

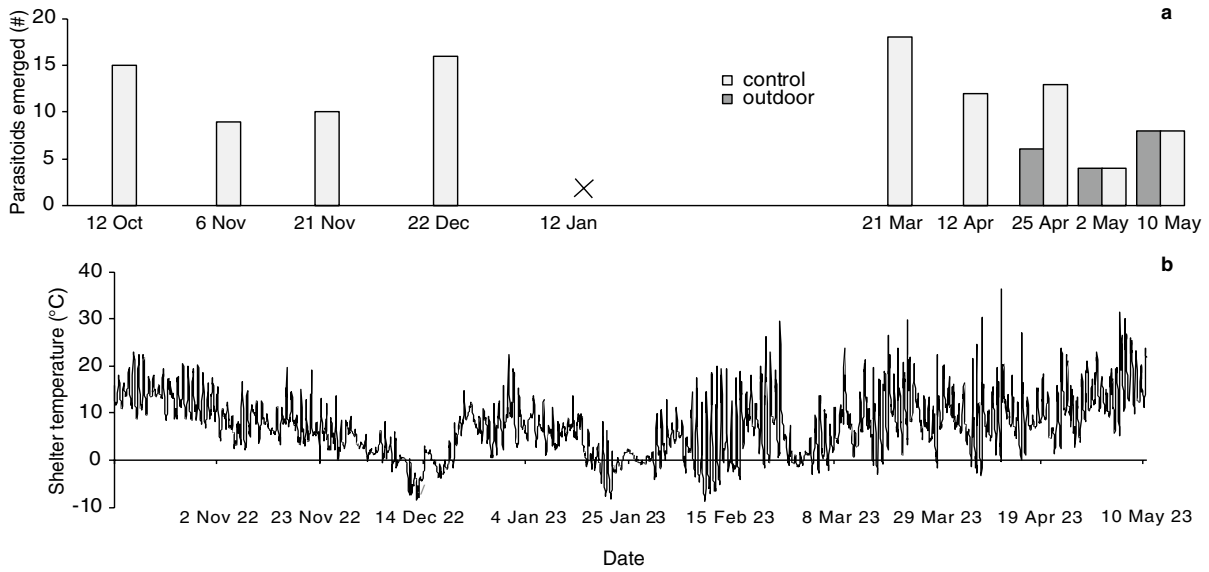

Fig. 5 Semi-field exposure in Northern Switzerland of *Anagyrus vladimiri* in the egg stage (**a**) emergence of adults from samples exposed outdoors under a shelter (grey bars) or kept in the laboratory as control (light bars), no control sample was placed on 12 January, (**b**) hourly measured temperatures during the exposure period

Discussion

Cold exposure negatively affected *A. vladimiri* at all life stages, yet the influence of cold on survival and hatching differed with the life-stage that was exposed, exposure time and exposure temperature. Bodies of adult, honey-fed *A. vladimiri* froze at approximately − 20 °C (− 18.9 °C for females and − 21.7 °C for males), while pupae froze at temperatures about 5 °C lower. The measured values are comparable to those reported for other parasitoids, e.g., *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae) with SCP values of − 18 °C for honey-fed adults, − 24.3 °C for unfed adults, and − 25.4 °C for pupae (Carrillo et al. 2005). According to Carrillo et al. (2005), honey-fed adult parasitoids show a noticeable increase in SCP, because the food inside their gut enhances ice nucleation. In our study, two outliers occurred for the SCPs of adult males, which were 6–7 °C lower than the mean. A possible explanation is that those individuals had not fed on the provided honey-solution before the measurements. Multiple studies have shown higher SCPs in feeding developmental stages of insects compared to non-feeding ones (Salt 1953; Chauvin and Vannier 1997; Koch et al. 2004; Carrillo and Cannon 2005). This could explain the higher SCP values of adult *A. vladimiri*, compared to the non-feeding pupal stages in our study. Further, sex differences in SCPs have been reported previously and may result from physiological differences such as the amount of ice nucleating agents in the hemolymph or size differences (female *A. vladimiri* are larger than males) due to the inverse relationship between supercooling capacity and volume (Renault et al. 2002).

Survival of adults cooled at a rate of 1 °C min^{-1} mostly ceased at temperatures above the SCP, with a few individuals surviving, at least for an hour after rewarming, at temperatures close to the SCP. It is possible that our estimates of adult survival are even overestimations, as mortality was only assessed 1 h after re-warming. In pupae, no hatching was observed after they had been cooled to temperatures well above their SCP. Thus, *A. vladimiri* do not tolerate tissue freezing and can be classified as freeze-intolerant. When *A. vladimiri* were exposed to cold temperatures for longer periods, we observed low cold hardiness. No adults could survive three days at 3 °C or lower, whereas 78% of adults still survived when they were exposed to 5 °C for the same duration. Moreover, in larvae and eggs almost no hatching occurred in any of the cold treatments beyond the acclimation. Pupae seem to be the stage with the highest cold tolerance

in *A. vladimiri*, with some individuals still emerging after 11 days at 5 °C or three days at 1 °C. While low temperatures affected survival and hatching ability of *A. vladimiri*, no sublethal effects on reproduction of surviving individuals were detected. Bale (1996) describes insects that survive in the temperature range of 0 °C to 5 °C but die after brief exposures to temperatures between − 5 °C and − 15 °C as chill-susceptible. We consider *A. vladimiri* as a species at the lower end of the chill-susceptible spectrum. In chill-susceptible insects, damages accumulate during exposure to cold due to loss of ion and water homeostasis, which ultimately may lead to their death (Overgaard and MacMillan 2017).

In several studies, the survival of parasitic wasps decreased when they were exposed to low temperatures. Low emergence was observed in *Aphidius colemani* Viereck (Hymenoptera: Braconidae) in 0–2-day-old aphid mummies already after a week of storage at 0 °C and more than two weeks at 4 °C or 7 °C (Hofsvang and Hagvar 1977). About two thirds of prepupae of *Aphidius rhopalosiphi* DeStefani-Peres (Hymenoptera: Braconidae) in aphid mummies died when they were stored at − 5 °C for ten days, but acclimation enhanced survival to 67% (Levie et al. 2005). Cold exposure in this species induced some sterility in females and high sterility in males. After a stepwise acclimation during three weeks, a significant proportion of eggs, larvae and pupae of *Trichopria drosophila* (Perkins) (Hymenoptera: Diapriidae) were able to survive one month at 0 °C, and some individuals even survived for the same period at − 5 °C (Amiresmaeili et al. 2020). *Ephedrus cerasicola* Stary (Hymenoptera: Braconidae) survived for several weeks at 0 °C in 0–2-day-old aphid mummies and still produced fertilized eggs after two weeks of storage.

Some insects are able to recover from chill injuries when exposed regularly to warmer temperature pulses (Colinet et al. 2006; Enriquez et al. 2020) and under natural winter conditions, temperatures are fluctuating throughout the day. For example, Häner et al. (2022) measured in Northern Switzerland average temperatures in the upper soil of 4.1 °C and 5.3 °C with an average daily range (maximal to minimal temperature) of 3.3° and 1.2 °C in orchards and forests, respectively during the winter 2019/2020. When we exposed pupae of *A. vladimiri* to fluctuating temperatures, we found an interaction effect between the baseline temperature and temperature fluctuation. At 5 °C baseline temperature, no significant difference was visible between fluctuating and constant temperature, whereas at 1 °C individuals exposed to daily warming to 10 °C fared worse than those at constant temperature. In contrast to our results, Colinet et al. (2006) showed that the aphid parasitoid *A. colemani* could recover from chill injuries experienced at 4 °C when exposed regularly to warmer temperature pulses, in this case 20 °C for 2 h. We therefore assume that *A. vladimiri* cannot repair the cold damage that accumulated at low temperatures during the limited periods of warming and that individuals at 1 °C experience enhanced stress from temperature fluctuation.

Although average winter temperatures may be around 4 °C in northern Switzerland, periods of several days with average and even maximum temperatures below 1 °C are not uncommon in different microhabitats such as in the upper soil layer or in the leaf litter in forests and orchards (Nina Häner and Jana Collatz, unpublished data). Even though, under natural conditions, adult parasitoids and parasitized mealybugs could seek out shelter, such as the bark of trees or leaf-litter with more favorable microclimatic conditions than the ambient temperature (Suggitt et al. 2011; Tougeron et al. 2016), we consider it unlikely that the studied population of *A. vladimiri* can survive in large numbers during the winter in Switzerland. This assumption is supported by the fact that none of the samples kept under semi-field conditions in our experiments survived. However, the individuals used in our experiments originate from a commercial mass rearing and have been kept under constant laboratory conditions over multiple generations. Genetic drift, bottleneck effects and artificial selection during mass rearing may result in domestication effects visible as changes in physiology and (cold) stress response (Hoffmann and Ross 2018). We can thus not rule out the possibility that other populations, in particular those that have been collected newly from the wild, may possess a higher cold tolerance.

As our study focused on the assessment of cold hardiness of different life-stages of the parasitoid we did not measure other indicators such as chill coma. As chill coma would render the free-living adults immobile, it might even decrease their chances of survival in the field at temperatures above the LLT

due to enhanced risk of predation and exposure to detrimental weather conditions (Sinclair et al. 2015).

Insects can adapt to their environment of introduction, either through phenotypic plasticity or evolution. We can thus not fully exclude the possibility that different experimental conditions might have led to higher emergence and survival in *A. vladimiri.* For example, it is known that gradual decrease in temperature, i.e., acclimation, before cold exposure enhances the survival of parasitoids because the risk of cold shock can be avoided by physiological adaptations (Singh and Srivastava 1988; Rigaux et al. 2000; Levie et al. 2005). In our experiments parasitoids were acclimated to low temperatures over several days. Under natural conditions, however, the acclimation to winter temperatures may occur over weeks and months. Further, if few insects survive the winter, populations with higher cold tolerance may gradually evolve. This phenomenon has been observed in the invasive *Lymantia dispar* (L.) (Lepidoptera: Erebidae) at its North American invasion front (Thompson et al. 2021). Similarly, in the invasive *Aedes albopictus* (Skuse) (Diptera: Culicidae) adaptations in thermal performance and diapause at higher latitudes in the invaded North America have been found (Batz et al. 2020). Finally, quiescence or diapause induction is a common physiological response to cold temperatures (Rahimi-Kaldeh et al. 2017). Exogenous-quiescence can appear after sudden drop of temperature (Keller 1986; Voegelé et al. 1988), while diapause is induced by environmental conditions such as cold temperatures or changes in photoperiod (Boivin 1994). In a study by Daane et al. (2004), mealybugs newly parasitized by *A. vladimiri* (parasitoids in egg stage) were placed outdoors during autumn in California. While *A. vladimiri* control individuals in the laboratory hatched within short time, the individuals that were kept outdoors only hatched the following spring. By the time their experiment started, ambient temperatures were on average close to the developmental threshold of *A. vladimiri* around 12 °C, yet the synchronized hatching in spring, regardless of varying accumulated day-degrees suggests the influence of some form of developmental diapause. To determine if unhatched mummies in our study were dead or in a living dormant state, we opened them under the stereomicroscope six weeks after adults were expected to hatch (cold storage period + developmental time). In all cases, opening the mummies revealed dried pupae, which were clearly dead. From these results, we conclude that the unhatched pupae died from cold exposure and were not in a dormant stage. We further set up an experiment exposing parasitoid eggs in a similar way to Daane et al. (2004), yet we did not find any emergence for samples exposed during winter and autumn but only from those exposed after mid-April (26/4), when temperatures did not fall below 5.2 °C anymore. We thus assume that all stages of the tested *A. vladimiri* population possess a limited cold tolerance and are unlikely to survive the winter under normal conditions in Northern Switzerland. On the one hand it is therefore unlikely that *A. vladimiri* could provide long-term control under outdoor conditions of Northern Switzerland against mealybug pests such as *P. ficus*. On the other hand, it can be used in augmentation biocontrol programs in greenhouses and is then unlikely to establish when escaping from this protected environment.

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Authors' contribution JC and JR conceived the study, FG, JC and JR developed the experimental design, FG conducted the experiments, FG and JC analyzed the data, FG and JC wrote the manuscript. JR reviewed and edited the manuscript.

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Data availability The raw data of the study are available at: https://doi.org/10.6084/m9.figshare.24526441.v1.

Declarations

Competing interests The authors declare they have no financial interests and no competing interests to declare that are relevant to the content of this article.

Ethical approval The authors approve the ethical guidelines of the journal.

Consent for publication All authors gave consent to the publication of the study.

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Florence Gilliéron has a strong interest in insect ecology and conducted her Master thesis on the establishment potential of *Anagyrus vladimiri* in Switzerland.

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