

Interactions Between Biotic and Abiotic Factors Affect Survival in Overwintering *Drosophila suzukii* (Diptera: Drosophilidae)

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Abstract

Drosophila suzukii Matsumura is an invasive species affecting berry crops and cherries throughout North America, South America, and Europe. Previous research suggests that in temperate climates, the overwintering success of *D. suzukii* is likely dependent on access to food, shelter, and adequate cold hardening. We performed a multi-state study under field conditions for two winters to determine whether *D. suzukii* sex, phenotype (summer-morphotype, winter-morphotype), and life stage (adults, pupae) affected survival over time while recording naturally-occurring spatial and temporal variation in temperature. Access to food was provided and the flies were buried under leaf litter. Baited traps were deployed to determine whether local populations of *D. suzukii* were active throughout the winter season. The duration of exposure, mean daily temperature, and cumulative time below freezing significantly affected survival. Below freezing, *D. suzukii* survival was significantly reduced, particularly in northern locations. In contrast, we observed sustained survival up to 10 wk in southern locations among adults and pupae. Biotic factors also significantly affected survival outcomes: female survival was greater than male survival, winter-morphotype survival was greater than summer-morphotype survival, and adult survival was greater than pupal survival. In the north, wild *D. suzukii* were captured only in early winter, while in the south they were found throughout the winter. These data suggest that although adult *D. suzukii* may overwinter in sheltered microclimates, this ability may be limited in regions where the ground temperature, or site of overwintering, falls below freezing for extended durations.

Key words: SWD, cold tolerance, habitat, survival, morphotype

First described in Japan in the early 20th century (Kanzawa 1939), *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) is an invasive pest of soft-skinned fruit that has historically been associated with cool temperate climates. Following its introduction in 2008, *D. suzukii* has spread rapidly across the continental United States, and has emerged as a major pest in temperate Europe and South America (Walsh et al. 2011, Cini et al. 2012, Asplen et al. 2015, dos Santos et al. 2017). Increasing population pressure in blueberries, cane fruits, strawberries, and cherries (Lee et al. 2011a, Lee

et al. 2011b) and the resulting economic losses (Bolda et al. 2010, Goodhue et al. 2011, Lee et al. 2011b) have led to interest in the overwintering biology of this species as a means to target vulnerable populations with novel methods of suppression.

Drosophila suzukii display differential seasonal morphology characteristic of temperate drosophilids in general. Summer-morphotypes (SM) are smaller and lighter in color than winter-morphotype flies (WM), which are characterized by darker abdominal banding, as well as longer wings and overall body size (David et al.

1994, Stephens et al. 2015, Shearer et al. 2016, Wallingford and Loeb 2016). These external morphological changes, which accompany development at cool temperatures, are associated with internal physiological changes leading to improved cold tolerance (Shearer et al. 2016). However, short-term cold acclimation appears sufficient to induce some degree of cold tolerance among adult flies, indicating that the biochemical changes associated with acclimation may occur during multiple life stages (Jakobs et al. 2015, Wallingford and Loeb 2016, Stockton et al. 2018). We recently reported physiological plasticity in the cold hardening response of *D. suzukii*, where cold tolerance increased as the temperature during larval and early adult development decreased (Stockton et al. 2018). Under gradual cooling conditions, which most closely mimics the thermal shifts that occur in nature, fully cold hardened adult *D. suzukii* may survive short durations up to -7.5°C .

However, in comparison to many overwintering arthropods (Neven et al. 1986; Bale et al. 1989; Gibert et al. 2000, 2001; Bale 2002; Hoffmann et al. 2003a; Rako and Hoffmann 2006; Bale and Hayward 2010) including the Holarctic drosophilid, *Chymomyza costata* Zetterstedt (Sinclair 1999, Košťál et al. 2003), *D. suzukii* appears relatively intolerant of extreme cold conditions (Kimura 2004, Dalton et al. 2011), and displays significant thermal susceptibility to extended durations below freezing (Kimura 2004, Dalton et al. 2011, Jakobs et al. 2015, Stephens et al. 2015). While acclimation appears to attenuate the susceptibility of both adults and pupae (Dalton et al. 2011, Wallingford and Loeb 2016, Stockton et al. 2018, Wallingford et al. 2018), and is associated with up-regulated carbohydrate metabolism (Shearer et al. 2016), the lower lethal limits of thermal susceptibility in *D. suzukii* remain more conservative than in most species expected to overwinter in harsh climates (Jakobs et al. 2015, Stockton et al. 2018). For this reason, it is likely that *D. suzukii* employs a freeze-avoidance strategy similar to *Drosophila melanogaster* (Diptera: Drosophilidae) (Izquierdo 1991, Hoffmann et al. 2002) and seeks shelter to aid in thermo-regulation rather than relying on physiological freeze-tolerance mechanisms (Lee 1989, Denlinger and Lee 2010, Rossi-Stacconi et al. 2016, Wallingford and Loeb 2016, Stockton et al. 2018, Toxopeus and Sinclair 2018, Wallingford et al. 2018).

Freeze avoidant strategies (Lee 1989, Sinclair 1999, Denlinger and Lee 2010, Chapman et al. 2015), allow animals to strategically exploit the environment to compensate for biological limitations (Bale et al. 2000, Bale and Hayward 2010). Overwintering insects may find a relatively warm, sheltered environment relative to the ambient condition, such as under a log, or beneath the soil surface. In *D. melanogaster*, latitudinal population stability in temperate climates (Mukai and Yamaguchi 1974, Inoue et al. 1984, Fabian et al. 2012, Machado et al. 2016) indicates that these flies likely do not migrate seasonally, but rather cope with low temperatures via the use of shelter (Izquierdo 1991, Hoffmann et al. 2002). Indeed, many researchers have suggested that *D. suzukii* may overwinter in wooded areas under leaf litter and snow pack (Kanzawa 1939, Leather et al. 1993, Bouléreau-Merle and Fouillet 2002, Bale and Hayward 2010, Zerulla et al. 2015, Jakobs et al. 2015, Stephens et al. 2015, Wallingford et al. 2016, Pelton et al. 2016, Rossi-Stacconi et al. 2016, Stockton et al. 2018, Wallingford et al. 2018). Snow acts as an insulator and ground temperatures under snow pack are typically kept close to 0°C (Bale and Hayward 2010). In several areas of the northern United States such as the Upper Midwest and Northeast, there is evidence that wooded areas are associated with earlier first capture and higher numbers throughout the field season, indicating access to resources or refuge in the forest, not afforded in well-maintained cropland (Pelton et al. 2016, Ballman and Drummond 2017).

Similar findings were recently reported in Europe (Briem et al. 2018). Moreover, there is speculation that this species uses anthropogenic structures, such as agricultural compost piles or structural debris, to avoid extreme temperatures (Bouléreau-Merle and Fouillet 2002, Rossi-Stacconi et al. 2016, Wallingford et al. 2016, Stockton et al. 2018). Compost piles and grape pomace are of particular interest because of the heat generated by large piles throughout the winter, in which *D. suzukii* could potentially feed and reproduce (Bal et al. 2017). Although there are no official reports of *D. suzukii* overwintering in compost or anthropogenic structures, other drosophilids are known to be highly adaptable and inhabit urban environments (Atkinson and Shorrocks 1977, Newbury et al. 1984, Watanabe et al. 1984).

In warmer climates, *D. suzukii* is captured year-round (Dean et al. 2013), while in colder climates, at least two studies have successfully captured adult *D. suzukii* during the winter despite temperatures well below freezing. A 4-yr study in Northern Italy detected *D. suzukii* each winter at temperatures as low as -2°C (Rossi-Stacconi et al. 2016). Similarly, in the northwestern United States, trap captures in a 4-yr field trial in Oregon and Washington state showed consistent captures throughout the winter despite lows below freezing (Thistlewood et al. 2018). This suggests that *D. suzukii* may enter a state of metabolic quiescence, rather than diapause, which is associated with reduced movement and encourages survival until warmer temperatures in the spring (Košťál 2006, Wallingford et al. 2016, Grassi et al. 2018). We recently reported that movement in cold hardened WM *D. suzukii* is often sustained at temperatures as low as -2.5°C (Stockton et al. 2018). Because acclimation improves mobility and survivorship of overwintering flies (Stockton et al. 2018), and flies are more often found close to known food sources (Wallingford et al. 2018), it is likely that *D. suzukii* emerges from its refuge to feed during intermittent periods of warm weather, thereby allowing for detection during warm periods throughout the winter months.

Our aim in this study was to determine the degree of overwintering survival in insulated areas, beneath leaf litter and snow pack, under field conditions representative of mild and harsh regions of North America. We controlled for biotic factors such as the sex of the insect, the degree of cold acclimation, and the insect life stage; and recorded ambient and ground temperatures throughout the sampling period, which allowed us to study the relationship between temperature and survival. We also monitored wild flies using baited drosophila traps at each field site. Collaborators from six states including Maine (ME), New York (NY), Michigan (MI), North Carolina (NC), Georgia (GA), and Oregon (OR) conducted this study over the course of two field seasons, between December and March, during 2016–2017 and 2017–2018.

Materials and Methods

Field Survival Trials

To assess how outdoor exposure affects *D. suzukii* survival during winter conditions, we carried out a large-scale field deployment of adult and pupal *D. suzukii* at six locations in the United States from November to March during 2016–2017 and 2017–2018. Our field sites were located in Northeastern (MI, NY, and ME), Southeastern (GA and NC), and northwestern (OR) United States, representing varying North American climates and crop systems (Table 1; Fig. 1A). NY had two field sites: one in Geneva, NY referred to as Vignoles (Vg), and another 30 miles south referred to as Silverthread (Slv). All other locations had one site.

Table 1. Winter collection sites for *D. suzukii*, GPS site coordinates, and adjacent crop system associated with each field site during 2016–2017 and 2017–2018

	Begin Exp ^a	End Exp ^b	Latitude	Longitude	Crop	Sex	Ph	Ls
2016–2017					<i>Vaccinium corymbosum</i>			
ME	15 Nov. 2016	25 Jan. 2017	44°55'46.70	68°41'47.6		+		
NY ^{vg} ^c	09 Nov. 2016	18 Jan. 2017	42°52'15.58	77°1'58.59	<i>Vitis vinifera</i>	+	+	
NY ^{slv} ^d	22 Dec. 2016	16 Feb. 2017	42°33'49.71	76°52'32.66	<i>Vitis vinifera</i>	+	+	
MI	22 Nov. 2016	13 Feb. 2017	42°41'21.41	84°29'23.30	<i>Vitis vinifera</i>	+		
NC	13 Jan. 2017	24 Mar. 2017	35°41'59.34	80°37'34.41	<i>Rubus Watson</i>	+	+	
OR	20 Dec. 2016	14 Feb. 2017	44°33'37.18	123°17'21.96	Various ^e	+		
2017–2018					<i>Vaccinium corymbosum</i>			
ME	07 Dec. 2017	15 Feb. 2018	44°55'46.70	68°41'47.6		+		
NY ^{vg}	05 Dec. 2017	16 Jan. 2018	42°52'15.58	77°1'58.59	<i>Vitis vinifera</i>	+		+
NY ^{slv}	12 Dec. 2017	16 Jan. 2018	42°33'49.71	76°52'32.66	<i>Vitis vinifera</i>	+		
MI	15 Dec. 2017	23 Feb. 2018	42°41'21.41	84°29'23.30	Various ^f	+	+	+
NC	26 Dec. 2017	07 Mar. 2018	35°41'59.34	80°37'34.41	<i>Rubus Watson</i>	+		
OR	03 Jan. 2018	26 Mar. 2018	45°41'7.33	121°31'0.01	<i>Prunus avium</i>			
GA	26 Nov. 2017	31 Jan. 2018	31°30'43.92	82°27'29.53	<i>Vaccinium ashei</i>	+		+

Biotic factors (sex, phenotype [Ph], and life stage [Ls]) assayed at each field site.

^aDate of experimental setup and the start of data collection.

^bLast day of temperature recording and final collection date.

^cNew York Vignoles (NYvg) vineyard field site.

^dNew York Silverthread (NYslv) vineyard field site.

^eMiscellaneous crops including vegetables and small fruit.

^fCorn (*Zea mays*), peach (*Prunus persica*), and grape (*V. vinifera*).

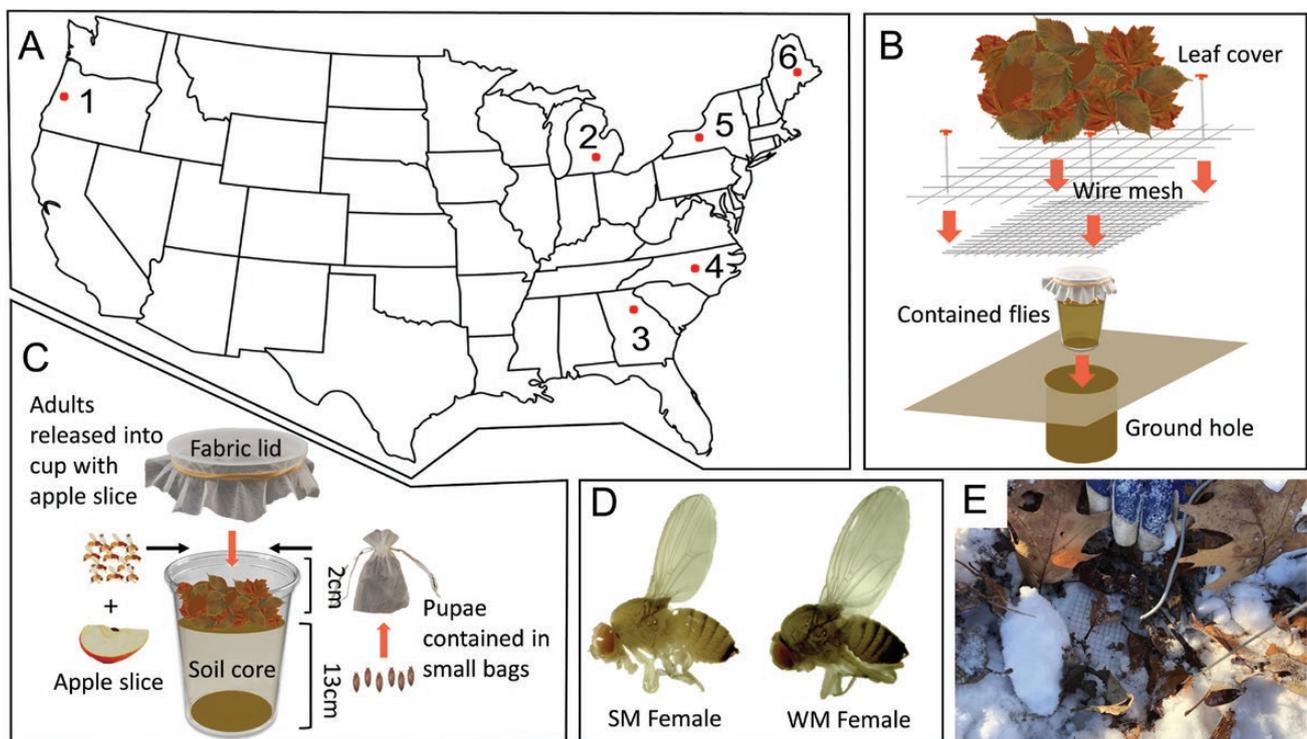


Fig. 1. Field sites sampled in OR (1), MI (2), GA (3), NC (4), NY (5), and ME (6) (A). Construction of the field assay: flies were contained in deli cups containing soil, leaf litter, and an apple slice, placed in the ground, secured with mesh, and covered with leaf litter (B). Method of fly containment for adult and pupal flies (C). Morphological differences in size and pigmentation among SM (left) and WM (right) female *D. suzukii* (D). Snow pack and leaf litter cover the flies hidden underground in the overwintering field assay (E).

We assessed the effect of three biotic factors: fly sex, phenotype (SM vs WM), and life stage (adult vs pupa) on survival. The effect of sex was assessed at all participating sites during winter 2016–2017 and 2017–2018. Phenotype was assessed in 2016–2017 in NY (Vg + Slv sites) and NC, in 2017–2018 in MI. Life stage was assessed in NY (Vg), MI, and GA in 2017–2018.

Insect Colonies

Summer-morphotype (SM) *D. suzukii* colonies were kept at $25 \pm 2^\circ\text{C}$ with a 16:8 (L:D) h light cycle and 65% relative humidity (RH). Cold-acclimated SM insects (Fig. 1D, left) were induced by moving SM adults to a $10 \pm 2^\circ\text{C}$, 12:12 (L:D) h chamber 1–3 d after eclosion for 5 d. WM colonies (Fig. 1D, right) were induced

by moving bottles containing 1–2 d old eggs and first instar larvae into a $15 \pm 2^\circ\text{C}$ growth chamber (model I-30BL; Percival Scientific, Inc.; Perry, IA), with a 12:12 (L:D) h light cycle at 65% RH, for the duration of their development. Within 48 h of emergence, adult WM flies were moved to new culture bottles at $10 \pm 2^\circ\text{C}$, 12:12 (L:D) h. In 2016–2017, all groups used WM flies for the primary release, while in 2017–2018, all groups used acclimated SM flies, unless otherwise stated, such as in tests directly involving phenotype. The flies were reared on 50 ml standard cornmeal diet (Wallingford et al. 2016) in 8 oz. (236 ml) polypropylene drosophila culture bottles with foam stoppers (#75813-140; VWR International, Radnor, PA). After eclosion, the diet bottles were replaced immediately, and every week thereafter, in both SM and WM colonies. Rearing practices varied at some locations (Table 2; Supp. Methods).

Site Selection and Preparation

Experimental locations were established along wooded margins adjacent to crop fields. We avoided low spots in the ground that could collect standing water. Golf hole cutters (20 cm depth \times 10.5 cm diameter; product # 1001-1; Par Aide Product Company, Lino Lakes, MN) were used to prepare the holes (15 cm depth \times 12 cm diameter) in which to place the release containers. We widened the holes approximately 1.5 cm by gently rocking the hole cutter while it was in the ground. The soil cores were used to fill the release containers (32 oz (944 ml) deli cups; 15 cm height \times 11 cm diameter; Fig. 1C) with 13 cm of compacted soil, leaving 3 cm open at the top of the container.

Fly Release

The flies were cold-sedated on ice inside an insulated cooler before being released at the field sites. Fifty male and 50 female flies were placed in each release container (32 oz transparent polypropylene deli cups, Fabri-Kal, Kalamazoo, MI) filled with compacted soil. Care was taken to move quickly and reduce the amount of exposure time to ambient air temperatures. A slice of apple (1/8 of fruit; Red delicious, *Malus pumila*) was included on top of the soil

core as a food source should the flies become mobile (Wallingford et al. 2018). After release, the sedated flies were immediately covered with 2 cm of locally collected leaf litter, and sealed within the release container using a 15×15 cm sheet of fine mesh fabric secured with a rubber band. The release containers were placed into the holes created by the golf hole cutter, covered with two layers of metal wire mesh sheeting secured with metal stakes on the corners to exclude wild animals, and were covered with more leaf litter (Fig. 1C). The first layer of mesh was 30×30 cm with 0.5 cm openings. The second layer of mesh was 50×50 cm with 1 cm openings. In ME, the flies were first anesthetized with CO_2 before being placed in a cooler with ice for transport. No mesh was placed over the release containers in ME and none were disturbed during the experiment.

Fly Recovery

Four randomly assigned arenas were collected from each field site at six time intervals following experiment initiation: 1 d, 2 wk, 4 wk, 6 wk, 8 wk, and 10 wk. In MI and OR, the 1 d sampling was omitted. Prior to recovery, any snow cover and leaf litter was carefully cleared from above the wire mesh cover. The cover was removed and the release containers were quickly transferred to an insulated cooler to prevent freezing.

The flies were then transported from the field to the laboratory and placed into individual cages (type varied by location) to evaluate survival. Once inside the cage, the mesh fabric and the apple slice were removed from the release containers, and the surface leaf litter was carefully removed and placed on the floor of the cage. This exposed the flies and allowed surviving *D. sukuzii* to recover. In ME and MI, while the apple slice was removed, the leaf litter in the release container was left undisturbed. A water source was placed in the cage for recovered flies. The cages were checked up to 14 d (in ME, MI, NC) to determine survival. In NY, survival was assessed only up to 7 d post recovery. Survival was noted by the presence of live flies moving around the cage, or the presence of fly bodies in previously clear parts of the cage.

Table 2. Method of rearing SM and WM *D. sukuzii* associated with each test location

	ME	NY	MI	NC	GA	OR
SM colony						
Temp 1 ^a	$25 \pm 2^\circ\text{C}$	$25 \pm 2^\circ\text{C}$	$25 \pm 2^\circ\text{C}$	$25 \pm 2^\circ\text{C}$	$25 \pm 2^\circ\text{C}$	
Temp 2 ^b	$10 \pm 2^\circ\text{C}$	$10 \pm 2^\circ\text{C}$	$10 \pm 2^\circ\text{C}$	$10 \pm 2^\circ\text{C}$	$10 \pm 2^\circ\text{C}$	
Light cycle 1	18:6 (L:D) h	16:8 (L:D) h	16:8 (L:D) h	16:8 (L:D) h	16:8 (L:D) h	
Light cycle 2	12:12 (L:D) h	12:12 (L:D) h	12:12 (L:D) h	12:12 (L:D) h	12:12 (L:D) h	
RH	65%	65%	85%	65%	50%	
WM colony						
Temp 1	$15 \pm 2^\circ\text{C}$	$15 \pm 2^\circ\text{C}$	$15 \pm 2^\circ\text{C}$	$15 \pm 2^\circ\text{C}$	$15 \pm 2^\circ\text{C}$	$15 \pm 2^\circ\text{C}$
Temp 2	$10 \pm 2^\circ\text{C}$	$10 \pm 2^\circ\text{C}$	$10 \pm 2^\circ\text{C}$	$10 \pm 2^\circ\text{C}$	$10 \pm 2^\circ\text{C}$	$10 \pm 2^\circ\text{C}$
Light cycle	12:12 (L:D) h	12:12 (L:D) h	10:14 (L:D) h	12:12 (L:D) h	12:12 (L:D) h	12:12 (L:D) h
%RH	65%	65%	85%	65%	50%	65%
Diet						
Formula	Carolina ^c	Cornmeal ^d	Cornmeal	Cornmeal	Cornmeal	Cornmeal
Amount	5–7 ml	50 ml	5–7 ml	50 ml	50 ml	2 ml
Housing ^e	7.3 \times 2.0 cm tubes	236 ml bottle ^f	9.5 \times 2.5 cm vials	236 ml bottle	236 ml bottle	Open colony ^g

^aThe developmental temperature at which the insects were reared.

^bThe temperature at which the insects were held following eclosion.

^cFormula 4–24 Instant Drosophila Medium (Carolina Biological Supply Company, Burlington, NC).

^dBased on Wallingford, Hesler, et al. 2016.

^eManner of fly containment within the colony.

^f236 ml polypropylene round bottom drosophila stock bottle.

^gFlies fed and oviposited on diet within Petri dishes placed in cages.

Pupal Survival

Pupal survival was compared with adult survival during 2017–2018 in MI, NY, and GA. Cold-acclimated pupae were created by rearing the larvae at 15°C until pupation. Two days after pupation, they were moved to a 5°C chamber and held in developmental arrest until their time of use. We carefully removed the pupae from the rearing media similar to methods previously reported (Stockton et al. 2018). Immediately afterward, they were rinsed with distilled water to remove agar from the respiratory horns, followed by a 70% ethanol rinse to reduce surface pathogens. A final distilled water rinse removed ethanol from the cuticle. The pupae were transferred to a paper towel to absorb excess moisture and transferred into small white organza bags (7 × 7 cm, model s-10647W; Uline, Pleasant Prairie, WI). Each bag represented a single experimental replicate. The pupae were stored at 5°C for up to 24 h until deployment in the field.

The number of pupae in each bag varied by location. In MI and GA 50 pupae were released per sample. In NY, 40 pupae were released per sample. The pupae were transferred to the field within release containers, as described previously in the adult fly assay. Four replicate samples were removed every 2 wk, held in Petri dishes with moistened filter paper under ambient laboratory conditions, and assessed for adult eclosion three times per week for 2 wk. In GA, the pupae were kept in the organza bags in which they were deployed and the number of eclosed adults was counted after 1 wk.

Temperature Data

The regional temperature data from each location were recorded using HOBO data loggers (Onset Computer Corporation, Bourne, MA; probe model varied by site). We measured ambient temperature 1 m above ground, as well as ground temperature 2 cm below the soil surface at each site. This depth corresponded to the approximate depth of the flies within the release cups. Temperature measurements were taken at least once per hour for the duration of the study. We

later modeled the relationship between abiotic temperature factors and *D. suzukii* survival.

Wild Fly Trapping

During the 2017–2018 winter field season, we monitored two traps (SC – SWD T; Scentry Biologicals, Inc.; Billings, MT) baited with an attractive lure (SC – SWD L; Scentry Biologicals Inc.) at each of the field sites in the study. In OR, red Drosotrap (Biobest, Westerlo, Belgium) lured with a 70% Merlot wine, 30% apple cider vinegar solution were used for trapping. The traps were placed 1–2 m above ground, along the perimeter of the wooded area in which the survival trial was run. The drowning liquid (10% saline [NaCl] solution) was replaced every 2 wk when trap captures were collected. In NC and GA, the saline solution was substituted for soapy water, prepared with clear, unscented dish soap. The drowning solution was filtered through fine mesh to collect any trapped insects, which were later evaluated under magnification to determine the number of *D. suzukii*.

Statistical Analysis

Differences in temperature pattern among the seven field sites (ME, NYvg, NYslv, MI, NC, OR, and GA) were compared with two-way analysis of variance (ANOVA) and general linear hypothesis testing (GLHT) using multiple means comparisons with Tukey contrasts ($\alpha = 0.05$). Separate analyses were performed for data collected in 2016–2017 and 2017–2018.

We used logistic regression ($glm(y \sim x, family = binomial)$) to determine whether environmental factors, such as temperature and exposure time, and biotic factors, such as sex, phenotype, and life stage affected survival. Our outcome variable, 'survival', reflected the number of living and dead flies at each observation point (1d, 2 wk, 4 wk, etc.), bound as a single factor ($cbind(alive, dead)$). Three models were run, one for each of the three biotic factors. In model 1, we used sex as the biotic factor and 5 covariates: field site, year (2016–2017,

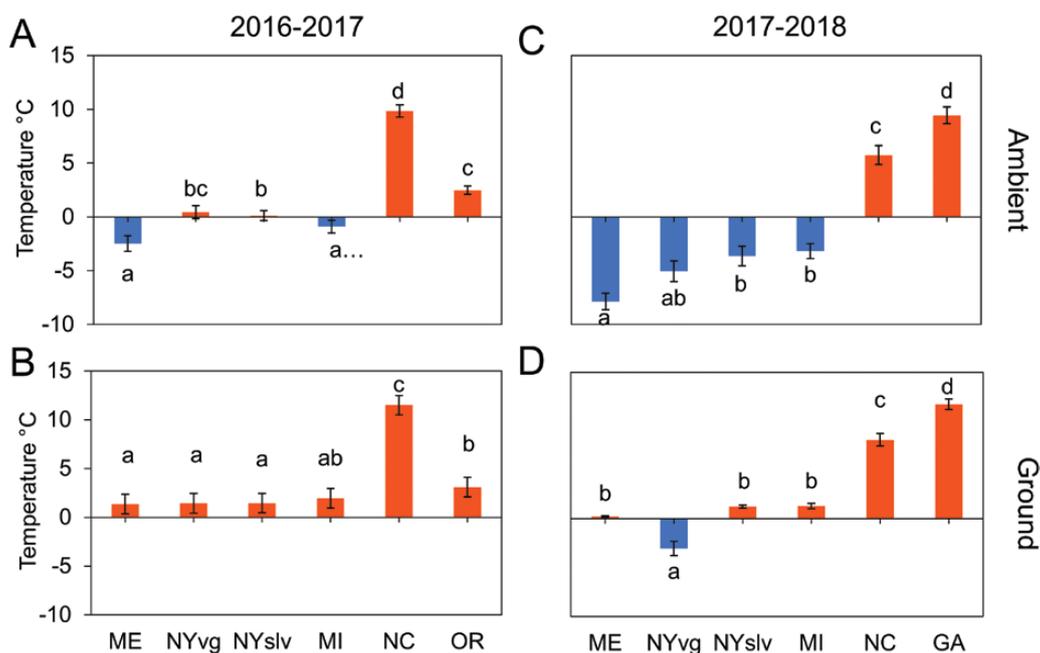


Fig. 2. Mean (\pm SEM) differences in ambient (A, C) and ground (B, D) temperatures among field locations during the 2016–2017 (A–B) and 2017–2018 (C–D) winter field seasons. Different letters indicate statistically significant differences ($\alpha = 0.05$).

2017–2018), total exposure time (weeks), cumulative time below zero (days), mean temperature (°C), and the interactions between sex and time, time below zero, and temperature. Although WM flies were used in the primary release in 2016–2017 and SM flies were used in 2017–2018, we did not include phenotype in model 1. In model 2, we used phenotype and sex as biotic factors, along with the five covariates (site, year, time, time below zero, and temperature). We also included the interaction between sex and phenotype. Only sites that collected data directly evaluating the role of phenotype on survival were included (NY vg + slv_{2016–2017}, NC_{2016–2017}, MI_{2017–2018}). In model 3, we used life stage as the biotic factor, along with the four covariates (site, time, time below zero, and temperature)—test year was excluded as all life stage collection occurred in 2017–2018. Model 3 only included data collected from sites that directly participated in testing life stage as a biotic factor in 2017–2018 (NY vg, GA, MI).

Differences in the proportion of live and dead flies at specific time points in relation to sex, phenotype, and life stage differences,

were estimated with chi-square tests. Wild fly trapping data were assessed using a GLM with Gaussian distribution. All analyses were performed in R i386 (version 3.4.0; the R Foundation for statistical computing [platform x86_64-w64-mingw32/x64]; Vienna, Austria).

Results

Survival Trial

Ambient ($F = 137.7$; $df = 1, 810$; $P < 0.001$) and ground ($F = 96.53$; $df = 1, 809$; $P < 0.001$) temperatures were significantly warmer during the 2016–2017 winter season, than during the 2017–2018 field season (Fig. 2A–D). Mean overall survival of *D. suzukii* (proportion alive) was significantly higher in 2016–2017 (29.3%) than 2017–2018 (12.2%; Table 3). Ground temperatures were significantly warmer than ambient temperatures at all locations ($t = -8.96$, $df = 1643$, $P < 0.0001$; Supp. Fig. S1). The northeastern sites (ME, NY, and MI) had the coldest temperatures among our field sites and experienced multiple days each winter with ambient temperatures

Table 3. Logistic regression evaluating the effect of sex (model 1), phenotype (model 2), and life stage (model 3) on *D. suzukii* survival

	df	OR ^a	SE ^b	z-value	Deviance	Res. Dev.	$P > \chi^c$
Model 1							
NULL	621					20307.3	
Sex	1, 620	-0.50	0.24	-2.01	248.7	20058.6	<0.001
Site (GA ref ^d)	6, 614				3198.6	16861.0	<0.001
Year	1, 613	-0.80	0.05	-15.24	623.4	16236.6	<0.001
Time	1, 612	-0.39	0.01	-33.93	9239.4	6997.2	<0.001
Time < 0°C ^e	1, 611	-0.23	0.01	-20.53	1281.6	5715.6	<0.001
Temperature	1, 610	-0.02	0.01	-1.89	25.0	5690.6	<0.001
Sex*Time	1, 609	-0.02	0.02	-1.12	3.4	5687.3	0.067
Sex*Temp	1, 608	-0.04	0.01	-2.73	0.7	5686.6	0.418
Sex*Time < 0°C	1, 607	-0.09	0.02	-4.01	19.5	5667.1	<0.001
Sex*Site	6, 601				22.0	5645.1	0.001
Model 2							
NULL	315					10151.6	
Phenotype	1, 314	0.61	0.21	2.84	37.4	10114.2	<0.001
Sex	1, 313	-0.92	0.07	-14.04	84.6	10029.6	<0.001
Site (MI ref)	3, 310				2811.2	7218.3	<0.001
Year	1, 309	-0.40	0.34	-1.16	6.3	7212.0	0.012
Time	1, 308	-0.34	0.02	-19.02	3717.2	3494.8	<0.001
Time < 0°C	1, 307	-0.21	0.02	-13.20	662.9	2832.0	<0.001
Temperature	1, 306	-0.22	0.02	-10.81	103.0	2729.3	<0.001
Phenotype*Sex	1, 305	0.76	0.09	8.30	68.5	2660.4	<0.001
Phenotype*Time	1, 304	-0.10	0.03	-3.87	205.4	2455.0	<0.001
Phenotype*Temp	1, 303	0.11	0.03	3.79	61.3	2393.7	<0.001
Phenotype*Time<0°C	1, 302	0.14	0.02	1.93	11.7	2382.1	<0.001
Phenotype*Site	3, 299				41.2	2340.9	<0.001
Model 3							
NULL	207					3702.2	<0.001
Life stage	1, 206	5.73	1.76	3.25	19.75	3682.4	<0.001
Site (ref)	2, 204				682.81	2999.6	<0.001
Time	1, 203	-0.56	0.05	-11.10	1292.60	1707.0	<0.001
Time < 0°C	1, 202	0.10	0.02	4.91	332.28	1374.8	<0.001
Temperature	1, 201	0.77	0.05	14.09	253.57	1121.2	<0.001
Life stage*Time	1, 200	0.49	0.06	7.59	147.76	973.4	<0.001
Life stage*Temp	1, 199	-0.55	0.12	-4.49	0.71	972.7	0.400
Life stage*Time < 0°C	1, 198	-0.56	0.16	-3.58	37.27	935.5	<0.001
Life stage*Site	2, 196				40.29	895.2	<0.001

^aOdds ratios.

^bStandard error.

^cChi-squared goodness of fit test.

^dReference level.

^eDuration of exposure below 0°C.

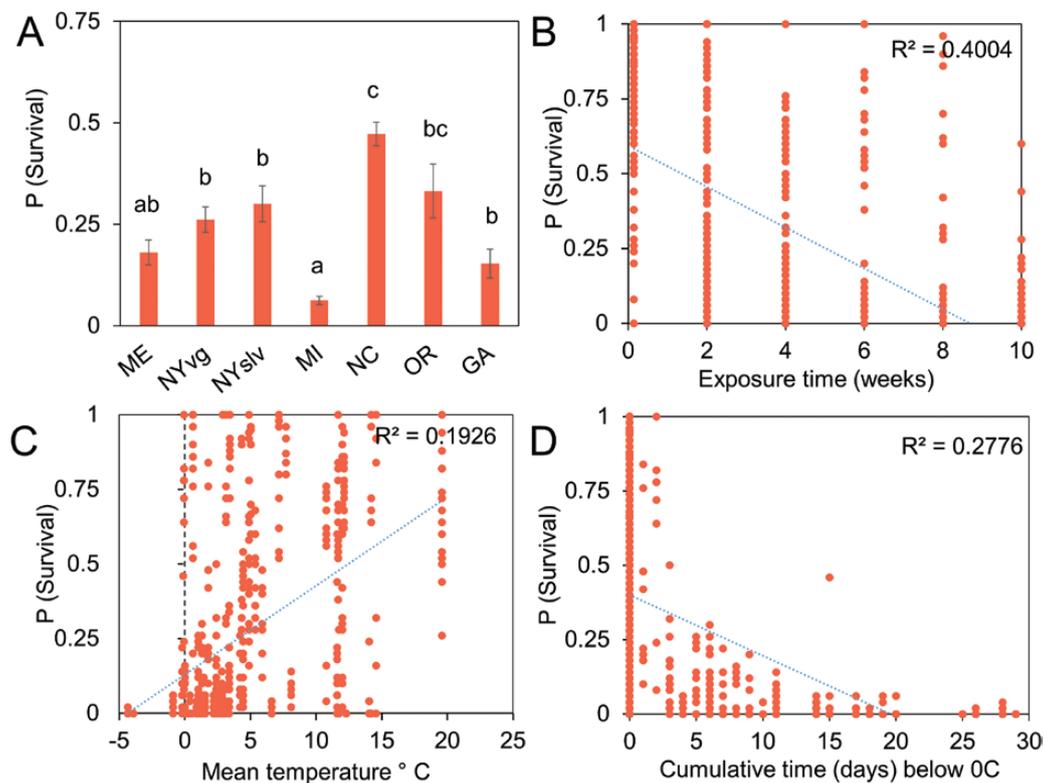


Fig. 3. Differences in overall survival (proportion alive) at each field site (A). Different letters indicate statistically significant differences in overall survival. Survival (proportion alive) plotted against total exposure time (B), cumulative time below zero (C), and mean exposure temperature (D) pooled for both test years and all field sites.

below freezing (Fig. 2). Location significantly affected *D. suzukii* survival (Fig. 3A, Table 3, Supp. Table S1), with lower survival in northeastern sites compared to southeastern (GA, NC) and northwestern sites (OR).

We observed an inverse relationship between the exposure time, defined as weeks in the field, and survival (Fig. 3B). Survival decreased as exposure time increased (Table 3; Fig. 3B). In warmer locations such as GA, NC, and OR, the slope of survival was more gradual and survival was observed up to 10 wk from the start of the experiment. In the most northern field sites (NY, MI, and ME) few insects were observed alive beyond 6 wk of exposure. Temperature was strongly correlated with survival of adult flies, with increased survival where winter temperatures were less extreme (Table 3; Fig. 3C). Few flies survived below freezing. There was also an inverse relationship between survival and the time below freezing—survival decreased as the cumulative time below freezing increased (Fig. 3D; Table 3). Variation in survival lessened after 2 wk below freezing, indicating that 2 wk was sufficient to induce mortality in a majority of the insects tested. Of the primary abiotic factors examined, the coefficient of determination was larger for exposure time ($R^2 = 0.40$; Fig. 3B) than mean daily temperature ($R^2 = 0.19$; Fig. 3C). The coefficient of determination for cumulative time below freezing was greater than for mean daily temperature ($R^2 = 0.28$; Fig. 3D).

There were several factors that affected survival at cold temperatures. The sex of surviving flies was recorded at each field location in both test years, resulting in a large sample size, useful for evaluating the effects of sex and overwintering success (Supp. Fig. S2A and B). Survival was significantly greater in female flies than males (Table 3; Fig. 4A) at each sampling point (Supp. Table S2). This was observed at each field site and during both test years. We also observed a difference in survival based on phenotype. WM flies survived in greater

proportions than SM flies during both test years, although this trend was limited to 4 wk exposure (Fig. 4B; Table 3; Supp. Table S2). This was significantly affected by location—there was greater survival among NC and NY flies than MI flies (Supp. Fig. S3A). While survival was greatest in NC overall, greater WM survival was limited to the initial weeks of the study, after which time there were no significant differences in survival among SM and WM flies.

Our results showed that adult flies lived longer than pupae during winter conditions (Fig. 4C; Table 3; Supp. Table S2). Location was a significant factor affecting survival—NY flies initially survived at higher rates than MI and GA flies, but this difference was limited to 4 wk exposure (Supp. Fig. S3B). In NY, 7.5% pupae survived up to 2 wk exposure, compared to 3.25% among adults. In MI, 2–4% of adults were recovered alive during week 10 of the study, all of which were female. In contrast, while 5% pupae were alive, but only up to week 2. Thereafter, there were no surviving pupae. In GA, adults and pupae were recovered alive up to week 10. During weeks 4–8, pupal survival was greater than adult survival, for reasons that are unclear. The most extreme difference in survival among adults (1.7% survival) and pupae (17.5% survival) occurred during week 8.

During the 2017–2018 test year, we observed 101 offspring flies in our NC field site; 50 were male, 47 were female, and 4 were non-enclosed pupae. This was the only site in our study in which reproduction was noted. Reproduction was also noted in NC samples during the 2016–2017 test year; however, the number of offspring produced was not recorded.

Wild Fly Trapping

Wild fly captures in 2017–2018 were variable and dependent on location (ANOVA: $F = 6.39$; $df = 5, 13$; $P = 0.003$; Table 4). There was no difference in capture based on month ($P = 0.45$) or

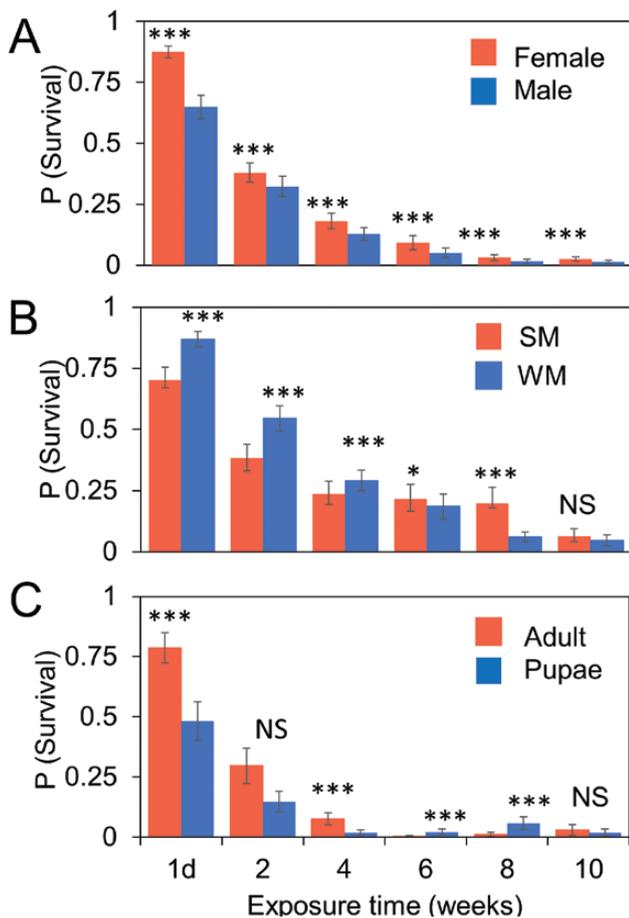


Fig. 4. Survival (proportion alive) among female and male (A), SM and WM (B), and adult and pupal (C) *D. suzukii* pooled for both test years and all field sites. Differences between biotic variables are indicated by asterisks: chi-square tests; $* > 0.05$, $** > 0.01$, $*** > 0.001$.

temperature ($P = 0.42$). In ME and MI we observed low numbers of wild flies in traps during December, but no flies were captured in January or February. A single male was caught in NC in January and February, but none were captured in March. In OR, low captures were observed in January and February. The greatest number of flies was captured in GA in 2017–2018. In GA, more than 100 flies were caught each month during December, January, and March. In February, 25 males and 48 females were caught. This decrease in

captures may be associated with a drop in temperatures below freezing prior to the February capture period.

Discussion

Our results illustrate that while abiotic thermal factors, such as the duration of exposure, and the cumulative time below freezing, were important factors affecting overwintering outcomes, physiological factors such as the sex, life stage, and phenotype of the insect were important as well. The duration of exposure in the field was the abiotic factor most strongly associated with survival outcomes. While previous research has focused on the lethal minimum temperature at which *D. suzukii* is susceptible (Dalton et al. 2011, Tochen et al. 2014, Jakobs et al. 2015, Shearer et al. 2016, Wallingford and Loeb 2016), survival outcomes in the field were more closely related to how long the insect was exposed to extreme temperatures. Similarly, the duration of time below freezing was a stronger predictor of survival than temperature as an isolated factor, possibly because short-term exposure to temperatures below freezing may not be lethal, particularly under snow. However, there were significant interactions between temperature and time, as we would expect because temperatures increased as the winter season progressed, which likely masked the effects of temperature as a single factor.

Females survived better than males at every field location and during both years of the study. We also found that WM flies survived better than SM flies. Overall there was a 16.8% difference in survival among WM and SM flies in the first 2 wk of exposure, indicating that for short-term cold stress events, acclimation is likely a significant factor affecting survival. These results are consistent with previous studies (Dalton et al. 2011, Jakobs et al. 2015, Stephens et al. 2015, Shearer et al. 2016, Wallingford and Loeb 2016, Wallingford et al. 2016, Stockton et al. 2018) and indicate that overwintering WM females may be the most important group to target for population suppression during the late dormant period, from late fall to early summer. Due to greater susceptibility among males, it is unlikely that males overwinter in large numbers. Rather, overwintering WM females may reproduce in the spring with sperm stored from mating in the fall, although we did not test differences in overwintering survival among mated and non-mated females. This is supported by a recent Italian study, which found that sperm was detectable in 30–50% of wild female *D. suzukii* collected during the winter (Grassi et al. 2018).

To assess the potential for juvenile overwintering in *D. suzukii* we compared survival among adults and pupae in the field. Brief

Table 4. Wild-type trapping of *D. suzukii* during the 2017–2018 winter field season at six locations in the United States

	N ^a	Dec. 2017			Jan. 2018			Feb. 2018			Mar. 2018		
		♂ ^b	♀ ^c	°C ^d	♂	♀	°C	♂	♀	°C	♂	♀	°C
ME	2	4	8	-6.80	0	0	-5.34	0	0	-10.45	—	—	—
NY	2	0	0	-4.33	0	0	-4.95	0	0	NA ^e	—	—	—
MI	2	5	5	-3.22	0	0	-3.82	0	0	-2.22	—	—	—
NC	2	—	—	—	1	0	4.94	1	0	11.84	0	0	11.20
OR	2	—	—	1.93	1	2	4.57	9	1	4.92	0	0	6.45
GA	2	319	472	1.00	124	360	-1.56	25	48	3.83	185	276	5.72

^aNumber of traps sampled.

^bTotal number of male fly captures during the month.

^cTotal number of female fly captures during the month.

^dMean monthly ambient temperatures.

^eUnavailable values.

exposure to freezing temperatures was associated with high levels of mortality among pupae. One drosophilid, *C. costata*, is known to diapause as larvae (Lankinen and Riihimaa 1992, Sinclair 1999, Košťál et al. 2003, Strachan et al. 2011). Once in diapause, the larvae can even reportedly survive submersion in liquid nitrogen, at temperatures as low as -196°C , making it one of the most freeze tolerant arthropods on Earth (Košťál et al. 2011). In contrast, most temperate drosophilids are freeze intolerant as juveniles and die after short-term exposure to even relatively mild temperatures (Dalton et al. 2011, Stephens et al. 2015, Enriquez and Colinet 2017, Stockton et al. 2018). For this reason, while leaf litter and snow pack may have an insulating effect and buffer against ambient thermal extremes, *D. suzukii* pupae likely cannot survive quiescence for extended periods in the leaf litter without a mechanism to initiate diapause.

While access to shelter conferred protection for quiescent adults, our data indicate that overwintering survival under leaf litter may be limited to years when ground temperatures remain above freezing. Because our study took place during 2 yr, we were able to see the differential effects of a mild and an extreme winter on survival. Survival was greater for all groups during the milder study year in 2016–2017 when temperatures were significantly warmer. In our most extreme winter climates (ME, MI, NY), approximately 75% of *D. suzukii* adults died within 2 wk exposure. In contrast, survival was recorded up to 10 wk under more moderate winter conditions. In NC we observed complete reproduction in natural conditions during the winter, indicating that off-season, winter-time reproduction may occur in mild climates.

During the 2017–2018 winter season, we did not observe high or consistent captures of wild flies at any northern sites. Captures were recorded in the initial weeks following subfreezing temperatures, but sustained captures ended by mid-winter at almost all locations. Only in GA did we capture flies throughout the winter field season, from December to March. Georgia also experienced the highest mean temperatures of all our field sites. Previous attempts at large-scale winter trapping have shown that while *D. suzukii* are present in such climates, overwintering movement and survivorship appears to be affected by the severity of the winter season (Rossi-Stacconi et al. 2016, Grassi et al. 2018, Thistlewood et al. 2018, Leach et al. in review).

Further research is needed to determine which habitats are most hospitable to overwintering *D. suzukii* adults. In OR, *D. suzukii* reportedly overwinter in Douglas-fir trees (*Pseudotsuga menziesii*) in aggregations, which were discovered in 2017 during tap sampling (D. Rendon, unpublished data). Although the current study focused on leaf litter, for which there has been considerable discussion as an overwintering habitat, (Boulétreau-Merle and Fouillet 2002, Hoffmann et al. 2003b, Kimura 2004, Dalton et al. 2011, Jakobs et al. 2015, Rossi-Stacconi et al. 2016, Shearer et al. 2016), future research may test other potential refugia. The Mediterranean fruit fly, *Ceratitidis capitata* Wiedemann (Diptera: Tephritidae), which like *D. suzukii* displays low freeze tolerance and does not undergo obligate diapause, is known to overwinter inside apples (Papadopoulos et al. 1996, 1998). Trapping studies and fruit recovery programs may be needed to determine whether fallen pome fruit could provide overwintering harborage for *D. suzukii*. In addition, some drosophilids are known to overwinter in anthropogenic structures (Newbury et al. 1984, Kimura 1988), but to our knowledge, no data exist currently linking *D. suzukii* to such environments. A recent study in MI has shown that *D. suzukii* reproduce on fallen fruit and pomeace and that captures are highest on farms with onsite fermentation areas, such as wine production or cideries (Bal et al. 2017). Like *D. melanogaster* and *D. virilis*, *D. suzukii* may inhabit urban islands

(Atkinson and Shorrocks 1977, Newbury et al. 1984) throughout the winter months, if only in isolated populations.

The idea of intraspecific resource partitioning is well known in *Drosophila*, including *D. suzukii* (Atkinson and Shorrocks 1977). In Japan, *D. suzukii* appears to employ a bet-hedging strategy with regard to movement. While some individuals remain local and enter a state of reproductive diapause in the lowlands of Japan during the late summer months, others use altitudinal movement to maximize resource acquisition, thermo-regulate, and shift their reproduction to later in the season (Mitsui et al. 2010). As such, there is speculation that invasive pockets of *D. suzukii* in Europe and North America may engage in migratory freeze avoidance, with some portion of the population relocating annually to warmer climates (Kimura 2004, Dalton et al. 2011). Among *Drosophila*, at least two other species are known for migration. In the *Drosophila immigrans* (Diptera: Drosophilidae) species group, *Drosophila curviceps* (Diptera: Drosophilidae) strategically manages thermal regulation via seasonal migration between the warmer lowlands and the cooler highlands of central Japan (Kimura and Beppu 1993), and *Drosophila simulans* (Diptera: Drosophilidae), which is more closely related to *D. suzukii* within the *D. melanogaster* group, seasonally migrates between Northern and Southern locations in North America (Machado et al. 2016). Low capture levels in the winter and early spring may reflect a partial absence of *D. suzukii* in Northern locations, until populations from warmer areas re-infest crop systems in the spring (Kimura 2004, Mitsui et al. 2010, Dalton et al. 2011, Jakobs et al. 2015). In Italy, recent mark-release-recapture of *D. suzukii* showed altitudinal migration similar to that previously reported in Japanese populations and indicated movement patterns extending up to 9,000 m seasonally (Tait et al. 2018). In North America, ongoing research (J. Chiu, personal communication) using single nucleotide polymorphism (SNP) analysis aims to determine seasonal population movement between southern and northern states.

Conclusions

Our study raises concerns that in unusually warm winters, which are predicted to occur more frequently due to climate change (Langille et al. 2017, Pecl et al. 2017), population suppression during this part of the season may become less predictable (dos Santos et al. 2017). As a result, understanding *D. suzukii* movement, habitat use, and resource requirements during the winter months may be increasingly important for predicting risk and for the development of novel pest management strategies. While our data suggest that 2 wk below freezing causes a substantial reduction in wild fly populations, this does not take into account other factors affecting the population such as habitat, refugia, migration, and access to dietary resources. Future research should attempt to identify how these factors affect overwintering success and investigate the economic impacts of off-season pest suppression.

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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