

# Stage-Specific and Seasonal Induction of the Overwintering Morph of Spotted Wing *Drosophila* (Diptera: Drosophilidae)

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## Abstract

*Drosophila suzukii* Matsumura (Diptera: Drosophilidae) is currently a major pest management challenge in berry and cherry production. This species has a winter morph phenotype with longer wings and increased melanization associated with survival in colder conditions. Measurements of wing morphology in Michigan *D. suzukii* collected during 2016 and 2017 showed that induction of this morph began in September and increased into December, correlated with decreasing temperature and day length. Importantly, we found that wing length increases along a continuous scale and there is overlap between the two morph types. We tested whether temperature or photoperiod elicited this phenotypic change using a factorial design with each preadult lifestage held at 10 or 25°C and 16:8 or 8:16 L:D. Our results support temperature as the main driver of transition to the winter morph for all immature stages. Comparing the reproductive capacity of winter morph flies in cold conditions and when previously acclimated to warm conditions, flies with the acclimation experience laid comparable numbers of eggs as the summer morphs at 25°C, indicating that winter morphs can reproduce after surviving cold periods. These results highlight the ability of *D. suzukii* to adapt to changing temperature conditions, allowing it to survive cold and also exploit warmer periods to build populations when conditions allow.

**Key words:** *Drosophila suzukii*, phenotypic plasticity, temperature, photoperiod

Insects use many strategies to survive seasonal changes including freeze tolerance (Sømme 1964, Zachariassen 1985, Bale 2002, Denlinger and Lee 2010), freeze avoidance (Leather et al. 1993, Bale 2002), chill susceptibility (Clough et al. 1990, Howling et al. 1994), chill tolerance (Bale 1996, Bale 2002), and opportunistic survival (Coulson and Bale 1991, Bale 2002). Seasonal polymorphism is often observed as a component of these adaptations, including increased melanization (Moczek 2010, Fedorka et al. 2013), larger body size (Atkinson and Sibly 1997, Hodek 2012, Nielsen and Papaj 2015), and longer wings (Larsen and Nault 1994, Endo 2006). In some cases, the adaptations are to survive the winter when low temperatures and dry or wet air conditions create a bottleneck for the population. Understanding the induction of adaptations to cold and how this affects important life-history traits should help with predicting populations and understanding gene flow. This could have implications for insecticide resistance management since survivors may carry resistance genes into the following spring (Caprio and Hoy 1994, Carrière et al. 2001, Baker et al. 2014, Gress and Zalom 2018).

The overwintering biology, physiology, and behavior of *Drosophila suzukii* (Diptera: Drosophilidae) is not well understood despite this pest successfully invading regions with cold winters.

It now persists in temperate berry and cherry producing regions of North America, South America, and Europe (Bolda et al. 2010, Dreves et al. 2011, Goodhue et al. 2011, Farnsworth et al. 2017). *Drosophila suzukii* are generally believed to overwinter as adults in leaf litter that provides protection from extreme winter temperatures (Wallingford et al. 2016, Rendon et al. 2018, Stockton et al. 2019). Kanzawa (1939) noted seasonal polymorphism among *D. suzukii* in cold regions, observing darker body color in the late summer and early winter. This winter morph has greater melanization and longer wing length than the summer morph, which may allow for survival in extreme cold temperatures (Shearer et al. 2016). Greater melanization in drosophilids is thought to decrease desiccation during cold periods (Rajpurohit et al. 2008). This is supported by recent reports of winter morph *D. suzukii* surviving winter conditions under leaf litter in various climates, including temperate climates of Michigan and New York (Stockton et al. 2019).

Winter morph *D. suzukii* can be induced in a laboratory setting when eggs or larva are exposed to colder temperatures and shorter day length, mimicking fall and winter conditions (Shearer et al. 2016, Everman et al. 2018, Rendon et al. 2018). These winter morphs can survive for extended periods of time at extreme cold temperatures

(Stephens et al. 2015), which suggests that *D. suzukii* winter morphs are cold tolerant. Additionally, winter morphs undergo a reproductive diapause not seen in summer morphs (Wallingford and Loeb 2016), though it is not understood whether this is driven by temperature, photoperiod, or morph type. The seasonal phenology of these morphs in the Midwest region and the environmental cues that elicit production of these morphs has not been investigated, though this will likely have important implications for the size of the endemic spring population.

Our objectives were to 1) determine the timing of induction of the winter morph under field conditions, 2) to determine the stage-specific temperature and photoperiod conditions required to elicit winter morph phenotype, and 3) to compare the oviposition capacity of winter morphs compared to summer morphs at three different ecologically relevant temperatures.

## Materials and Methods

### Wing Removal and Measurements

To measure wing length, the right wing of each specimen was removed by cutting near the regula using the tip of a small metal syringe (8 mm BD 3/10 ml/cc Insulin Syringe, Becton, Dickinson and Company, Franklin Lakes, NJ). Each wing was placed flat on a glass slide (VWR Micro Slides, Cat. No. 48300-037, VWR International LLC, Radnor, PA). All flies were stored in 80% ethanol prior to and during wing removal. Up to 10 female and 10 male wings on slides were arranged and fixed to the slide using a protein fixative (ClearMount Mounting Solution, Life Technologies, Frederick, MD) and slide cover (VWR Plastic Cover Slip, Cat. No. 48376-049, VWR International LLC). Additionally, the whole abdomen melanization of each specimen was recorded as 'light', 'medium', or 'dark'. This was a subjective assessment and lab-reared summer morphs were used as the baseline 'light' category. All slides were then photographed at identical magnification parameters (Leica S6D and Microscope Camera MC120 HD, using the Leica Application Suite V 4.6.0, Leica Microsystems Inc., Buffalo Grove, IL) and measured using ImageJ (imagej.nih.gov/ij/). Two length measurements were made for each wing along the IV vein, as described in Shearer et al. (2016). For analysis, these two lengths were summed and considered as total wing length.

### Field-Caught Flies: Wing Length

Wild populations of *D. suzukii* were collected using a single yeast-sugar trap (Van Timmeren and Isaacs 2013) year-round in 2016 and 2017 across six sites in Allegan County, MI. All sites were within a 35 km radius of the Trevor Nichols Research Center in Fennville, MI (42.594383, -86.155558). In 2016, flies were caught from June to December, with no flies caught from January to May during the coldest months of the year. In 2017, flies were caught from May to December. In both years, 10 flies of each sex, when available, were randomly selected from the flies captured at each site. Their body color was assessed as described previously, and the right wing was then removed and mounted, as described above. To relate wing length to environmental changes, the daily temperature, humidity, and solar flux were taken from the weather station closest to the collection sites of the flies in Fennville, MI ([www.enviroweather.msu.edu/](http://www.enviroweather.msu.edu/)).

### Lab-Reared Flies: Wing Length

To determine how temperature and photoperiod affect morph induction of different life stages of *D. suzukii*, a factorial design was used with high or low temperature (25 or 10°C) and long or short-day photoperiods (16:8 or 8:16 L:D). Forty fly rearing vials were

established containing either eggs, or first, second, or third instar larvae or pupae. Ten vials of each life stage were placed in each of the four treatments. Each life stage was obtained by placing 10 male and 10 female *D. suzukii* in diet for 12 h to mate and lay eggs. At 12 h, adults were removed and vials were examined daily for the instar stage. Eggs were obtained at 12 h when adults were removed, and at 24 h, first instars were placed into each treatment. Second instars were obtained 3 d after adult exposure, third instars at 5 d, and pupae at 7 d after adult exposure. Adult emergence in each of these vials was checked and recorded every 2 d. After emergence surpassed 10 flies per vial, flies were anesthetized with CO<sub>2</sub> and placed into 80% ethanol for wing dissection.

### Oviposition

We compared oviposition by lab-reared colonies of winter and summer morph *D. suzukii*. Summer morphs were reared at 25°C, 16:8 L:D, and 60–70% RH while winter morphs were reared at 10°C, 12:12 L:D, and 60–70% RH. In addition to these two morphs, male and female winter morphs were placed in a rearing chamber at 25°C, 16:8 L:D, and 60–70% RH for 48 h prior to the experiment. This allowed them to mate and acclimate to warmer temperatures. Ten mated 3–7-d-old female *D. suzukii* of the summer morphs, winter morphs, and acclimated winter morphs were placed into separate 60 × 15 mm Petri dishes containing 10 ml of *Drosophila* diet (cornmeal recipe, *Drosophila* Species Stock Center, San Diego, CA) that was dyed green using food-grade green dye (McCormick and Company, Baltimore, MD), for better contrast to see the white eggs. The Petri dishes were then placed in environmental chambers at either 10, 15, or 25°C for 48 h with a 16:8 photoperiod and 60–70% RH. After 48 h, the number of eggs that were laid in each Petri dish was counted by placing the dishes under a stereomicroscope (Nikon SMZ-2B, Nikon Instruments Inc., Melville, NY) and counting the pairs of spiracles above the surface of the diet.

### Statistical Analyses

The wing length values for field-collected flies were analyzed using a generalized linear mixed model with date nested within site and included as random effects. The lab data for time to emergence and wing length were analyzed using a linear mixed effect model. Oviposition data were analyzed using a generalized linear mixed model with the logit link function. All models were followed by Tukey's Honestly Significant Difference test for post-hoc comparisons. All data were analyzed using R (3.5.2., R Core Team, R Foundation for Statistical Computing, Vienna, Austria).

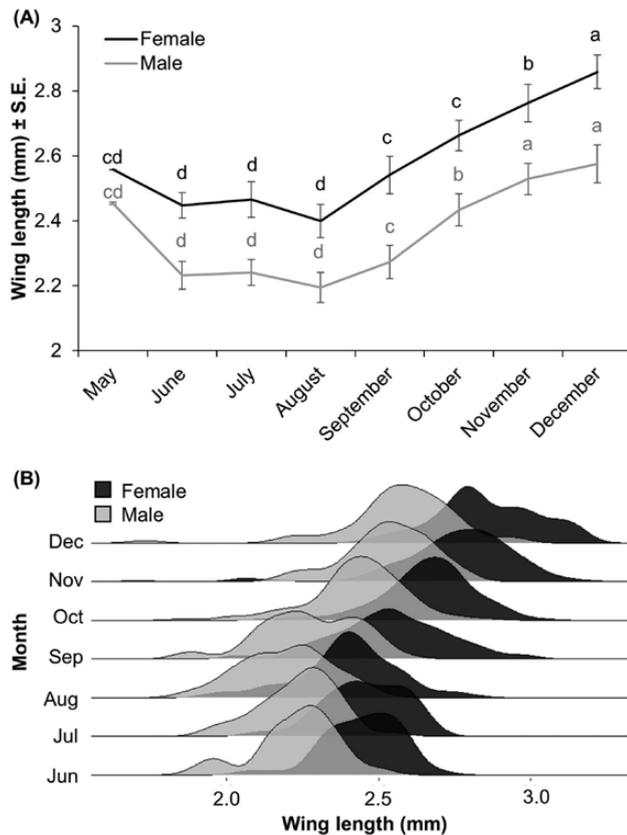
## Results

### Field-Caught Flies: Wing Length

In May, wing lengths were 2.454 ± 0.003 mm for male flies and 2.558 ± 0.0004 mm for females. Wing lengths declined in the middle of the season with the smallest size in August (male: 2.19 ± 0.04; female: 2.39 ± 0.05), then increased until they reached maximum length in December (male: 2.57 ± 0.06; female: 2.86 ± 0.05) (Fig. 1). In both years, the average daily temperature began to decline in September while the solar flux declined starting in August (Fig. 2). Humidity fluctuated throughout the season, but generally increased as the season progressed towards winter (Fig. 2B). There was a significant effect of month on wing length of male ( $F_{7,635} = 78.54, P < 0.0001$ ) and female ( $F_{7,688} = 85.47, P < 0.0001$ ) flies. For both sexes, flies considered having a 'medium' or 'dark' body color category had statistically longer wings compared to flies in the 'light' body color category ( $F_{2,1344} = 152.2; P < 0.001$ ).

### Lab-Reared Flies: Wing Length

Flies reared at 25°C emerged up to  $64.4 \pm 1.1$  d earlier as adults compared to those at 10°C, regardless of photoperiod (25°C 8:16:  $F_{4,45} = 155.0$ ,  $P < 0.001$ ; 25°C 16:8:  $F_{4,45} = 35.6$ ,  $P < 0.001$ ; 10°C 8:16:  $F_{4,42} = 301.3$ ,  $P < 0.001$ ; 10°C 16:8:  $F_{4,41} = 282.3$ ,  $P < 0.001$ ) (Fig. 3). This difference between treatments was greatest in the early life stages compared to the later life stages. Additionally, there was a significant interaction between temperature and photoperiod in



**Fig. 1.** (A) Average wing length ( $\pm$  SE) of field-caught female (black) and male (gray) *D. suzukii* from May through December in 2016 and 2017. Different letters between each month indicate statistically different values for each sex. (B) The monthly distribution of wing lengths of the sampled population for males and females.

time to emergence ( $F_{1,172} = 407.2$ ,  $P < 0.0001$ ). In the warmer conditions, photoperiod did not influence emergence time at any of the life stages ( $F_{1,18} = 1.0$ ,  $P = 0.3$ ). However, in the 10°C conditions, flies in the short day photoperiod took statistically less time to emerge as adults in all life stages (egg:  $F_{1,16} = 17.7$ ,  $P < 0.001$ ; first:  $F_{1,17} = 64.9$ ,  $P < 0.001$ ; second:  $F_{1,16} = 70.2$ ,  $P < 0.001$ ; third:  $F_{1,16} = 57.3$ ,  $P < 0.001$ ; pupae:  $F_{1,18} = 26.3$ ,  $P < 0.001$ ).

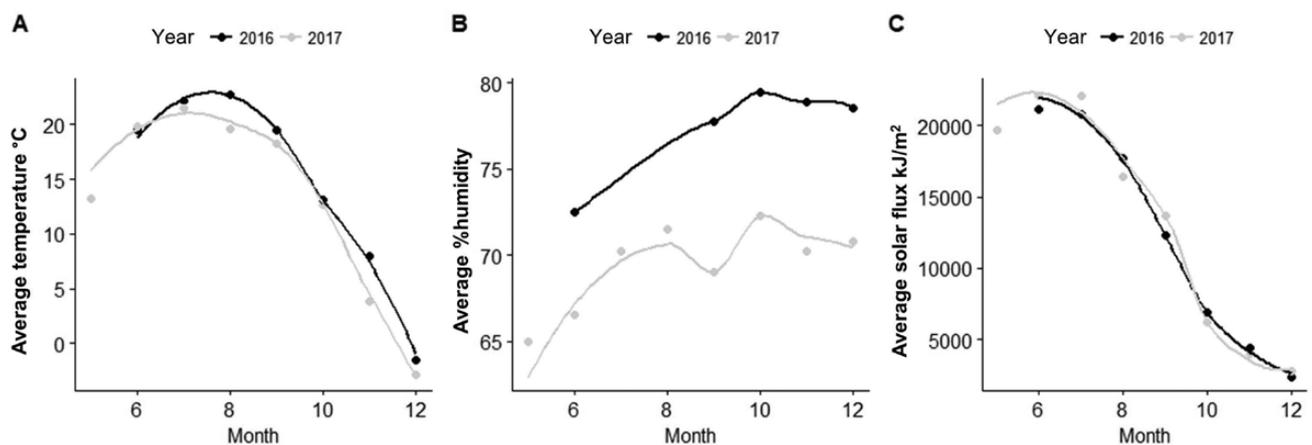
Temperature had a stronger effect on wing length than photoperiod. The flies raised in cold temperatures had the longest wings regardless of photoperiod or life stage exposed ( $F_{1,383} = 1520.6$ ;  $P < 0.0001$ ) (Fig. 4). However, there was a significant interaction between temperature and photoperiod ( $F_{1,383} = 36.3$ ;  $P < 0.0001$ ). In warm conditions, eggs, first, second, and third instar flies emerged with longer wings under short day conditions than when they were reared in long day conditions (egg:  $F_{1,27} = 7.4$ ,  $P = 0.01$ ; first:  $F_{1,27} = 30.6$ ,  $P < 0.001$ ; second:  $F_{1,27} = 8.3$ ,  $P = 0.008$ ; third:  $F_{1,27} = 15.2$ ,  $P < 0.001$ ). There was no difference in wing length for flies emerging from pupae exposed to either photoperiod in warm conditions (pupae:  $F_{1,27} = 3.4$ ,  $P = 0.08$ ). In cold conditions, only first and second instars had longer wings in long day conditions compared to short day (first:  $F_{1,27} = 4.2$ ,  $P = 0.049$ ; second:  $F_{1,27} = 4.5$ ,  $P = 0.04$ ). Flies emerging from eggs, third instars, and pupae exposed to different conditions all had similar wing lengths in cold conditions regardless of photoperiod (eggs:  $F_{1,27} = 1.3$ ,  $P = 0.3$ ; third:  $F_{1,27} = 0.4$ ,  $P = 0.5$ ; pupae:  $F_{1,27} = 2.0$ ,  $P = 0.2$ ).

### Lab-Reared Flies: Oviposition

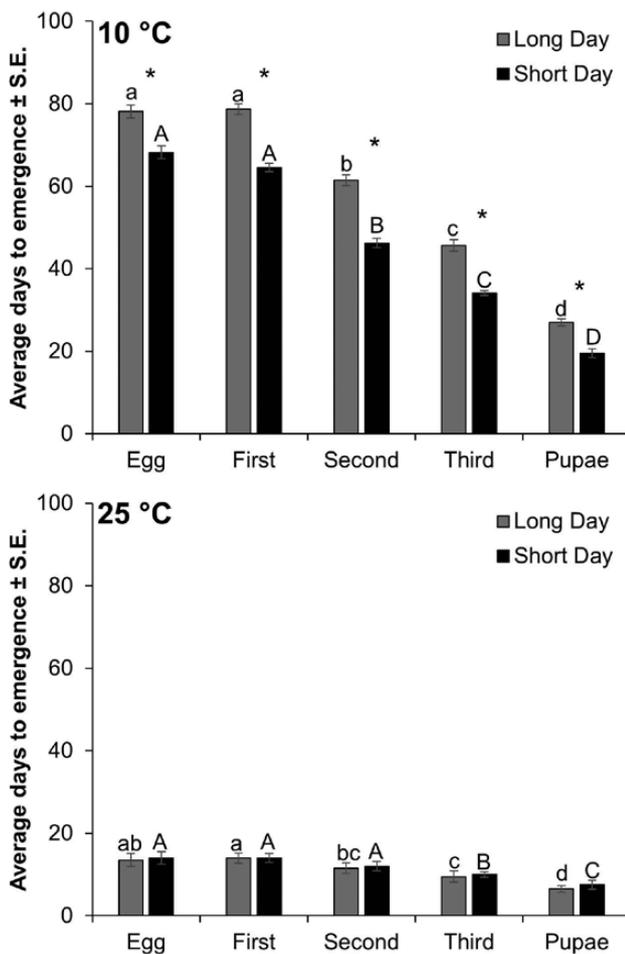
Summer morph flies laid significantly more eggs compared to either the winter morphs or the acclimated winter morphs at 10°C ( $F_{2,9} = 22.34$ ;  $P < 0.001$ ) and 15°C ( $F_{2,9} = 38.78$ ;  $P < 0.001$ ) (Fig. 5). At 25°C, there was no difference in the number of eggs laid between the summer and acclimated winter morphs, though the unacclimated winter morphs laid significantly fewer eggs ( $F_{2,9} = 7.49$ ;  $P = 0.01$ ). There was no statistical difference in the number of eggs laid between the acclimated and unacclimated winter morphs across the three temperatures.

### Discussion

This study shows that *D. suzukii* respond to changing weather conditions in the Midwest region of the United States with an increased proportion of adult flies that exhibit the winter morph phenotype, confirming the general findings from recent studies in other regions



**Fig. 2.** (A) Average temperature (°C), (B) percent humidity, and (C) solar flux ( $\text{kJ}/\text{m}^2$ ) per month from May through December in 2016 (black) and 2017 (gray) from the nearest weather station (Fennville, MI, MSU Enviroweather).



**Fig. 3.** Average days to emergence  $\pm$  SE of *D. suzukii* adults when put into cold (10°C, top) or warm (25°C, bottom) conditions in either long day (16:8 L:D) or short day (8:16 L:D) photoperiods. The egg, first instar, second instar, third instar, and pupal life stages were tested separately. Lowercase letters above the bars denote significant differences among life stages for the long day, and capital letters denote significant differences among life stages for the short-day emergence duration values for each temperature. Asterisks denote significant differences between short and long days within each life stage for cold and warm temperatures.

of the United States (Shearer et al. 2016, Wallingford and Loeb 2016, Rendon et al. 2018, Stockton et al. 2019). This induction increases gradually through the fall months, and all of the immature life stages are sensitive to the environmental changes. We found that the response of immature stages was primarily a response to temperature rather than day length, which we suspect may be an adaptation providing flexibility to adjust to early cold fall temperatures rather than being constrained by day length that would be more tied to calendar date.

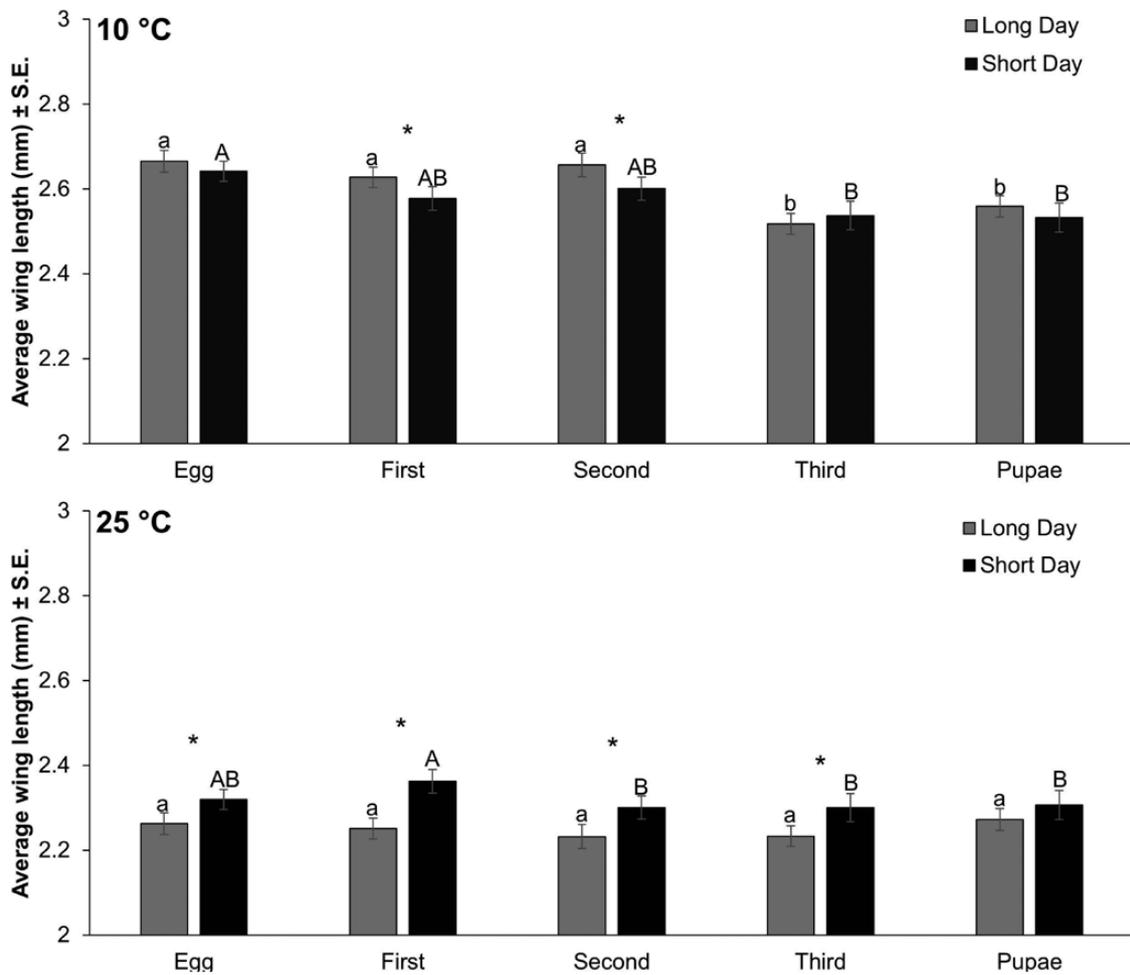
Our findings on seasonal wing length for flies caught in Michigan are very similar to those found in Oregon by Shearer et al. (2016). Both temperature and photoperiod appear to be highly correlated with the shift in wing length and corresponding body melanization. Humidity was fairly different between the two study years (Fig. 2B) and this could indicate that humidity is not an important factor in the development of winter morphs. Importantly, the development of longer wings is gradual within the population and takes up to 4 mo until the population of flies reach the maximum wing length based on the results of our study. The distribution of wing length as the season progresses (Fig. 1B) suggests that more long-winged flies are

entering the population, rather than there being a gradual increase in wing length across individuals. However, there is variability among flies within the population at any given time point (Fig. 1) and further research should target whether the longest winged individuals indicate the greatest ability to overwinter. We determined that wing length is represented across a continuum produced by a changing environment and we emphasize that the winter and summer morphs do not represent a dichotomy.

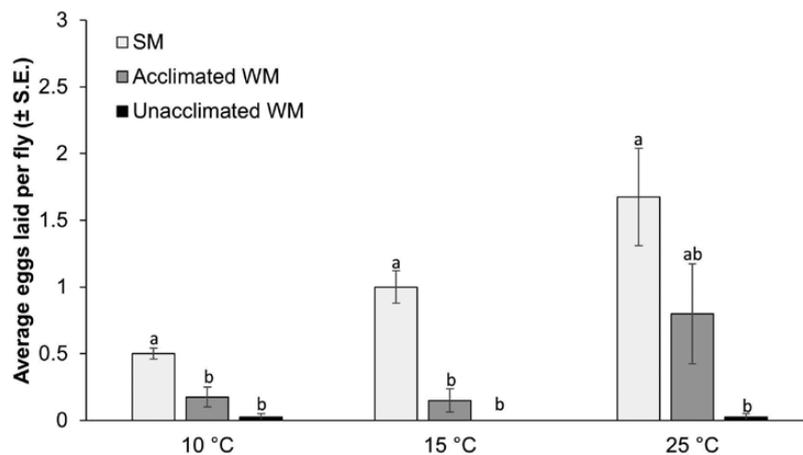
In both years of this study, no flies were captured in traps earlier than May. In the May samples, however, some flies with longer wings were observed, though these were statistically similar to the flies caught in the summer. These early-caught flies may be winter morphs that survived through the cold months, though a larger sample size and better understanding of winter survival is needed to fully evaluate this.

Interestingly, while the 'medium' and 'dark' body color occur on flies with longer wings, there are also flies with a 'light' body color and longer wings, and 'light' colored flies occurred in every collection month. These flies were collected and assessed within 1 mo of capture and we expect the body color integrity would be unchanged. However, it is possible that the ethanol caused bleaching or fading of the body color, and a follow-up study could further clarify this. The darker melanization described by Shearer et al. (2016) was not associated with short wings in our study and was found only in October, November, and December, though light colored flies are also found during these months. This indicates that body color may not be a reliable indicator of winter morphs and may further support that winter and summer morphs don't represent a distinct dichotomy. In other *Drosophila* species, increased melanization is associated with reduced desiccation (Parkash et al. 2009, Brisson et al. 2010, Parkash et al. 2010). Longitudinal gradients of melanization have been observed, including for *Drosophila nepalensis*, *D. jambulina*, *D. takahashii*, *D. ananassae*, and *D. immigrans*, where darker flies are located in arid environments (Brisson et al. 2010, Rajpurohit et al. 2008, Parkash et al. 2009). *Drosophila americana*, however, has increased melanization in more humid environments (Wittkopp et al. 2011), suggesting that this mechanism for reduced desiccation is not found among all *Drosophila* species. Though the longitudinal variation in melanization of *D. suzukii* has not been evaluated, we expect that this darker pigmentation plays a role in increasing longevity and survival during seasonal changes. Additionally, increased melanization is thought to contribute to protection from UV radiation for *D. melanogaster* (Wang et al. 2008), but this is unlikely to be adaptive for *D. suzukii* in this scenario, where solar flux is decreasing as melanization increases, and flies are unlikely to be exposed to sun during this period.

Wing length changes in other insects in response to seasonal changes, including aphids (Dixon 1985) and gerrids (Zera 1985). This is also observed in hemipterans in response to photoperiod changes (Zera et al. 1983, Tanaka and Wolda 1987). For *D. suzukii*, it is undetermined whether the longer wings increase winter survival, though current findings suggest that *D. suzukii* are not active during the winter and are likely found under protected areas (Stockton et al. 2019). The wing length in our study may also serve as a proxy to body size which could increase survival at cold temperatures (Robertson and Reeve 1952, Sokoloff 1966, Gilchrist et al. 2001, Shearer et al. 2016), however, this was not measured in our study. Additionally, body size and wing length may also be related to host quality, size, competition among other larvae, or age of the fly. However, Jaramillo et al. 2016 found no difference in wing length or body size when *D. suzukii* were reared in a blueberry versus *Drosophila* larval diet.



**Fig. 4.** The average wing length (mm)  $\pm$  SE of *D. sukii* adults when put into cold (10°C, top) or warm (25°C, bottom) conditions in either a long day (16:8 L:D) or short day (8:16 L:D) photoperiods in the egg, first instar, second instar, third instar, or pupal life stage. Lowercase letters denote significant differences among life stages for the long day, and capital letters denote significant life stages for the short day for each temperature. Asterisks denote significant differences between short and long days within each life stage.



**Fig. 5.** Average number of eggs laid per fly ( $\pm$  SE) within 24 h at 10, 15, or 25°C for summer morphs (SM), winter morphs (unacclimated WM), and winter morphs acclimated to warmer temperatures (25°C) for 48 h prior to experiment (acclimated WM). Different letters above bars within a temperature indicate significant differences among the morph treatments at  $\alpha = 0.05$ .

In the laboratory, we found that the time to reach eclosion was strongly affected by temperature (Fig. 1), with pupae at 10°C having up to double the time to emergence than those at 25°C. Eggs or first

instars at 10°C took an average of almost 80 d to eclosion. This slow development could be a benefit for initial seasonal diapause during at least part of the fall and early winter season. Fall conditions with low

nighttime temperatures could assist larvae in acclimating to colder temperatures before the onset of winter. However, freezing conditions for multiple days are thought to kill larvae in fruit (Kanzawa 1939) and pupae are also unlikely to survive outside in freezing conditions (Stockton et al. 2019); so, *D. suzukii* would likely need to reach adulthood to survive sustained freezing conditions. When *D. suzukii* was reared in cold temperatures they developed faster under short-day conditions compared to long-day conditions. Similar findings have been observed in *D. montana*, where development time decreased with the onset of decreasing photoperiod to simulate autumn conditions (Salminen et al. 2012). This could be another adaptation to help *D. suzukii* reach the adult stage for overwintering, as the cold conditions combined with shorter daylength would be a more reliable signal of approaching winter.

We determined that seasonal morph induction can occur at 10°C with either a long-day or short-day photoperiod (Fig. 4). While there was increased wing length for flies under the short-day photoperiod at 25°C, increased melanization was not seen in these flies. Additionally, the wing length increases seen in the laboratory were less than the wing length of field-collected flies in late season. However, the capacity of these flies to survive at colder temperature was not evaluated and should be investigated further. Additionally, it is important to note that the first 10 emerging flies were analyzed in the lab-reared conditions, and there could be a difference in wing length between early and late emerging flies and the individual age of the flies. In a recent multistate study, survival of winter morphs was greater than summer morphs, and winter morphs were capable of surviving up to 10 wk in winter conditions (Stockton et al. 2019). Their survival duration was significantly shorter in colder climates including Michigan and New York. Survival by winter morph *D. suzukii* in the laboratory has been recorded for up to 13 mo at 10°C (H. Leach, unpublished data), but survival of *D. suzukii* in sustained freezing field conditions is expected to be much shorter. This should be further evaluated under relevant temperature and humidity conditions, with and without snow cover, and in the different habitats that this fly may be found in the winter. However, there is still limited information on the range of overwintering habitats used by this species (Stockton et al. 2019).

We found that *D. suzukii* winter morphs are capable of laying a small number of eggs, even at 10°C. However, when acclimated to warmer conditions and given the opportunity to mate, their capacity to lay eggs increases. These results reflect those found in Wallingford et al. (2016), where egg maturation was significantly reduced when placed in 10°C. However, they also found an increased capacity for winter morphs to mature eggs given a short-day period compared to a long-day period, especially when held in these conditions for a long period of time. Our study reflected the activity of these flies for 48 h only, and we expect there may be greater physiological considerations when keeping these flies at these varied conditions for longer. Additionally, Wallingford and Loeb (2016) found that it took 5–6 d for field-collected winter morphs to begin laying eggs in simulated summer conditions. Regardless of photoperiod, *D. suzukii* has an ability to modulate its reproductive potential according to temperature, which suggests that this insect would be able to rapidly exploit suitable conditions during spring warm-up periods.

Our data highlight that *D. suzukii* exhibits temperature-induced phenotypic plasticity, which is thought to benefit their survival at lower temperatures (Shearer et al. 2016, Stephens et al. 2015, Wallingford et al. 2016, Stockton et al. 2019). While we found that temperature is the main driver of winter morph induction, both our lab and field studies indicate a gradient of wing length, depending on life stage exposure and photoperiod. Further experiments are

warranted to fully understand the role of increased wing length and melanization, among other phenotypic and physiological changes for winter survival. This would provide a better understanding what environmental cues may be important for overwintering habitat and survival (Kirkpatrick et al. 2018, Stockton et al. 2019). In colder regions, harsh winters are likely to serve as a population bottleneck, and more focus on this sensitive period may offer important information to improve management of this pest.

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## Author Contributions

All authors conceived the research. S.V.T. and J.S. conducted the experiments, H.L. analyzed the data, and R.I. secured the funding. All authors wrote, read, and approved the manuscript.

## Conflict of Interest

The authors declare no conflict of interest.

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