

Research

Behavioral and Physiological Resistance to Desiccation in Spotted Wing *Drosophila* (Diptera: Drosophilidae)

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Abstract

With a high surface to volume ratio, small organisms must carefully regulate their internal water status. Spotted-wing drosophila, *Drosophila suzukii* (Matsumura), is an invasive frugivorous insect distributed across a wide range of geographical regions that can have periods of dry and hot weather, suggesting that this species has strategies to avoid stressful environments and reduce water loss. It also survives winter as an adult fly, indicating that it has adaptations to the low air humidity of this season. To determine the importance of water stress to *D. suzukii*, we studied their survival in environments of low humidity, which was manipulated using Drierite, and their survival and water loss in response to desiccation. Survival of both sexes was lower in drier conditions, and while female winter morph *D. suzukii* had higher mortality early on, remaining flies were able to survive longer in the drier conditions than the summer morphs. A bioassay method was adapted from [Enjin et al. \(2016\)](#) using 48-well plates to videotape the location of flies and quantify their behavioral responses to humidity. Male and female *D. suzukii* avoided dry conditions within the bioassay system, but only when there was at least 25% differential between humidity extremes. This response was observed for both summer and winter morphs of *D. suzukii* and our results provide guidance for attempts to manipulate crop environments to reduce the economic impact of this pest.

Key words: water balance, *Drosophila*, humidity sensing, water loss

Maintaining water balance is a critical aspect of insect physiology ([Arlian and Veselica 1979](#)), with important implications for geographical distribution and habitat use ([Eckstrand and Richardson 1981](#), [Naidu and Hattingh 1986](#), [Hoffmann and Harshman 1999](#), [Marron et al. 2003](#)). Insects that are arid-adapted primarily maintain water balance through exhibiting reduced rates of water loss when compared with nonadapted species ([Hadley 1994](#)). This reduced rate of desiccation has been observed in spiders ([Hadley et al. 1981](#)), beetles ([Hadley and Schultz 1987](#), [Zachariassen et al. 1987](#)), ants ([Hood and Tschinkel 1990](#)), and fruit flies ([Eckstrand and Richardson 1981](#), [Gibbs and Matzkin 2001](#)).

Small-bodied insects with a high surface to volume ratio are at greater risk of desiccation than large insects ([Hadley 1994](#)). This is particularly important for *Drosophila* flies that are only a few millimeters long and have been found in some of the driest regions of the world ([Eckstrand and Richardson 1980, 1981](#), [Gibbs and Markow 2001](#), [Gibbs and Matzkin 2001](#), [Marron et al. 2003](#)). In *Drosophila melanogaster* Meigen (Diptera: Drosophilidae), there is no absorption of water from the atmosphere and net water loss (assessed by fresh weight change) occurs even >90% relative humidity (RH), and increased with decreasing RH ([Arlian and Eckstrand 1975](#)). [Enjin et al.](#)

(2016) showed that *Drosophila* sense humidity via sensillae located in the first two chambers of the sacculus, and three different species exhibited varying preferences for RH. The flies showed innate and predictable preferences for specific humidities based on their native climate, suggesting that humidity sensing is a plastic trait ([Enjin et al. 2016](#)). The ability to sense humidity and respond to these gradients allows them to avoid desiccation, as drosophilids lose significant quantities of water and dry mass during desiccation events ([Lehmann et al. 2000](#), [Marron et al. 2003](#)). In addition to behavioral and sensory adaptations, many small insects have physiological adaptations to reduce water loss, including lowered metabolic rate leading to reduced respiration and thus water loss through the spiracles ([Zachariassen et al. 1987](#), [Addo-Bediako et al. 2001](#)) and modifications of the fatty acid composition of the cuticle reducing permeability and thus water loss ([Quinlan and Hadley 1993](#), [Williams et al. 1998](#)). [Folk et al. \(2001\)](#) found that desiccation resistance in selected flies was positively correlated with hemolymph volume and to a lesser extent with the intracellular water content of the flies.

Drosophila flies have various water conservation mechanisms that have evolved to improve their survival in challenging environments ([Gibbs 2003](#)). Previous research has focused on physiological

mechanisms: lower water excretion, loss through the cuticle, and respiratory loss through the spiracles. Gibbs et al. (1998) found that cuticular wax composition did not affect water loss rates in *Drosophila mojavensis*, and suggested that avoidance of unfavorable humidity conditions would be more important for survival.

Drosophila suzukii (Matsumura), commonly referred to as spotted-wing drosophila, is an invasive frugivore that has become a severe pest in soft skinned berries and cherries globally (Walsh et al. 2011, Cini et al. 2014, Asplen et al. 2015). This species can occupy regions of the world with hot and dry periods during summer and some regions where cold and dry conditions occur during winter. However, the extent of winter cold and the extremes of summer heat can limit overall population growth (Eben et al. 2018, Thistlewood et al. 2018); this pest has been exceptionally successful over the past decade in expanding its range to most of the major fruit production regions of the world. Environmental conditions, including temperature and humidity, are important to the development and survival of *D. suzukii* (Dalton et al. 2011, Tochen et al. 2014, Tochen et al. 2016, Eben et al. 2018). Humidity can affect the survival of female *D. suzukii* adults, which ranged from 20 to 27 d in high RH conditions (71–94% RH) to 1–3 d in lower humidity ranges (20–33% RH) (Tochen et al. 2016). Humidity is strongly correlated with captures of this species in field conditions (Tochen et al. 2016) and infestation of fruit within blackberry canopies (Diepenbrock and Burrack 2017). Through the annual activity cycle, high heat and low humidity can result in lower population density of *D. suzukii* (Eben et al. 2018).

While numerous studies have examined the effect of humidity on the development and survival of *D. suzukii*, however, there has been very little study of the different morphs of *D. suzukii*. Adults of *D. suzukii* become darker and the wing length increases during the autumn in response to lower temperature and shorter photoperiod, and these changes can be induced artificially (Shearer et al. 2016, Wallingford et al. 2016). These modifications are expected to be adaptive for survival in adverse conditions (Wallingford et al. 2016), and they are expected to be associated with adaptive behavioral and physiological changes to survive the winter. Adults with these modified characteristics were described as the winter morph by Shearer et al. (2016), with those developing under high-temperature conditions and longer photoperiod conditions termed the summer morphs. While induction of these morphological differences is clear and repeatable, there is much less known about other changes that may contribute to *D. suzukii* survival during the 3–4 mo of winter conditions, involving subfreezing temperature and low humidity in some regions where it persists.

Observations of *D. suzukii* under field conditions have shown that its activity and fruit infestation are affected by environmental conditions (Van Timmeren et al. 2017). However, temperature and humidity conditions vary at the same time, so there is a need to isolate each factor independently. Concentrated salt solutions are a well-tested approach to manipulate the RH of enclosed spaces (Winston and Bates 1960, Greenspan 1977), and these have been used previously to explore insect adaptation to dry conditions (Buxton 1931). These saturated solutions maintain a very constant RH in the head space above them, even under changing moisture conditions because a gain of water causes some of the solid to go into solution and a loss of water causes some of the dissolved material to precipitate. In this study, we determine the effect of desiccation on the survival and weight of both sexes of the different morphotypes of *D. suzukii*. Additionally, we assessed a choice bioassay method to determine whether *D. suzukii* exhibits a preference for different humidity levels and used this to ascertain whether winter and summer morphs of *D. suzukii* avoid environments with low humidity.

Materials and Methods

Cultures of *D. suzukii*

Adults of *D. suzukii* in this experiment were from a colony established using flies collected across western Michigan in 2016. Summer morph flies were cultured using a standard drosophila diet (Drosophila Stock Center, San Diego, CA) and maintained in growth chambers at 25°C, photoperiod of 16:8 (L:D) h, and 75% RH. Adults of the *D. suzukii* summer morph used in experiments ranged from 2- to 6-d old in drosophila vials (2.5 × 9.5 cm; Lab-Express, Ann Arbor, MI) with foam stoppers (Genesee Scientific, San Diego, CA). Winter morph flies were reared from the summer morph colony using the method described in Wallingford et al. (2016). Mature summer morph adults (~15 males and 15 females) were placed into vials with drosophila diet for 24 h under simulated summer conditions (25°C, photoperiod of 16:8 [L:D] h). Adults were removed from the vials, and the vial with new eggs were placed in a growth chamber maintained at 10°C, photoperiod of 12:12 (L:D) h, and 75% RH for several months until adults were used in experiments.

Desiccation Survival and Weight Loss

The ability of *D. suzukii* to survive in low humidity was determined by adapting the method described by Folk et al. (2001). Desiccation vials were made by adding 4.5 g of Drierite to 50-ml plastic vials; foam stoppers were then placed directly above the Drierite and 20 flies (10 males, 10 females) were anesthetized with CO₂ and placed in the vials, which were sealed with Parafilm. Each morph of *D. suzukii* was replicated 10 times. The number of dead male and female flies were visually scored every 20 min until all flies in the vials were dead. The typical RH was measured in these desiccation vials using an EK-H4 multiplexer equipped with SHT71 sensors (Sensirion, Stäfa, Switzerland) and recorded with the EK-H4 viewer software Sensirion 2015 and vials were maintained at room temperature (~24°C), with the lights of the laboratory on, throughout the experiment.

To determine how desiccation affected water loss (as determined by the fresh weight of flies), and the loss of cations and anions (as determined by the dry weight of flies) over time, females for each morph of *D. suzukii* were desiccated in vials as described above. In total, 21 vials were prepared per morph of *D. suzukii*, and five female flies were added into each vial. All flies were removed from three randomly selected vials at 20-min intervals from the starting point to a maximum duration of 120 min, which was the duration that did not result in mortality based on previous experiments. At each timepoint, flies were removed from the desiccation vials, immediately transferred to Eppendorf vials, and flash frozen in liquid nitrogen. Flies were weighed individually on a Cahn C-35 Ultra-Microbalance (Cahn Scientific, Irvine, CA), and then they were dried over the following 3–4 d until their weight became stable in dehumidifying chambers filled with Drierite. Once dried, individual flies were reweighed to obtain their dry weight.

Humidity Preference

Super saturated salt solutions have previously been used to alter humidity in studies of *D. suzukii* (Tochen et al. 2016, Wong et al. 2018). In this study, to alter the RH, four super salt solutions were prepared by boiling 100 ml of deionized water, adding 50 g of the following salts: LiCl (Fisher-Scientific, Fairlawn, NJ), MgCl₂ (Fisher-Scientific), Mg(NO₃)₂ (Sigma-Aldrich, St. Louis, MO), or KNO₃ (Fisher-Scientific), and mixed using a stirring plate until dissolved. Once solutions cooled, more salts were added to the solutions until

solutions were saturated. Humidity produced in the headspace above these solutions was measured using an EK-H4 multiplexer equipped with SHT71 sensors and recorded with the EK-H4 viewer software (Sensirion). Solutions resulted in RH of 11% (± 0.72) above the LiCl solution, 38% (± 0.77) above the MgCl₂ solution, 54% (± 0.32) above the Mg(NO₃)₂ solution, and 90% (± 0.16) above the KNO₃ solution. The solutions maintained steady humidity levels during the 180-min duration of the experiments and remained at room temperature of $\sim 24^\circ\text{C}$ (Fig. 1A).

To compare the preferences of adult flies between the different humidity levels, choice arenas were modified from those described in Enjin et al. (2016). Arenas were made of 48-well plates, and the 24 wells on each side of the plates were filled with 0.75 ml of salt solution, leaving 9 mm between the flies and the salt solution, with a different solution placed in each half of the plate. After salt solutions were loaded into plates, a thin mesh was placed over the base of the 48-well plates to prevent the flies from drowning. Once the arenas were prepared, 30 *D. suzukii* (15 males and 15 females) were anesthetized with CO₂, then added to the center of the plate. To prevent the flies escaping, the humidity arenas were closed with the 48-well plate lid, and this provided a 3 mm high space in which the flies could move around freely (Fig. 1B). Arenas were filmed from above for 3 h after fly release, using a video camera (Canon Vixia HR R700) to record the behaviors. Video of the first 180 min of the flies' activity in the choice arena was analyzed, and the number of flies in each of the two humidity conditions in each treatment comparison was manually recorded at 15-min intervals.

All combinations of the four salt solutions were compared (11 vs 38%, 11 vs 54%, 11 vs 90%, 38 vs 54%, 38 vs 90%, and 54 vs 90%), and each comparison was replicated four times for both morphs of *D. suzukii*. The order of the treatments and their relative positions were randomized, and a white sheet was used to diffuse external light to control for potential effects of orientation on the direction of stimuli in the choice arenas.

Statistical Analysis

All data were tested for normality and homogeneity of variances using Kolmogorov–Smirnov and Levene's test, respectively. Transformations of the data were attempted, and assumptions

rechecked. When assumptions were met, parametric statistical tests were conducted, and when assumptions were not met, equivalent nonparametric tests were used. Desiccation effects on the survival of different sexes of summer and winter morphs of *D. suzukii* were analyzed with a generalized linear model, with a binomial distribution. Proportion dead was the response variable, and morph, gender, and time were included as fixed effects. Changes in the weight of females of both morphs of *D. suzukii* in response to desiccation were analyzed using a generalized linear mixed model, with fresh and dry weight as the response variables and desiccation time and morph as fixed variables and vial as a random factor.

The preference of adult *D. suzukii* for different humidity levels was determined using a preference index, calculated as follows: (no. of flies on the moister side – no. of flies on drier side)/total no. of flies. This returned values ranging from 1 to –1, where 1 signifies a complete preference for the higher humidity and –1 signifies complete preference for the lower humidity in the choice arena. A value close to 0 is returned when there is little or no preference for either humidity.

To determine whether the summer morph of *D. suzukii* showed a preference for one of the humidity regimes, due to non-normal data a nonparametric Wilcoxon–signed rank test was conducted with a hypothesized mean of 0, and with $\alpha < 0.05$ considered significant. The difference in preference index between morph types was determined using a generalized linear mixed model, with the preference index as the response variable and morph and time as fixed effects, and replicate arenas were included as a random factor in the model. All the analyses were conducted using JMP, Version 13.0 (SAS Institute Inc., Cary, NC).

Results

The RH inside desiccation vials while maintained at room temperature ($\sim 24^\circ\text{C}$) averaged $23 \pm 7.75\%$ throughout the course of desiccation experiments, and ranged from $38 \pm 0.02\%$ at the start and $12 \pm 0.06\%$ at the end of the assays. This difference is due to exposure of the inside of the desiccation vials to the outside environment during the transfer of flies into the vials. Survival of the different morphs and sexes of *D. suzukii* in response to desiccation was significantly

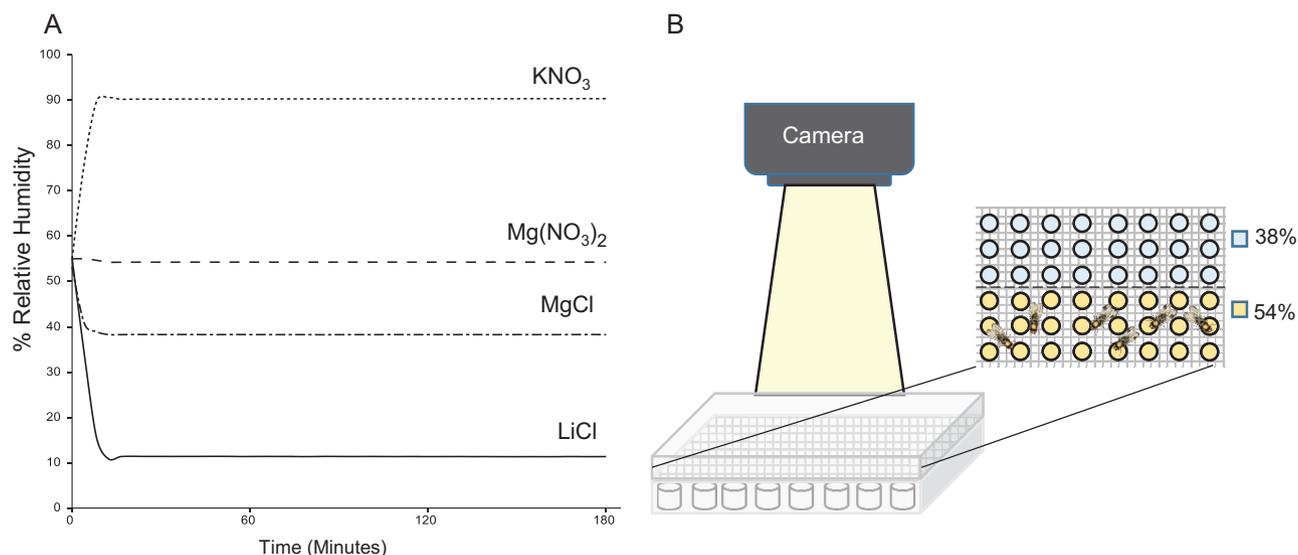


Fig. 1. (A) Average RH provided by super saturated salt solutions over time and (B) humidity preference assay setup for assessing behavioral response of *Drosophila suzukii*.

different ($\chi^2 = 699.11$, $df = 7$, 712 ; $P < 0.0001$; Fig. 2). Males of summer and winter morphs had a lower overall survival rate than females. Summer morph females initially survived better than the winter morph females; however, after 120 min, their survival decreased rapidly. Although winter morph females died at a faster rate initially, a small number (4%) of the winter morphs survived longer than all other individuals tested, with the last female dying after 320 min of desiccation.

In addition to morph-specific differences in survival, females of the morphs also differed in the amount of fresh weight ($F_{(1,190)} = 27.32$; $P < 0.0001$) and dry weight ($F_{(1,190)} = 14.16$; $P = 0.0002$) lost in response to desiccation. Winter morphs lost significantly less water, as determined by the loss in fresh weight in response to desiccation, than summer morphs (Fig. 3A). The winter morphs also lost significantly less dry weight in response to desiccation compared with summer morphs (Fig. 3B).

Both morphs were less likely to be in the sections of choice arenas with lower humidity (Fig. 4A). There was a trend toward fewer flies in the dry side of the assay in all tests, and for the summer morphs, this preference was significant ($t = 2.79$, $df = 3$; $P < 0.05$) only when the assay compared 90 and 11% RH at room temperature ($\sim 24^\circ\text{C}$). For the winter morphs, there was a greater response to RH, with a significant response of *D. sukukii* to 38% ($t = 3.32$, $df = 3$; $P = 0.04$), 54% ($t = 4.44$, $df = 3$; $P = 0.02$), and 90% ($t = 5.62$, $df = 3$; $P < 0.01$) RH when compared with 11% at room temperature ($\sim 24^\circ\text{C}$). Winter morphs of *D. sukukii* exhibited greater preference index values than summer morphs, with these values being significantly higher in the 11 versus 38% ($F = 18.85$, $df = 1,86$; $P < 0.0001$), 11 versus 54% ($F = 19.71$, $df = 1,97$; $P < 0.0001$), and 11 versus 90% ($F = 107.80$, $df = 1,97$; $P < 0.0001$) comparisons; however, there was no significant difference in 38 versus 54% ($F = 3.00$, $df = 1,97$; $P = 0.086$), 38 versus 90% ($F = 1.87$, $df = 1,97$; $P = 0.1745$), and 54 versus 90% ($F = 1.37$, $df = 1,97$; $P = 0.2442$) comparisons (Fig. 4B).

Discussion

In this study, the effect of desiccation on survival and weights of different morphs of *D. sukukii*, and their preference for different humidity levels were assessed. Water balance is particularly important for *Drosophila* and maintaining this water balance can be particularly challenging during the winter months in temperate climates, where the air is dry, and the opportunities to move towards moisture

are limited. In this study, the winter morphs rate of water loss was significantly lower over the 120-min desiccation event than the summer morph females, suggesting that the winter morph is adapted to survive at lower humidity. This could be a result of the increased melanization as reported by Shearer et al. (2016), that results from development in lower temperatures ($<10^\circ\text{C}$) and from short photoperiod prior to eclosion (Wallingford et al. 2016). Wallingford et al. (2016) also found that these physiological changes resulted in the adults being acclimated to survive at lower temperatures (-1°C for 24 h) than nonacclimated *D. sukukii*. In addition to water, drosophilids can lose significant quantities of dry mass during desiccation (Arlian and Eckstrand 1975, Lehmann et al. 2000, Marron et al. 2003). This loss is represented by the permanent excretion of Na^+ , Cl^- , and K^+ due to osmoregulatory strategies (Folk and Bradley 2003). Desiccation resistance of flies selected in the laboratory was tied to Na^+ loss, particularly from hemolymph and the tissues. The desiccation resistant flies lost ~ 60 and $\sim 70\%$ of hemolymph volume and Na^+ content, respectively, in a 24-h period, whereas tissue water and Na^+ content were not significantly reduced. In comparison, control flies susceptible to desiccation lost $\sim 60\%$ of volume and $\sim 80\%$ of the Na^+ content in the hemolymph in an 8-h period. Furthermore, the control flies lost significant water and sodium content from the tissues within the 8-h period (Folk and Bradley 2003). In this study, there was no reduction in the dry mass of the winter morph of *D. sukukii*; however, there was significantly greater reduction in dry mass for the summer morph. The ability of the same selected population of desiccation resistant flies to conserve these inorganic ions, particularly Na^+ , also imparts an ability to recover from sublethal desiccation (Folk and Bradley 2004).

Desiccation eventually caused mortality in all specimens exposed to this stress, with no specimens living longer than 320 min. A small percentage (4%) of the winter morph females survived longer than the other morphs. However, while males of both morphs died faster than females, winter morph males died faster than summer morph males. Winter morph females similarly died at a faster rate initially than summer morph females but survived for a longer duration than the summer morphs. This high rate of mortality over the longer desiccation period suggests that while the rate of desiccation was lower in the winter morph, these morphs can desiccate, resulting in mortality. Previous studies by Toxopeus et al. (2016) concluded that there was no difference in desiccation resistance between winter morphs and summer morph. Unlike this study, they used silica gel and sealed

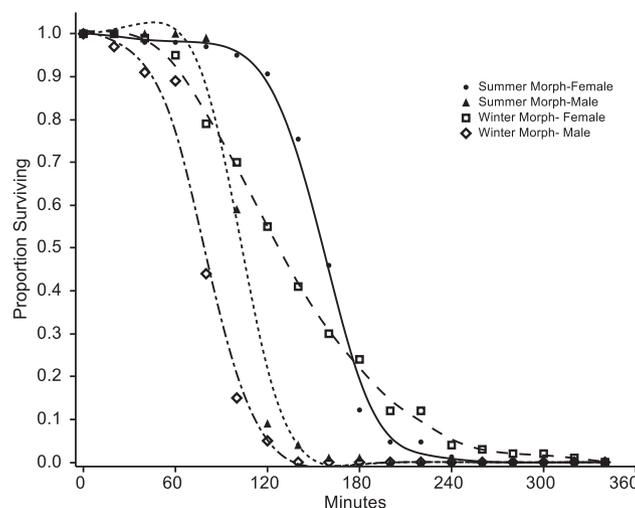


Fig. 2. Effect of desiccation on survival of male and female of summer and winter morphs of *D. sukukii*.

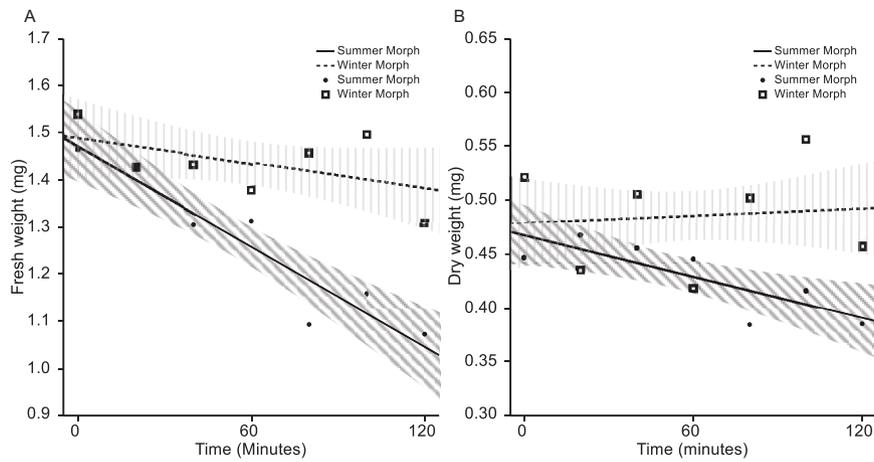


Fig. 3. Effect of desiccation on the fresh and dry weights of female (A) summer and (B) winter morphs of *D. sukukii*.

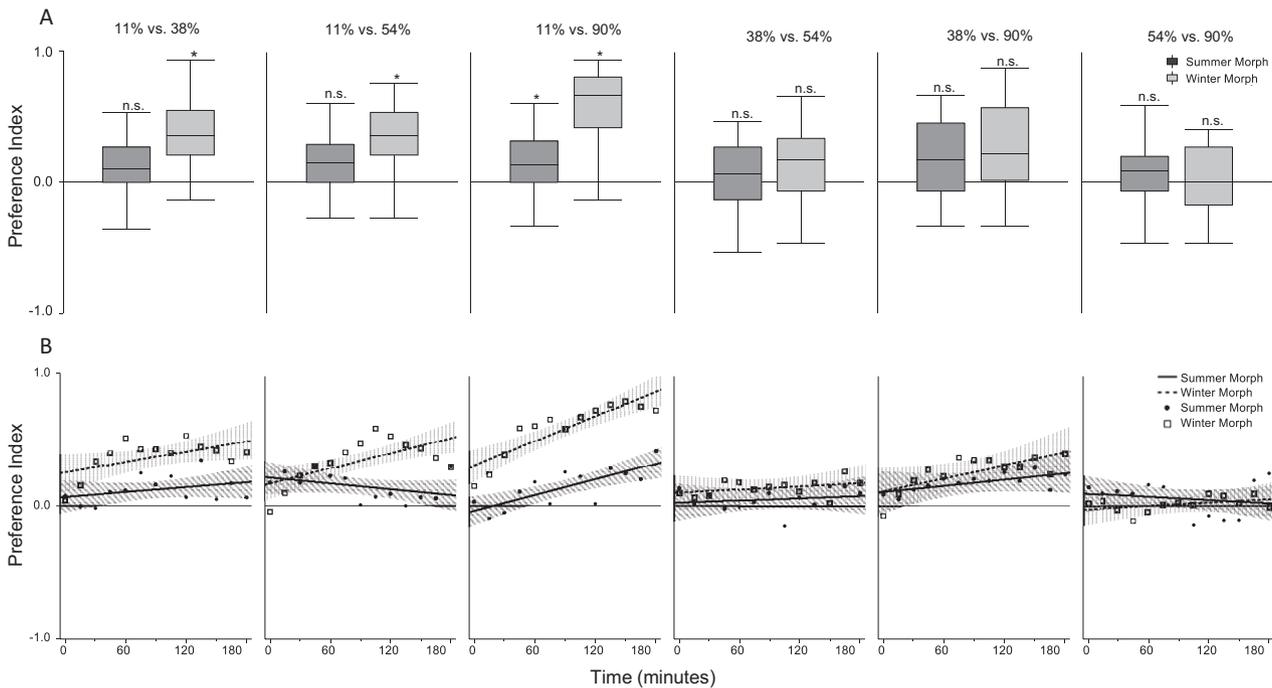


Fig. 4. (A) Preference of summer and winter morph of *D. sukukii* for different pairs of humidity conditions in a choice arena. Bars headed with * were significantly ($\alpha = 0.05$, Wilcoxon-signed rank test) different from 0. Bars topped with n.s. were not significantly different from 0. (B) Preference of summer and winter morph of *D. sukukii* for different humidity pair combinations in a choice arena over a 180-h observation period.

vials with a foam stopper instead of Para-film, with the last mortality in that study being observed 24 h after the experiment commenced. Water loss and dry weight was not assessed by [Toxopeus et al. \(2016\)](#). The high desiccation of winter morph individuals with their melanized cuticle could be due to increased loss through the spiracles from respiration in response to an increased metabolic rate. Previous studies have highlighted the greater relative importance of respiratory water loss in xeric or arid adapted species due to reduced cuticular permeability ([Zachariassen et al. 1987](#), [Lighton et al. 1993](#)). In this study, experiments were conducted at room temperature ($\sim 24^{\circ}\text{C}$), as was the study by [Toxopeus et al. \(2016\)](#), which is lower than the rearing temperature of the summer morphs but higher than that of the winter morphs, thus winter morphs were much more active than would be expected in field conditions. Temperature has

previously been shown to affect locomotion in *D. melanogaster* ([Gibert et al. 2001](#)). Studies by [Hoffmann and Parsons \(1993\)](#) suggest that reduced locomotor activity and metabolic rate contribute to desiccation resistance. Therefore, it is possible that if these experiments were repeated at lower temperatures, survival of the winter morphs in response to desiccation would be higher due to the associated reduction in metabolic rate.

Humidity is sensed in the sacculus of the drosophilid antenna ([Sayeed and Benzer 1976](#), [Helmut and Loftus 1985](#), [Enjin et al. 2016](#)), and triggers a behavioral response to environmental humidity. This response is likely to avoid adverse humidity and thus desiccation, rather than being an innate attraction to the high humidity; however, both serve the same ecological purpose. The results of our preference bioassays indicated that, while both morphs of *D. sukukii*

sensed humidity, winter morphs had a different behavioral response in preference bioassays than the summer morphs, displaying a greater preference to the side of the choice arena with higher humidity than the drier side. This suggests that winter morphs may have a greater behavior response to adverse humidity. However, repeating these preference bioassays at lower temperatures would allow for more thorough investigation of whether the higher temperature, and likely higher metabolic rate of the winter morphs, was a confounding factor in these comparisons.

Considering the pest status of *D. suzukii*, it is important to consider the significance of these results for ecologically based pest management of this species. There has been considerable research to determine the overwintering survival of *D. suzukii* and the significance of the winter morphs. Wallingford et al. (2016) highlighted the importance of winter morphs in surviving low temperatures. This study indicates that the winter morph is an adaptation to survive and avoid desiccation and that the conditions during the spring warm up may be critical for the survival of winter morphs into the spring, and further expands our understanding of how humidity affects the level of risk from this species (Tochen et al. 2016).

The results also shed light on the potential for pruning as a cultural control for *D. suzukii*. Both reducing the density of crop canopies (Hamby et al. unpublished) and using mulch under bushes (Rendon et al. in press) or changing irrigation practices (Rendon and Walton 2018) can manipulate environmental conditions within crop fields to make them less hospitable to *D. suzukii*. The preference index bioassays indicate that the humidity differential to significantly influence the behavior of *D. suzukii* in the field may not be achievable in locations with high ambient humidity.

In conclusion, our results show that winter morph of *D. suzukii* may survive longer in low humidity than the summer morphs, reflected in their reduced weight loss. While survival of the two morphs in desiccation vials did not differ greatly a small portion of the winter morph females survived longer than all other specimens. In addition, preference bioassays revealed that winter morph *D. suzukii* better orientated to areas with higher humidity than the summer morph flies. While winter morphs showed a reduced rate of water loss in response to desiccation, adults did eventually desiccate, possibly because of higher water loss due to the temperature of the experiments. Future studies using more humidity levels could be used to better understand the effects of future patterns of humidity and temperature on populations of *D. suzukii*. Understanding how these abiotic factors affect the behavior of *D. suzukii* is important for determining whether cultural controls for *D. suzukii* can cause a sufficient differential in humidity to elicit a behavioral response.

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