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Research

Ovary Development and Cold Tolerance of the Invasive Pest *Drosophila suzukii* (Matsumura) in the Central Plains of Kansas, United States

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Abstract

Environmental challenges presented by temperature variation can be overcome through phenotypic plasticity in small invasive ectotherms. We tested the effect of thermal exposure to 21, 18, and 11°C throughout the whole life cycle of individuals, thermal exposure of adults reared at 25°C to 15 and 11°C for a 21-d period, and long (14:10 hr) and short (10:14 hr) photoperiod on ovary size and development in *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae) cultured from a recently established population in Topeka, Kansas (United States). Examination of the response to temperature and photoperiod variation in this central plains population provides insight into the role of phenotypic plasticity in a climate that is warmer than regions in North America where *D. suzukii* was initially established. We found both low temperature and short photoperiod resulted in reduced ovary size and level of development. In particular, reduced ovary development was observed following exposure to 15°C, indicating that ovary development in females from the central plains population is more sensitive to lower temperature compared with populations examined from the northern United States and southern Canada. We also provide evidence that *D. suzukii* reared at 25°C are capable of short-term hardening when exposed to −6°C following 4°C acclimation, contrary to previous reports indicating flies reared at warm temperatures do not rapidly-cold harden. Our study highlights the central role of phenotypic plasticity in response to winter-like laboratory conditions and provides an important geographic comparison to previously published assessments of ovary development and short-term hardening survival response for *D. suzukii* collected in cooler climates.

Key words: spotted wing drosophila, ovarian development, cold tolerance, acclimation

The ability of invasive species to persist in novel environments despite limited genetic variation within the founding population remains an evolutionary paradox (Frankham 2005), though there are a number of hypothesized mechanisms for successful invasion following a founding event (Dlugosch and Parker 2008, Stapley et al. 2015). Phenotypic plasticity for fitness-related traits is one mechanism that can facilitate invasion, allowing a small number of genotypes to thrive in a novel environment. Davidson et al. (2011) illustrated that invasive plants have greater phenotypic plasticity for many fitness related traits when compared to their noninvasive congeners. Similar patterns have also been documented in arthropods (Lardies and Bozinovic 2008, Kleinteich and Schneider 2011). Taken together, these results suggest that plastic species have increased capacity to respond to a range of environmental stressors and more successfully survive and persist in novel environments.

One important source of environmental stress in temperate habitats is daily and seasonal temperature fluctuation, during which temperatures can regularly drop below freezing between day and night (Supp Fig. 1 [online only]). The ability of organisms to tolerate moderate to large shifts in temperature is often predictive of geographic distribution and activity (Addo-Bediako et al. 2000, Kellermann et al. 2012, Overgaard et al. 2014, Andersen et al. 2015) as environmental temperature is often a source of strong natural selection (Huey and Kingsolver 1993). In small ectothermic species, exposure to temperatures below freezing can result in chilling injury and ultimately death (mechanisms reviewed in Teets and Denlinger 2013); however, these consequences can be avoided through plastic responses such as hardening or acclimation (Wilson and Franklin 2002, Bowler 2005, Angilletta 2009, Colinet and Hoffmann 2012, Fallis et al. 2014, Gerken et al. 2015).

Short-term hardening is a plastic response that can occur over very short timescales (minutes to hours) wherein brief exposure to cold (or hot), nonlethal temperatures increases survival following acute exposure to more extreme stressful temperatures (Chen et al. 1987, Lee et al. 1987, Czajka and Lee 1990, Worland and Convey 2001, Wang and Kang 2003, Bowler 2005, Loeschcke and Sørensen 2005, Sinclair and Chown 2006). Acclimation occurs over longer timescales (days to weeks) and can be induced by temperature variation associated with seasonal change (Denlinger 1991, Bowler 2005). In *Drosophila melanogaster*, acclimation and short-term hardening can act synergistically. For example, short-term hardening of acclimated individuals can increase survival relative to unacclimated individuals (Kelty and Lee 2001, Gerken et al. 2015). Understanding how an invasive species employs both plasticity strategies will elucidate their overall potential for range expansion in a novel environment as temperature tolerance is directly related to an organism's overall fitness and reproductive success.

Drosophila suzukii (Matsumura) (Diptera: Drosophilidae), an invasive pest species endemic to Asia, was first detected in the continental United States in California in 2008 (Walsh et al. 2011). Within 3 yr of initial detection, specimens were collected throughout the west and east coasts of the United States and in southern parts of Canada (Burrack et al. 2012, Freda and Braverman 2013). Concurrently, *D. suzukii* was introduced and expanded its range throughout Europe beginning in 2008 (Cini et al. 2012). Recently, stable populations were documented in the central plains of the United States (Everman et al. 2015). *D. suzukii* introductions are significant because unlike the vast majority of drosophilids, *D. suzukii* is an agricultural pest (Burrack et al. 2012). Female *D. suzukii* have a serrated ovipositor that allows eggs to be laid in un-ripened, soft-skinned fruits including a variety of berries, stone fruits, and grapes (Atallah et al. 2014, Harris et al. 2014, Hamby et al. 2016, Baser et al. 2018). Shortly following the detection of *D. suzukii* in Kansas, it was noted as an agricultural pest species of concern (Cloyd and Copland 2015). However, a thorough survey of the central plains and broader Midwestern region of the United States has not been completed, so while *D. suzukii* is considered an agricultural risk species, the extent of the spread and impact of this species throughout this region is not well documented. Previous studies have suggested that *D. suzukii* is active during winter months (Rossi-Stacconi et al. 2016) but is unlikely to survive harsh winters of Ontario and the northern United States without access to refugia associated with humans (Dalton et al. 2011, Jakobs et al. 2015, Stephens et al. 2015, Toxopeus et al. 2016); therefore the milder winters of the central plains may increase the likelihood of establishment and thus intensify crop risk.

For invasive species like *D. suzukii*, the ability to respond to diverse thermal regimes through phenotypic plasticity via short-term hardening and acclimation can facilitate overwintering in novel temperate environments. Successful overwintering can be achieved via a combination of increasing basal levels of cold tolerance (Bergland et al. 2014, Shearer et al. 2016) and/or limiting reproduction by entering adult quiescence or diapause (Kimura 1988, Hoffmann et al. 2003a) where ovaries are reduced in size and contain few to no mature eggs (Saunders et al. 1989, Denlinger 1991). Female drosophilids such as *D. pseudoobscura*, *D. melanogaster*, and *D. suzukii* can store sperm until thermal conditions become permissive for development (Denlinger 1991, Price et al. 1999, Giraldo-Perez et al. 2016, Rossi-Stacconi et al. 2016, Ryan et al. 2016). Then, as average temperature rises, ovarian development resumes, and eggs are fertilized and oviposited (Zerulla et al. 2015, Wallingford et al. 2016). *D. suzukii* may employ a similar strategy in North America and Europe (Ryan et al. 2016). Ovarian diapause phenotypes can be induced via environmental cues that occur with changing seasons such as shorter photoperiod and/or lower overall temperature (Kimura 1988, Zhai et al. 2016).

Environmental cues in the form of temperature and photoperiod variation can alter the likelihood of survival through acclimation and induce a diapause-like phenotype in *D. suzukii* as well. For example, winter morphs of *D. suzukii* that develop at low temperatures (e.g., 10–15°C) typically exhibit relatively high tolerance of cold stress (Shearer et al. 2016, Wallingford and Loeb 2016, Wallingford et al. 2016, Enriquez and Colinet 2017), whereas summer morph flies reared under warm conditions (e.g., 25°C) tend to be much less cold tolerant with low capacity for phenotypic plasticity (Dalton et al. 2011, Jakobs et al. 2015, Wallingford and Loeb 2016). Similarly, reduction in ovary development was observed in winter morph females that had been reared at cool temperatures under a short photoperiod (Zerulla et al. 2015, Toxopeus et al. 2016, Wallingford and Loeb 2016, Zhai et al. 2016). However, much of this insight comes from studies that tested individuals collected from populations established in Ontario, Canada, Oregon, New York, and Michigan or from populations established in Europe.

The recently detected population of *D. suzukii* in Kansas provides the opportunity to assess seasonal plasticity in diapause and thermal tolerance across the increasing invasive range of this species. Variation in temperature in Kansas can be pronounced during seasonal transitions, especially during early spring and late fall. Overall, Kansas is warmer compared with New York, Michigan, and Canada but typically has greater seasonal variability compared with Oregon (Supp Fig. 1 [online only]). Regional climate differences are known to influence the evolution of thermal physiological response and tolerance of ectothermic organisms (Janzen 1967, James et al. 1997, Addo-Bediako et al. 2000, Hoffmann et al. 2002, Deutsch et al. 2008, Gunderson and Stillman 2015), and the combination of higher average temperature and increased thermal variability of the Kansas climate provides a novel seasonal thermal environment for *D. suzukii* in which altered physiological response to seasonal environmental cues can evolve (Parmesan 2006, Gomez-Mestre and Jovani 2013, Tonina et al. 2016). In addition, variation in thermal selection regime may also influence capacity for phenotypic plasticity (Kingsolver and Huey 1998, Ayrinhac et al. 2004, Beaman et al. 2016, Noh et al. 2017). For invasive species like *D. suzukii* that have agricultural impacts, understanding how altered thermal regimes across introduced geographic ranges influences sensitivity to thermal and photoperiod cues will provide insight into the likelihood that recently established populations will persist (Porter et al. 1991, Shearer et al. 2016, Hulme 2017). Study of a recently established *D. suzukii* population facilitates both a more comprehensive understanding of its success as an invasive species and the prediction of the degree to which overwintering strategies determine geographic boundaries of this species.

In this study we tested the ability of *D. suzukii* sampled from a population in the central plains (Topeka, Kansas, United States) in 2014 to respond to diverse environmental conditions including photoperiod and temperature. During 2014 (and on average), temperatures tended to be warmer and/or more variable throughout the year in Topeka, Kansas compared with northern and coastal populations where the effects of thermal stress and photoperiod on ovary development have been extensively examined in populations of *D. suzukii* (Dalton et al. 2011, Hamby et al. 2014, Jakobs et al. 2015, Stephens et al. 2015, Ryan et al. 2016, Shearer et al. 2016, Toxopeus et al. 2016, Wallingford and Loeb 2016, Wallingford et al. 2016, Wiman et al. 2016). As this invasive pest becomes established across an increasingly broad geographic range, it is essential to understand the response of *D. suzukii* to winter-like conditions in diverse thermal habitats. This is particularly true for populations that become established in locations with higher average temperatures (such as Kansas and the central plains). To assess response to thermal and photoperiod variation, we first tested whether adult exposure (AE) and whole life cycle (WLC) exposure to low, nonlethal temperatures and alteration to photoperiod induced a female reproductive diapause-like phenotype. Second, we quantified the effect of short-term hardening (RCH) to nonlethal temperature on adult cold tolerance of individuals reared at warm temperatures. We hypothesized that short photoperiod, AE, and WLC exposure would result in reduced ovary development similar to diapause in female *D. suzukii* and that adult short-term hardening to nonlethal temperature prior to cold shock would increase overall survival in both male and female adult *D. suzukii*. Observation of plastic responses to these treatments in *D. suzukii* illustrates the importance of phenotypic plasticity for the survival and population persistence in geographically diverse novel environments of this species.

Methods

Stock Maintenance

Adult female *D. suzukii* were collected from an orchard in Topeka, Kansas (39°12′10.4″ N, 95°44′31.4″ W) in July, 2014 near raspberry and blackberry plants using banana bait traps as described in Everman et al. (2015). A culture was initiated with four females and was maintained at 25°C on a 12-hr light:dark cycle for 30 generations prior to experiments. Flies were maintained in 8 oz polypropylene stock bottles on standard cornmeal–molasses–yeast agar supplemented with raspberries to stimulate oviposition. While food was still warm and unset, a frozen raspberry was added to the media. When food cooled, the thawed raspberry was embedded in the media. Because flies were maintained as a large population following the initial establishment of the culture, we assume that changes in physiological response to temperature are minimal between successive generations. *D. suzukii* individuals used in each experiment described below were obtained by placing five male and five female flies on raspberry-supplemented media in polystyrene vials at 25°C on a 12:12-hr photoperiod and allowing females to lay eggs for 48 hr before being removed. Vials of eggs were then moved to the treatment conditions, as described below for the photoperiod and temperature experiments.

Temperatures for each experiment were chosen based on recent studies that measured reduction in ovary development and shifts in cold tolerance of summer and winter morphs under similar conditions (Stephens et al. 2015, Hamby et al. 2016, Toxopeus et al. 2016, Wallingford and Loeb 2016, Zhai et al. 2016). WLC exposure to temperatures ranging from 10 to 15°C has been demonstrated to be sufficient to induce the winter morph in *D. suzukii* females in populations in Ontario, Michigan, Oregon, and New York (Stephens et al. 2015, Shearer et al. 2016, Toxopeus et al. 2016, Wallingford and Loeb 2016, Wallingford et al. 2016), and previous studies suggest *D. suzukii* does vary in sensitivity to temperature and photoperiod depending on the population considered (Hamby et al. 2016, Tonina et al. 2016, Zhai et al. 2016). For example, rearing *D. suzukii* at 25°C induces differences in physiological response to thermal stress compared to rearing at 21°C (Enriquez and Colinet 2017). We chose experimental conditions that allow detection of subtle differences in sensitivity to stimuli that are known to influence ovary development in other populations that are likely under thermal selection regimes unique from that of Kansas (Fig. S1).

Effect of Photoperiod on Ovary Development

In the photoperiod experiment, we tested the influence of a long (14:10 hr) and short (10:14 hr) photoperiod on ovary development in females that experienced a WLC or AE thermal treatment. For the photoperiod experiment, WLC females were reared and maintained at either 18 or 21°C on long and short photoperiods. These temperatures were chosen to reflect relatively warm (21°C) and relatively cool (18°C) average temperatures experienced by natural populations in the fall, when diapause initiation would naturally occur. In contrast, AE females were reared at a relatively warm temperature (25°C) and transferred to a relatively cold temperature (15°C) on either a long or short photoperiod (Table 1; Fig. 1A). We used this wider range of temperatures to provide a starker contrast between rearing (egg to adult) and adult conditions in order to test whether conditions experienced by adults are of primary importance in determining diapause initiation.

Females were collected over light CO_2 anesthesia within 8 hr of emergence for each treatment (Saunders et al. 1989, Zhai et al. 2016). Long- and short-photoperiod treatments were conducted in separate incubators. To ensure conditions were comparable between incubators, a light-proof control box was placed in the short-photoperiod 15°C incubator. A group of females in the control box experienced a long-photoperiod 15°C treatment. Females in both the WLC and AE treatments were maintained for an additional 21 d at their respective treatment temperatures to ensure full maturation of females (Table 1; Fig. 1A) (Toxopeus et al. 2016).

Effect of Temperature on Ovary Development

In the temperature experiment, we compared the effects of WLC and AE treatments at relatively cool temperatures on ovary development

Table 1. Sample sizes for each of the temperature and photoperiod treatments

Exposure period	Experiment	Rearing temp (°C)	21-d exposure temp (°C)	Photoperiod (hr)	No. females
AE	Photoperiod	25	15	14:10	32 total (14 in CB)
AE	Photoperiod	25	15	10:14	13
WLC	Photoperiod	21	21	14:10	42
WLC	Photoperiod	21	21	10:14	7
WLC	Photoperiod	18	18	14:10	63
WLC	Photoperiod	18	18	10:14	29
WLC	Temperature	25	25	12:12	20
AE	Temperature	25	11	12:12	20
WLC	Temperature	15	15	12:12	19
WLC	Temperature	11	11	12:12	17

CB = control light box, AE = adult exposure, WLC = whole life cycle exposure.

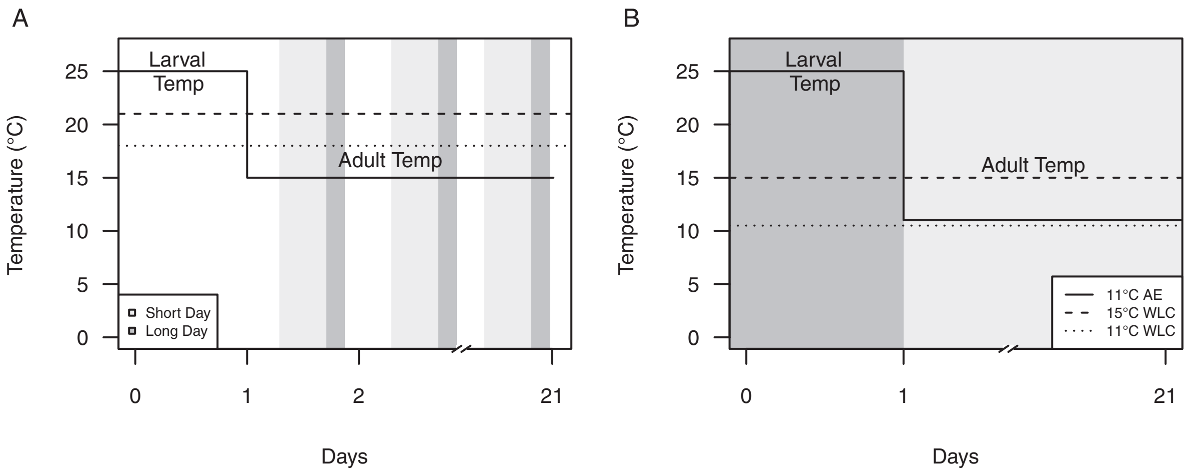

Fig. 1. Treatments used to test the effect of (A) photoperiod and (B) temperature on ovary development. (A) Females were exposed to a short (10 hr; light grey shading) or long (14 hr; light and dark grey shading) photoperiod. Females were exposed to three different temperatures in the photoperiod experiment: AE to 15° (solid line), WLC exposure to 21°C (dashed line) or WLC exposure to 18°C (dotted line). (B) Females experienced WLC exposure to 15°C (dashed line) or 11°C (dotted line), or experienced AE to 11°C (solid line) for 21 d. Dark grey shading indicates the rearing conditions; light grey indicates the post-eclosion conditions. In both experiments, photoperiod began at 07:00 hr each day.

on a 12:12-hr photoperiod. Females that were exposed to a WLC thermal exposure treatment were reared at either 11 or 15°C (Table 1; Fig. 1B). Females that were exposed to an AE thermal exposure treatment were reared at 25°C and transferred within 8 hr of emergence to 11°C (Table 1; Fig. 1B). As with the photoperiod experiment, females were maintained at their respective treatment temperature and photoperiod for 21 d post-emergence. An additional set of females was reared at a constant 25°C from egg to 21 d post-eclosion (Fig. 1B). We did not test flies at 21 or 18°C in the temperature experiment as ovary development was not influenced by photoperiod at these temperatures (see Results).

Ovary Dissection and Classification

After the 21-d post-eclosion period in both the photoperiod and temperature experiments, all experimental females were preserved at −80°C until dissection. Intact (unflattened) ovaries were dissected from frozen females in a 1× PBS/0.14% Triton solution using Dumont No. 5 Inox alloy forceps. Ovary width and length were measured using an ocular micrometer, and ovary development was classified according to three developmental categories (Zhai et al. 2016). Immature ovaries were 0–0.40 mm wide and 0–0.50 mm long with fewer than two maturing eggs visible within the ovarioles. Developing ovaries were 0.41–0.55 mm wide and 0.51–0.70 mm long with more than two maturing eggs. Developed ovaries were 0.56–0.95 mm wide and 0.71 mm or longer and nearly all ovarioles contained maturing eggs. We refer to females with immature ovaries as having a diapause-like phenotype, as reduction in size and development of ovaries represents only part of the full reproductive diapause phenotype.

Effect of Temperature on Adult Rapid Cold-Hardening

To determine the effect of short-term cold temperature stress on survival through RCH, experimental flies were reared at 25°C, and adults were collected on the third day of eclosion. Experimental flies were sorted by sex with light CO_2 anesthesia. Flies were allowed to recover and mature for 5 d at 25°C with 10 same-sex individuals per vial.

Survivorship was measured following exposure of flies to one of two experimental treatments. Rapidly cold-hardened flies were exposed to 4°C for 2 hr immediately prior to a 1-hr −6°C cold stress (Fig. 2A). Non-rapidly cold-hardened flies were exposed to the 1-hr −6°C cold stress without a pretreatment (Fig. 2B). Exposure to 4°C is a mildly stressful temperature similar to temperatures (0–5°C) that are hypothesized to induce chill injury in *D. suzukii* following extended exposure (Jakobs 2014, Enriquez and Colinet 2017); however, brief exposures to 4°C have been shown to have a temporary beneficial acclimation effect that can increase survival following harsher stress in *D. melanogaster*(Gerken et al. 2015; Everman et al. 2017; Noh et al. 2017). Cold stress at temperatures similar to −6°C or slightly lower (−7.5°C) have also been used to test susceptibility of *D. suzukii* to cold temperature (Jakobs 2014, Jakobs et al. 2015, Enriquez and Colinet 2017). Once the cold stress treatment was completed, flies were transferred to fresh raspberry supplemented media and allowed to recover for 24 hr at 25°C, at which point survivorship was determined as the number of flies alive in each vial. Flies were determined to be alive if they were capable of walking and/or flying. A total of 1,060 flies were tested (530 RCH and non-RCH flies).

Data Analysis

Both ovaries were measured for each female and were used to calculate the average ovary length and width per female. We calculated the product of these measures to approximate ovary size. The photoperiod and temperature experiments were assessed in separate analyses (Tables 1 and 2). We found that ovary-size residuals were not normally distributed for either the photoperiod ($W = 0.98$, $P < 0.05$) or temperature experiment ($W = 0.90$, $P < 0.0001$), nor were they homoskedastic in the photoperiod experiment (photoperiod: $F_{5,180} = 4.41$, $P < 0.001$; temperature: $F_{3,72} = 0.11$, $P = 0.95$). Therefore, ovary size was square-root-transformed to resolve normality issues (photoperiod: $W = 0.995$, $P = 0.84$; temperature: $W = 0.97$, $P = 0.05$).

For the photoperiod experiment, the effects of photoperiod and WLC or AE were tested using a two-way analysis of variance with temperature, photoperiod, and their interaction treated as fixed effects. Because the ovaries of females from the photoperiod control box were not different from ovaries of females treated in the long photoperiod incubator ($F_{1,30} = 0.13$, $P = 0.72$), these ovaries

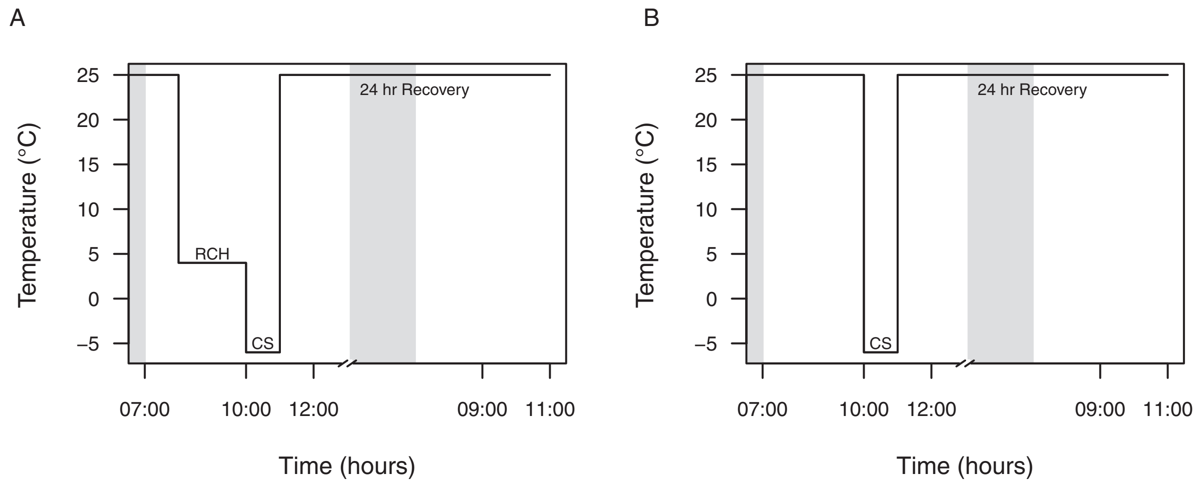

Fig. 2. Treatments used to test the effect of (A) RCH and (B) cold shock without RCH on survival. (A) Adult flies were exposed to an RCH treatment (4°C) for 2 hr prior to cold shock (−6°C) for 1 hr and were allowed to recover for 24 hr at the rearing temperature (25°C) before survival was assessed. (B) Adult non-RCH flies were exposed to the cold shock temperature (−6°C) for 1 hr and were allowed to recover for 24 hr at the rearing temperature (25°C) before survival was assessed. In both A and B, shading indicates the timing of the 12:12 photoperiod.

were combined for this analysis. For the temperature experiment, the effect of WLC and AE temperature exposure on ovary size at a constant 12:12 LD was tested using a one-way analysis of variance with temperature as a fixed effect. Post hoc comparisons of photoperiod and temperature treatments for analyses of both the photoperiod and the temperature experiment were assessed using Tukey's HSD with an experiment-wide α = 0.05.

Because quantitative differences in ovary size due to photoperiod and thermal exposure treatments may not fully reflect the level of ovary development under the treatment conditions, we also compared the frequency of ovaries classified as developed, developing, or immature following each photoperiod and thermal exposure treatment. Differences in frequency of ovaries in each developmental category were tested using contingency table analyses, and as before, the photoperiod and temperature experiments were analyzed separately in a pairwise manner (Tables 1 and 2).

To test the effect of photoperiod on ovary development, we performed a two (long versus short photoperiod) by three (developed, developing, and immature ovaries) contingency table analysis for each of the thermal exposure treatments (WLC 21°C, WLC 18°C, or AE 25–15°C) with an alpha level adjusted for the three tests using the Bonferroni correction (α = 0.016).

To test the effect of temperature without altered photoperiod on ovary development, we performed a four (WLC 25°C, WLC 15°C, WLC 11°C, and AE 11°C) by three (developed, developing, and immature ovaries) contingency table analysis. To determine if differences in the frequency of developed, developing, and immature ovaries existed between different levels of temperature exposure, we performed pairwise analyses of each temperature treatment with an experiment-wide alpha level adjusted for the seven comparisons using the Bonferroni correction (α = 0.0071).

We analyzed the fixed effects of sex, treatment, and the interaction on the survival response to temperature with and without rapid cold-hardening (RCH) using a mixed effects general linear model with a binomial error distribution and logit link function in the R package lme4 (Bates et al. 2015). Vial replicate was also included in the model as a random effect. Post hoc comparisons were assessed using Tukey's HSD with an experiment-wide α = 0.05. All analyses were conducted in R (Wickham 2009, Hope 2013, R Core Team 2017), and all data are available from Dryad (doi:10.5061/dryad.kj26br0).

Results

Effect of Photoperiod on Ovary Development

Photoperiod did not significantly influence ovary size independent of temperature among the females that experienced WLC exposure to 21°C, 18°C, or AE to 15°C (Table 3; Fig. 3A). However, WLC exposure to 21°C and 18°C, and AE to 15°C independent of photoperiod did influence ovary size (Table 3), as did the interaction between the thermal exposure treatment and photoperiod (Table 3). This interaction was driven by differences in ovaries of females that were exposed to different temperatures on the same photoperiod (e.g., ovaries of females reared at 21 and 18°C on a long and short photoperiod were different; Fig. 3A).

A slight effect of photoperiod was detected in the categorical levels of ovary development following contingency table analysis (Table 2; Fig. 3B). Immature ovaries were more common in females following AE to 15°C on a short photoperiod compared with females that experienced the long photoperiod for the same thermal exposure treatment (Table 2; Fig. 3B). Analysis of ovary developmental category (immature, developing, developed) did not illuminate any differences due to photoperiod for females that experienced WLC exposure to 21 or 18°C (Table 2; Fig. 3B). Together, these results suggest that the reduction of the photoperiod to a 10:14 hr light cycle experienced by the females following AE to 15°C had a small effect on ovary size that was not independent of the thermal exposure treatment.

Effect of Temperature on Ovary Development

Temperature significantly influenced ovary size in females following WLC exposure to 25, 15, or 11°C and AE to 11°C on a 12:12 hr photoperiod (Table 3; Fig. 4A). Post hoc comparisons of the treatments indicated that reduction in ovary development and size was primarily due to exposure of females to temperatures at or lower than 15°C (Tukey's HSD $P < 0.0001$). Ovaries of females following WLC exposure to 15°C and 11°C and AE to 11°C were not significantly different in size (Fig. 4A). The significant effect of thermal exposure suggests that change in temperature was sufficient to induce a diapause-like phenotype without reduced photoperiod. This result suggests that female *D. suzukii* from the Kansas population are more sensitive to temperature than photoperiod, which

Table 2. Chi-square post hoc comparisons of ovary developmental stage (photoperiod corrected α = 0.016; temperature corrected α = 0.0071)

Experiment	Comparison	X^2	df	*P* value
Photoperiod	21°C WLC long × 21°C WLC short	0.53	2	0.77
	18°C WLC long × 18° WLC short	0.78	2	0.68
	15°C AE long × 15°C AE short	15.38	2	**0.0005**
Temperature	25°C WLC × 15°C WLC × 11°C WLC × 11°C AE	76.98	6	**<0.0001**
	25°C WLC × 15°C WLC	31.72	2	**<0.0001**
	25°C WLC × 11°C WLC	37.0	2	**<0.0001**
	25°C WLC × 11°C AE	36.19	2	**<0.0001**
	15°C WLC × 11°C WLC	5.02	2	0.08
	15°C WLC × 11°C AE	10.56	2	0.005
	11°C WLC × 11°C AE	3.39	2	0.18

Significant *P* values are indicated in bold.

WLC = whole life cycle exposure; AE = adult exposure; long = 14:10 hr photoperiod; short = 10:14 hr Photoperiod.

Table 3. Analysis of variance of ovary size for the photoperiod and temperature experiment

Experiment	Source	df	SS	MS	*F* value	*P* value
Photoperiod	Thermal exposure	2	3.54	1.77	65.72	**<0.0001**
	Photoperiod	1	0.0002	0.0002	0.0067	0.93
	Thermal exposure × photoperiod	2	0.37	0.18	6.77	**<0.01**
	Residuals	180	4.85	0.027		
Temperature	Thermal exposure	3	1.96	0.65	41.06	**<0.0001**
	Residuals	72	1.14	0.016		

Significant P values are indicated in bold.

may allow this population to remain reproductively active into the fall when temperatures are still permissive but day length begins to shorten.

We also found that ovary development differed among females in the WLC and AE treatments on a 12:12 hr photoperiod (Fig. 4B; Table 2). Pairwise comparisons of each exposure treatment showed that WLC exposure to 15 and 11°C resulted in significantly reduced ovary development relative to WLC exposure of females to 25°C (Fig. 4B; Table 2) as did AE to 11°C (Fig. 4B; Table 2). WLC exposure to 15°C did not alter ovary development relative to WLC exposure to 11°C (Fig. 4B; Table 2), nor did WLC exposure to 11°C alter ovary development relative to AE to 11°C (Fig. 4B; Table 2). Following adjustment for multiple testing, the level of ovary development compared between WLC exposure to 15°C and AE to 11°C was also not significant (Fig. 4B; Table 2).

Effect of Temperature on Survival

The effect of short-term thermal acclimation via RCH on survival was highly significant; the non-RCH cold stress resulted in poor survivorship, while the RCH cold stress significantly improved survival (fixed effect odds ratio coefficient $\beta_{TRT} = 2.34 \pm 0.5$, $z = 5.1$, $P < 0.001$; Fig. 5). While the effect of sex was significant ($\beta_{SEX} = 1.63 \pm 0.7$, $z = 2.3$, $P < 0.05$; Fig. 5), there was no sex by treatment interaction ($\beta_{TRT:SEX} = -0.58 \pm 0.9$, $z = -0.61$, $P = 0.54$; Fig. 5). Thus, *D. suzukii* adults, when reared at 25°C similar to summer conditions, had very low cold tolerance; however, the marked increase in survival following the short-term hardening treatment demonstrates that adults have the capacity to respond plastically to cold temperature variation.

Discussion

Populations of *D. suzukii* in the northern United States and southern Canada respond to seasonal cooling and the shortening of photoperiod through diapause-like ovarian developmental arrest and increased cold tolerance via acclimation (Dalton et al. 2011, Jakobs 2014, Jakobs et al. 2015, Stephens et al. 2015, Ryan et al. 2016, Shearer et al. 2016, Toxopeus et al. 2016, Wallingford and Loeb 2016, Wallingford et al. 2016). Similar physiological responses have been observed in populations in Italy, France, and China as well (Zerulla et al. 2015, Plantamp et al. 2016, Rossi-Stacconi et al. 2016, Tonina et al. 2016, Zhai et al. 2016, Enriquez and Colinet 2017). The purpose of our study was to examine the effect of variation in photoperiod and WLC and AE to cool temperatures on ovary size and development in a recently established population from Topeka, Kansas (Everman et al. 2015). In doing so, we provide insight into the capacity of a recently established population to adapt and respond plastically to seasonal and daily temperature variation in a mid-latitude temperate region.

We found that both ovary size and development were significantly influenced by exposure to cool temperature. In our study, when females experienced WLC exposure to 11, 15, and 18°C, ovary size was reduced, similar to results reported by Toxopeus et al. (2016) for flies originally collected from a population in Ontario, Canada, and to reports for other *D. suzukii* populations that together demonstrate that delayed ovarian development in this species is closely tied to environmental temperature in natural populations (Fig. 4A; Zerulla et al. 2015, Plantamp et al. 2016, Rossi-Stacconi et al. 2016, Tonina et al. 2016, Toxopeus et al. 2016, Wallingford and Loeb 2016, Wallingford et al. 2016, Zhai et al. 2016). However, in contrast to these previous studies, we demonstrate that temperatures as mild as 18°C are sufficient to influence ovary size independent of photoperiod in females collected from the Kansas population, suggesting that substantial variation between populations exists in sensitivity to cues that are known to induce diapause-like phenotypes. The change in ovary size following WLC to 18°C may reflect quiescence (temperatures below the ovarian development threshold) rather than diapause (programmed developmental suppression that persists when temperatures increase). In either case, increased sensitivity to thermal cues may be especially beneficial for invasive populations during seasonal

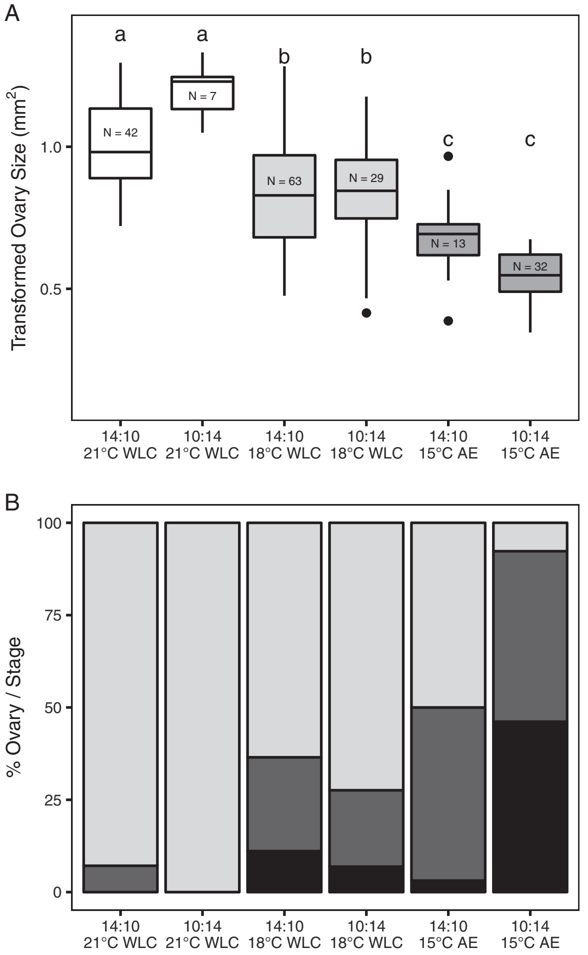

Fig. 3. The effect of photoperiod at different thermal exposure treatments on ovary size (A) and development class (B). In A, all measurements are given in mm^2 following square root transformation, and shading and letters indicate statistically similar groups. In B, immature ovaries are represented in dark gray, developing ovaries are represented in medium gray, and developed ovaries are represented in light gray. Sample sizes are given for each group. In both plots, WLC = whole life cycle exposure; AE = adult exposure.

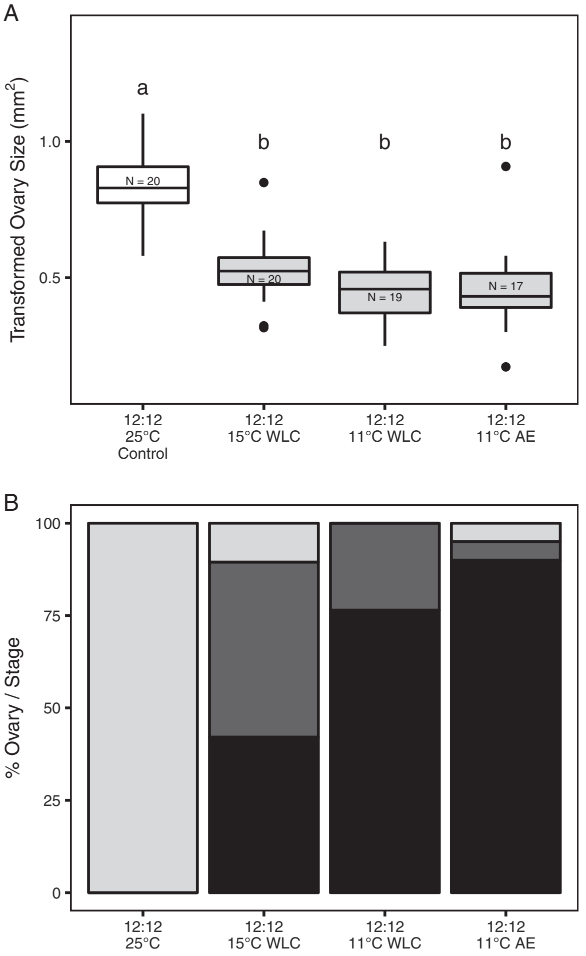

Fig. 4. The effect of temperature independent of photoperiod on ovary size (A) and development class (B). In A, all measurements are given in mm^2 following square root transformation, and shading and letters indicate statistically similar groups. In B, immature ovaries are represented in dark gray, developing ovaries are represented in medium gray, and developed ovaries are represented in light gray. Sample sizes are given for each group. In both plots, WLC = whole life cycle exposure; AE = adult exposure.

shifts from spring to summer and from summer to fall as temperatures can fluctuate drastically during seasonal transitions in Kansas and the central plains more broadly (Noh et al. 2017).

Alternatively, the effect of WLC exposure to cool temperatures on ovary development observed in our study may have been influenced by developmental delay. Ovary development typically takes 4–5 d in *D. suzukii* females when reared at warm temperatures (e.g., 25°C) (Jaramillo et al. 2015, Hamby et al. 2016). However, when females are reared at temperatures that are cool enough to induce the winter morph, *D. suzukii* females can take as long as 20 d for ovary maturation to be complete at 15°C and as long as 30 d at 11°C (Toxopeus et al. 2016). The influence of low temperature on ectotherm development has long been established (Powsner 1935), and it is possible that the frequency of ovaries observed in each developmental class in our study was influenced by development rate (Atkinson 1994, Toxopeus et al. 2016). However, evidence from Zhai et al. (2016) and Wallingford et al. (2016) that temperature- and photoperiod-induced reduction in ovary development at 11 and 15°C can be quickly reversed and egg-laying resumes when females are moved to a warmer and/or longer photoperiod suggests that developmental delay is playing a minor role in the patterns reported here. Regardless of the possible influence of development rate on ovary development at 11°C, WLC exposure to 15°C does appear to have had a stronger influence on ovary development compared with females originating from Ontario under similar conditions; following WLC exposure to 15°C, we detected several instances of immature ovaries at 21-d post-eclosion compared with full ovarian development reported at 20 d by Toxopeus et al. (2016) in flies originating from the cooler climate of Ontario.

A similar temperature-dependent change in ovary development was also observed in AE *D. suzukii* originating from native populations from the Shandong Province in China (Zhai et al. 2016). According to field temperatures reported in Zhai et al. (2016), the Shandong Province climate is warm-temperate, and falls at a latitude that is intermediate between Kansas and populations of *D. suzukii* that occur in northern United States and southern Canada. Zhai et al. (2016) demonstrated that the proportion of females with immature ovaries increased when adult females were exposed to gradually decreasing temperatures. In this native population, AE of females to 15°C under a 12:12 photoperiod resulted in levels of reduced ovary development that are similar to that of females that experienced WLC exposure to 15°C in our study. In contrast, *D. suzukii* originating from populations

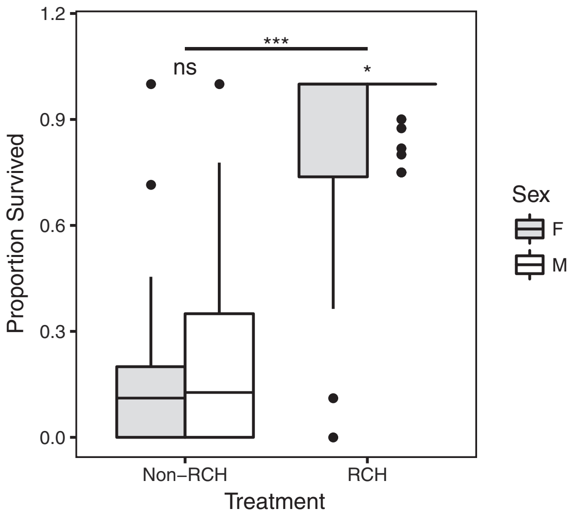

Fig. 5. The proportion survived of adult *D. suzukii* reared and maintained at 25°C was significantly influenced by RCH. While flies were generally highly susceptible to the non-RCH stress, RCH greatly improved survival for both sexes. The y-axis shows the proportion of the 10 individuals per vial that survived. Females are shown as filled boxplots; males are shown as open boxplots. Significance between groups is indicated in the plot: ns indicates not significant, * indicates <0.05, *** indicates <0.0001.

in New York and Oregon at higher latitude did not exhibit reduction in ovary development when females experienced WLC exposure to 15°C relative to controls on a 12:12 photoperiod (Wallingford and Loeb 2016, Wallingford et al. 2016), nor did females collected in Ontario, Canada that experienced AE or fluctuating environment conditions that simulated fall temperatures and photoperiod (Jakobs 2014). The decrease in ovary size in WLC and AE to 15°C in our study provides further evidence suggesting that reproduction of *D. suzukii* collected from the Kansas population is more sensitive to shifts in temperature compared with more northern populations. The degree to which ovary size or developmental reduction varies among the invasive populations sampled suggests that, while a diapause-like phenotype is likely to occur across latitude when females experience cooler temperatures and shorter photoperiod, the temperatures at which the initial shift occurs may be influenced by latitude.

In our study, ovary size and development was similar in females that experienced WLC exposure to 11°C and AE to 11°C with a 12:12-hr photoperiod (Fig. 4A and B; Table 2), suggesting that ovary development may depend on the adult thermal environment moreso than that of larvae. Exposure of newly eclosed adults to a shift to cooler temperature is a strong cue known to induce diapause in *D. melanogaster* (Saunders et al. 1989), and this result is consistent with Zhai et al. (2016) who demonstrated that exposure of the adult lifestage to 10°C on a short photoperiod (8:16 hr) was sufficient to induce diapause independent of thermal exposure of other lifestages. Taken together, these and other studies (Wallingford and Loeb 2016, Wallingford et al. 2016) suggest that exposure of maturing adult females to cool temperatures is sufficient to reduce ovary development across a range of latitudes. The reduction in ovary development in females following AE to 11°C provides insight into how natural populations respond to the transition between summer and fall. For example, females in natural populations during this transition would likely have developed under warm summer-like conditions, but may be faced with much cooler temperatures as adults. Phenotypic plasticity in ovary development could allow these females to survive increased fluctuation in daily temperature and potentially overwinter as diapausing adults (Stephens 2015, Shearer et al. 2016, Wallingford and Loeb 2016, Wallingford et al. 2016). This hypothesis is consistent with reports that populations are primarily composed of diapausing females during colder months of the year (Zerulla et al. 2015, Rossi-Stacconi et al. 2016, Wallingford et al. 2016).

In addition to temperature-sensitivity of ovary size, we also found that ovary size and development were influenced to a degree by photoperiod following AE to 15°C. While ovary size was not significantly different in females following 15°C AE between the long and short photoperiod, the frequency of immature and developing ovaries was much higher for females following AE at 15°C on a short photoperiod compared with females that had experienced AE to 15°C on a long photoperiod (Fig. 3B). The slight influence of short photoperiod on females following AE to 15°C in our study contrasts with Toxopeus et al. (2016) who showed that flies reared at 15°C on long and short photoperiods did not differ in ovary development as was observed in our study (Fig. 3B). Although the *D. suzukii* females from the Kansas population appear to be slightly more photoperiod sensitive compared with populations in Ontario, Canada, they are generally much less sensitive to photoperiod compared with populations examined in China and New York. For example, in *D. suzukii* populations in China, females are highly sensitive to photoperiod. Zhai et al. (2016) showed that short photoperiod combined with WLC exposure to 25°C resulted in reduced frequency of developed ovaries, and when diapausing females following AE to 10°C were transferred a longer photoperiod, they broke diapause and ovary maturation was observed (Zhai et al. 2016). Additional research is necessary to determine whether females from the Kansas population would respond to more drastically reduced photoperiods (such as 8:16), but broadly, our results suggest that sensitivity to photoperiod may be reduced in the recently established Kansas population.

Capacity for diapause alone is likely insufficient for population persistence following seasonal temperature changes. An increase in cold tolerance accompanies most examples of reproductive quiescence in *D. suzukii* (Shearer et al. 2016, Toxopeus et al. 2016, Wallingford and Loeb 2016, Wallingford et al. 2016, Zhai et al. 2016), and it is likely that this increase in cold tolerance is due to a combination of acclimation and adaptation to seasonal shifts in temperature (Bergland et al. 2014). Acclimation and short-term hardening have been repeatedly shown to increase survival following cold stress in many ectothermic species (Kimura 1988, Coulson and Bale 1992, Hoffmann et al. 2003b, Sinclair and Roberts 2005, Geister and Fischer 2007, Basson et al. 2012, Jakobs et al. 2015, Stephens 2015, Toxopeus et al. 2016). However, when Jakobs et al. (2015) examined the RCH response in lab-reared summer morph *D. suzukii* flies originally collected from Ontario, Canada, exposure of females to a 1-hr hardening treatment did not improve survival following 1-hr exposure to −7.2°C. A similar result was observed for summer morph *D. suzukii* examined in Toxopeus et al. (2016). These previous assessments of short-term hardening suggest that summer morph *D. suzukii* have little capacity for rapid acclimation at the level of daily temperature fluctuations. However, in our study, *D. suzukii* individuals that were reared at warm temperatures similar to summer morphs examined in Jakobs et al. (2015) and Toxopeus et al. (2016) had significantly higher survival following cold stress following RCH at 4°C relative to non-RCH individuals (Fig. 5). The lack of a short-term hardening response in a northern population and presence of a strong RCH response coupled with low cold tolerance in our central plains population may by indicative of latitudinal variation in the capacity to plastically respond to short-term cold-hardening under warm environmental conditions.

Conclusions

Responses to thermal variation are not constant over geographic distributions of species (Hoffmann and Watson 1993, Hoffmann et al. 2002), and have been shown to be particularly variable for insect species (Sinclair et al. 2012). *D. suzukii* has become a highly successful invasive pest that appears to have established populations throughout the United States and Canada that are capable of overwintering in temperate regions. For these populations to persist, individuals must either adapt to novel thermal environments that vary in seasonally predictable ways, respond through phenotypic plasticity, or use a combination of these strategies. In order to fully understand the success of invasive species that are hypothesized to be temperature-limited (Kimura 1988, Stephens et al. 2015), it is important to account for variation in the adaptive and phenotypic plasticity strategies that characterize populations over the span of their distribution. We have shown that *D. suzukii* in the recently established population in Kansas are generally less photoperiod-sensitive and more temperature sensitive, both in reproduction and capacity for short-term hardening. Greater sensitivity to temperature is likely to be highly beneficial for these populations in the central plains climate where they face milder winters compared with Canada and the northern United States but greater daily and seasonal thermal variability. Additional insight into the establishment and persistence of invasive populations will come from continued study of physiological responses to temperature and photoperiod in climatically distinct populations across the expanding range of *D. suzukii.*

Supplementary Data

Supplementary data are available at *Environmental Entomology* online.

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Author Contributions

E.R.E., P.F., G.J.R., and T.J.M. designed the experiments. E.R.E., M.B., and A.S. performed the investigation. E.R.E. performed the statistical analyses. All authors contributed to the construction of the manuscript.

Data Availability Statement

Data from this study are available from the Dryad Digital Repository: doi:10.5061/dryad.kj26br0.

Conflict of Interest

No competing interests declared.

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