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### Title

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### Permalink

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### Journal

Evolution: International Journal of Organic Evolution, 74(7)

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### Publication Date

2020-07-01

### DOI

10.1111/evo.14029

Peer reviewed

# Rapid adaptive evolution of the diapause program during range expansion of an invasive mosquito

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Received September 18, 2019

Accepted May 25, 2020

In temperate climates, the recurring seasonal exigencies of winter represent a fundamental physiological challenge for a wide range of organisms. In response, many temperate insects enter diapause, an alternative developmental program, including developmental arrest, that allows organisms to synchronize their life cycle with seasonal environmental variation. Geographic variation in diapause phenology contributing to local climatic adaptation is well documented. However, few studies have examined how the rapid evolution of a suite of traits expressed across the diapause program may contribute to climatic adaptation on a contemporary timescale. Here, we investigate the evolution of the diapause program over the past 35 years by leveraging a “natural experiment” presented by the recent invasion of the Asian tiger mosquito, *Aedes albopictus*, across the eastern United States. We sampled populations from two distinct climatic regions separated by 6° of latitude (~700 km). Using common-garden experiments, we identified regional genetic divergence in diapause-associated cold tolerance, diapause duration, and postdiapause starvation tolerance. We also found regional divergence in nondiapause thermal performance. In contrast, we observed minimal regional divergence in nondiapause larval growth traits and at neutral molecular marker loci. Our results demonstrate rapid evolution of the diapause program and imply strong selection caused by differences in winter conditions.

**KEY WORDS:** Adaptation, diapause, life history evolution, phenotypic plasticity.

Understanding how organisms respond to thermal heterogeneity is a fundamental goal of biology (Selye 1956; Hoffmann and Parsons 1991; Johnston and Bennett 1996). The impacts of daily and seasonal thermal variability can be particularly acute for small ectotherms like insects, whose body temperature closely tracks ambient conditions (Huey and Kingsolver 1989; Huey and Berrigan 2001; Bradshaw et al. 2004; Sunday et al. 2011; Kingsolver et al. 2013). In temperate climates, winter challenges insects with

an extended period of low temperatures unsuitable for growth and reproduction. In response, many temperate insects have evolved diapause, an alternative developmental program characterized by a genetically controlled developmental arrest initiated in advance of unfavorable seasonal conditions (Denlinger 2002). Here, we refer to diapause in the broad sense of a “diapause program”: an alternative developmental trajectory with distinct molecular, physiological, developmental, and behavioral characteristics that precede the onset of developmental arrest and modify post-arrest growth and reproduction (sensu Andrewartha 1952; Denlinger 2002; Košťál 2006; Košťál et al. 2017).

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It is well established that the phenology of the diapause program varies across environmental clines. For example, a wide variety of insects initiate diapause earlier in the year at higher latitudes in response to the earlier onset of winter (Andrewartha 1952; Danilevsky 1965; Tauber et al. 1986). Diapause duration also tends to increase with latitude, presumably in response to longer winters (Masaki 1965; Kimura 1988; Koveos et al. 1993; Wang et al. 2012; Chen et al. 2013). Similarly, the physiology of diapause has been shown to differ among populations from different climates, although most studies have not determined whether these differences are genetic or plastic in nature. For instance, populations from regions with colder winters tend to have greater cold tolerance during diapause than populations from warmer regions (Tanaka 1997; Kalushkov and Nedvěd 2000; Zani et al. 2005; Xie et al. 2015; Medley et al. 2019). Nevertheless, a major knowledge gap exists regarding how diapause contributes to climatic adaptation. This gap is due to the lack of studies that examine how multiple traits expressed across the trajectory of the diapause program can evolve in response to climatic selection pressures, as well as the timescale on which such evolution may occur (but see Lehmann et al. 2015).

Addressing this knowledge gap has important theoretical and practical implications. From a theoretical perspective, physiological studies typically consider diapause as a dynamic process rather than simply a static shutdown of development. However, within the ecological literature, a historical view of diapause as a static developmental arrest (i.e., “escape in time”) is more prevalent. Evidence that suites of traits throughout the diapause program can rapidly evolve in response to novel environmental conditions would provide a new perspective on the role of diapause in contemporary climatic adaptation. From a practical perspective, biological invasions and climate change regularly expose insect populations to novel climate and winter conditions that are a critical determinant of insect distributions (Messenger 1959; Bale and Hayward 2010; Robinet and Roques 2010). Thus, accurate projections of species’ ranges will require integrating information about the rate of evolution of multiple components of the diapause program (Hoffmann and Sgró 2011; Bush et al. 2016; Diamond and Yilmaz 2018).

Here, we investigate geographic variation across a suite of traits spanning the diapause program among invasive populations of the Asian tiger mosquito, *Ae. albopictus*, from eastern North America. This species first established in the United States in 1985 and rapidly expanded across approximately 15° of latitude. Across this range, adult females produce diapause eggs in response to short day lengths. Diapause eggs complete embryonic development and then enter developmental arrest as pharate larvae inside the chorion of the egg. Previous studies have demonstrated rapid evolution of both diapause incidence and diapause timing (i.e., critical photoperiod) among these recently estab-

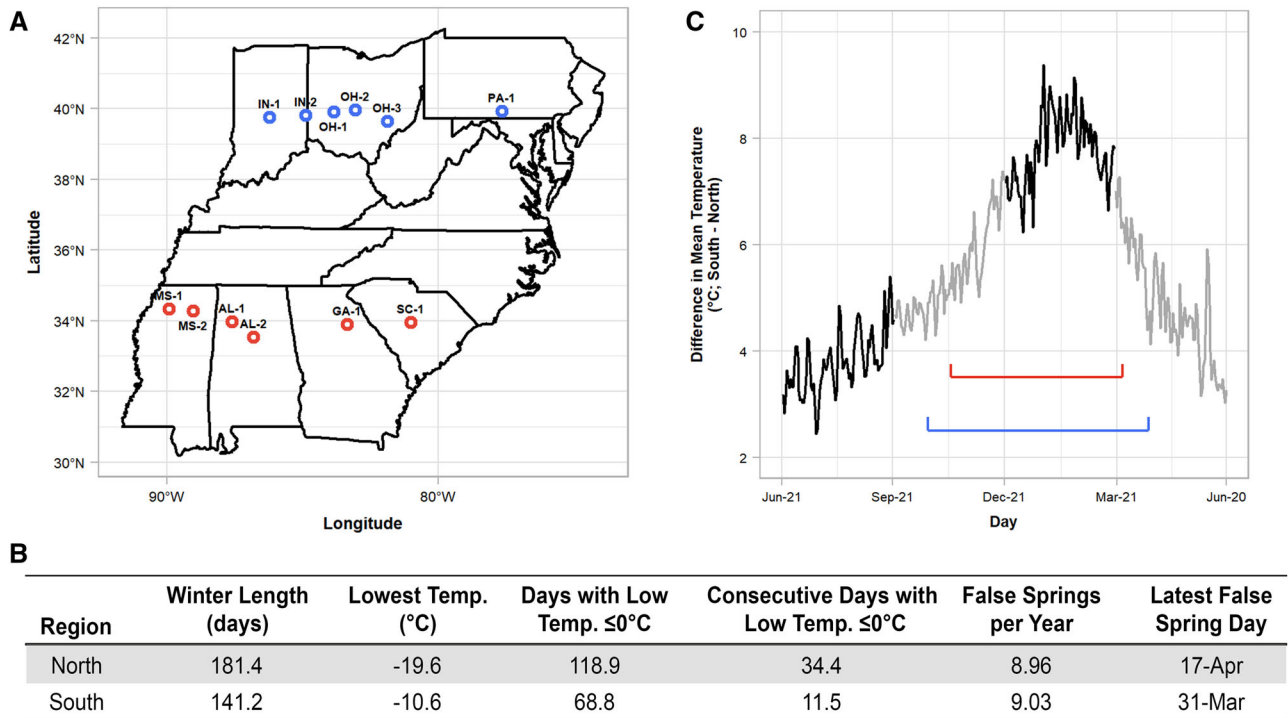
lished populations (Lounibos et al. 2011, Urbanski et al. 2012). Here, we further leverage this contemporary invasion by sampling six replicate populations from both high (40°N) and low (34°N) latitude regions. We performed common-garden experiments to quantify genetically based variation in a suite of traits reflecting physiological conditions throughout the diapause program. We also measured nondiapause traits (adult thermal performance, larval growth) to compare the evolution of nondiapause traits with traits expressed throughout diapause. Finally, we quantified population structure based on neutral molecular marker loci (microhaplotypes) to rule out the possibility that differences in traits between populations in the northern and southern regions could be caused by independent invasion histories. Our results provide novel insight into the role of the diapause program in rapid climatic adaptation.

## Material and Methods

### POPULATION ESTABLISHMENT AND LABORATORY REARING

We established laboratory colonies (populations) of *Ae. albopictus* from larvae collected at 12 field sites in eastern North America between 27 July 2017 and 30 August 2017 (Fig. 1; Table 1). We established six populations from the northern region (mean latitude: 39.8°N), which represents the approximate northern range limit of *Ae. albopictus* in North America (Benedict et al. 2007; Hahn et al. 2016). Additionally, we established six populations from the southern temperate region (mean latitude: 34.0°N). The range of *Ae. albopictus* in North America extends south to approximately 25°N (Hahn et al. 2016), but previous research demonstrated that populations of *Ae. albopictus* south of 34°N have reduced diapause incidence (Lounibos et al. 2011; Urbanski et al. 2012). Here, we explicitly investigated traits expressed throughout the diapause program, thus populations south of 34°N were not suitable for our experiments. All sites were separated by at least 70 km and regions were separated by at least 700 km.

At each site, at least 81 larvae (range: 81–210; Table 1) were collected from at least five artificial containers, primarily discarded tires, and reared as described previously (Armbruster and Conn 2006). Briefly, larvae were maintained in 15 cm petri dishes at a density of approximately 30 larvae per 90 mL deionized (DI) water under long-day (LD) photoperiod (16 h light:8 h dark) at 21°C. Every Monday–Wednesday–Friday (M–W–F), larvae were transferred to a new petri dish with clean DI water and fed approximately 1 mL of a slurry composed of 120 g dog food (Nutro Ultra Small Breed Puppy, Nutro Products Inc., Franklin, TN) and 40 g of frozen brine shrimp (Sally’s Frozen Brine Shrimp, San Francisco Bay Brand, Newark, CA) homogenized in 1 L



**Figure 1.** Population sites experience drastically different winter conditions. (A) Map of population collection sites. Blue dots identify northern sites; red dots identify southern sites. (B) Summary of mean winter conditions at collection sites. Winter length and False Springs as defined in text. (C) Absolute difference in mean daily temperature between northern and southern sites based on Daymet data, 1985–2017. The shade of the line alternates by season. Blue and red brackets represent mean time between first and last frost in northern and southern regions, respectively.

of DI water. Upon pupation, at least 40 males and 40 females were transferred to adult cages under LD photoperiod at 21°C and 80% relative humidity (RH). Adults were provided with organic raisins (Newman’s Own, Westport, CT) ad libitum for sugar feeding and females were offered a human bloodmeal approximately once per week. The Georgetown University Institutional Review Board (IRB) has determined that mosquito blood feeding is not human research and thus does not require IRB approval; however, the blood feeding protocol has been approved by the Georgetown University Occupational Health and Safety Office. After blood feeding, females were provided an oviposition cup lined with an unbleached paper towel and half-filled with DI water. F<sub>1</sub> eggs were collected every M-W-F, maintained on a wet paper towel for approximately 48 h, then air dried, and stored in a Tupperware container under LD photoperiod at 21°C and 80% RH. Following egg collection, 30 live F<sub>0</sub> adults from each population were anaesthetized with CO<sub>2</sub>, individually transferred to 1.5-mL Eppendorf tubes, and frozen at –80°C for population genetic analysis (see below).

For all subsequent generations and experiments, except the larval starvation assay and growth rate experiments (see below), larval rearing conditions were modified as follows: eggs from a single population were placed in a 5.5-L Sterlite container and

stimulated to hatch by submersion in approximately 2.5 L of DI water and addition of 5 mL of larval food slurry (described above). After hatching, populations were maintained at a density of approximately 250 larvae per 2.5 L DI water under LD photoperiod at 21°C and 80% RH. Every M-W-F, the larval water was poured through a fine mesh net and larvae were transferred to a clean 5.5-L Sterlite container with approximately 2.5 L DI water and 5 mL of larval food slurry. Pupae were transferred to adult cages maintained at 21°C with 80% RH under LD photoperiod as described above. Methods for bloodfeeding, egg collection, and egg storage were as described above. Each population was reared to the F<sub>4</sub> generation to eliminate field effects, synchronize populations, and generate sufficient egg stocks to complete all experiments. For all assays described below, either 10 or 12 populations we used according to egg availability.

**CLIMATE CONDITIONS AT COLLECTION SITES**

The distance between weather stations and collection sites varied. We therefore assessed the climate conditions at each site via Daymet, a database that provides meteorological data interpolated to a 1-km grid (Thornton et al. 2016). Climate data were extracted for the 32 years between the initial invasion of North America by *Ae. albopictus* and field collection (1985–2017)

**Table 1.** Overview of experiment sample sizes by population. Numbers represent the total number of samples in an experiment; numbers in parentheses are total egg counts summed across samples. Population labels correspond to those presented in Figure 1A.

Pop.	Latitude (°N)	F <sub>0</sub>		F <sub>4</sub>		F <sub>5</sub>						
		Colony founding	Pop. genetics	Larval growth	Adult chill coma	Diapause incidence	Diapause duration	Rapid cold shock	Simulated winter	Larval starvation		
<i>North</i>												
IN-1	39°45'	210	30	♂ 70	♀ 55	196	3 (478)	33 (4139)	16 (2164)	26 (3463)	96	
IN-2	39°48'	121	30	64	57	159	3 (371)	34 (3779)	16 (1907)	–	–	
OH-1	39°55'	128	30	73	50	185	3 (405)	33 (3862)	10 (1323)	6 (694)	72	
OH-2	39°58'	150	30	60	59	154	3 (331)	32 (3673)	20 (3343)	34 (4245)	96	
OH-3	39°39'	128	30	70	56	129	3 (387)	31 (3665)	20 (2788)	20 (2552)	96	
PA-1	39°55'	200	30	64	59	185	3 (367)	32 (3841)	14 (1719)	14 (1770)	96	
<i>South</i>												
MS-1	34°20'	133	30	62	62	208	3 (401)	39 (4763)	22 (3031)	34 (4113)	96	
MS-2	34°17'	88	30	69	51	163	3 (317)	33 (4192)	24 (3631)	20 (2350)	96	
AL-1	33°59'	150	30	69	54	204	3 (297)	33 (4147)	30 (3699)	26 (3053)	96	
AL-2	33°33'	82	30	76	44	189	3 (339)	30 (3755)	10 (1181)	–	–	
GA-1	33°53'	81	30	60	42	160	3 (358)	33 (3750)	14 (1761)	6 (736)	96	
SC-1	33°58'	109	30	66	46	196	3 (357)	35 (4215)	16 (1868)	14 (1590)	96	

using the *daymetr* package (Hufkens et al. 2018). We calculated the absolute difference in mean daily temperature across the entire calendar year between northern and southern sites. Next, for each region, we calculated the mean length of winter (days from first frost to last frost), lowest absolute temperature, number of days with temperatures below freezing, and longest run of consecutive days with a low temperature below freezing. Finally, we enumerated “false springs” as days between 1 January and 1 May with measurable precipitation and a maximum temperature above 10°C that were subsequently followed by a day with minimum temperatures below 0°C. We selected these criteria because rainfall stimulates *Ae. albopictus* eggs to hatch and 10°C represents the approximate developmental threshold for larvae (Delatte et al. 2009), implying that these conditions present opportunities for spring reemergence. Because hatched *Ae. albopictus* larvae have minimal ability to survive temperatures below 2.5°C (Chang et al. 2007; Delatte et al. 2009), individuals that hatch during false springs are unlikely to survive subsequent subzero exposure and false springs may represent an important selective factor.

#### DIAPAUSE INCIDENCE

To measure diapause incidence, F<sub>4</sub> larvae from each population were reared in 5.5-L Sterilite containers as described above and pupae were transferred to adult cages under unambiguous diapause-inducing, short-day (SD) photoperiod (8 h light:16 h dark) at 21°C (Table 1). Three adult cages (biological replicates) were established for each population. F<sub>5</sub> eggs were collected as described above then stored in a Tupperware container under SD photoperiod at 21°C. Two days postoviposition (dpov), we divided all egg papers into samples each containing approximately 120 eggs (range: 60–198), air dried these samples, and stored them in 10 cm petri dishes at 21°C under SD photoperiod.

We quantified diapause incidence for each population as previously described (Urbanski et al. 2012) using one egg paper from each of three biological replicate adult cages. Each egg paper contained ~120 eggs (range: 88–180). Briefly, at 14 dpov, we submersed eggs in DI water and approximately 1 mL of larval food slurry. We recorded the number of larvae hatched per egg paper, air dried the remaining eggs, and then repeated the process one day later. After the second hatch attempt, remaining eggs were bleached to clear the chorion (Trpiš 1970) and all embryonated, unhatched (i.e., diapause) eggs were enumerated. Diapause incidence was calculated for each replicate according to the following formula: (number of embryonated, unhatched eggs)/(number of hatched eggs + number of embryonated, unhatched eggs). To determine if diapause incidence differed between northern and southern regions, we constructed a linear mixed effect model with diapause incidence as the response variable, region as a fixed factor, and population nested within region

as a random factor. We tested the significance of the region effect via ANOVA using population within region as the error term.

### SIMULATED WINTER COLD TOLERANCE

Next, we assessed cold tolerance under conditions similar to those experienced in the field during a typical mid-latitude winter in eastern North America. We measured cold tolerance as survival following simulated winter exposure in five northern and five southern populations (Table 1) using  $F_5$  diapause eggs generated as described above. For each population, eggs were collected from three biological replicate adult cages, divided into samples of  $\sim 120$  eggs each (range: 63–211), and haphazardly assigned to a simulated winter treatment closely corresponding to winter duration (Fig. 1B) in either the southern (140 d) or northern region (185 d). In pilot experiments,  $\sim 90\%$  of diapause eggs hatched (i.e., had terminated diapause) by 115 dpov under simulated winter conditions (Table S3). Thus, to ensure that nearly all eggs had terminated diapause, samples were maintained for at least 136 dpov before exposure to hatching stimuli. In total, 50 samples (i.e., egg papers) per region were assigned to each treatment including at least one sample per biological replicate (Table S1). All samples were placed in a Percival I-36VL incubator (Percival Scientific, Perry, IA) and exposed to simulated winter conditions as described in Figure S1. After simulated winter exposure, eggs were stimulated to hatch as described above; the precise age of eggs at hatch varied by up to 16 days within a treatment (Table S2) but the mean age of eggs did not differ by region in either the southern simulated winter ( $F_{1,5.77} = 2.27$ ,  $P = 0.185$ ) or the northern simulated winter ( $F_{1,7.48} = 2.44$ ,  $P = 0.159$ ) treatment. To assess survival, we counted hatched larvae; remaining unhatched eggs were air dried, returned to  $21^\circ\text{C}$  under LD photoperiod, and re-stimulated to hatch seven days later. Finally, eggs were bleached and embryonated; unhatched larvae were scored as dead. Survival was calculated as (number of hatched larvae)/(number of hatched larvae + number of unhatched, embryonated larvae).

Simulated winter survival was tested using a linear mixed-effects model for each treatment (southern winter, northern winter) with percent survival as the response variable, region as a fixed effect, and population nested within region as a random effect. We tested the significance of the region effect via ANOVA, using population within region as the error term. We directly compared survival of cold-exposed samples among regions without adjusting for survival in parallel control samples (i.e., constant  $21^\circ\text{C}$ ), because long-term cold exposure decreases the time required for diapause termination in *Ae. albopictus* (Table S3; Pumpuni 1989). Egg mortality is not discernable from unternated diapause in this species, so variation in the rate of diapause termination would confound comparisons of survival between cold-treated samples and non-cold-treated control samples.

### RAPID COLD SHOCK TOLERANCE

To assess the physiological limits of cold tolerance, we measured rapid cold shock tolerance in all 12 populations in two blocks, with three southern and three northern populations in each block (Tables 1 and S4). Three biological replicates of each population were reared in 5.5-L Sterlite containers as described above and then pupae were transferred to adult cages under a diapause-inducing SD photoperiod.  $F_5$  eggs were collected, divided into samples each containing approximately 130 eggs (range: 59–270), and randomly assigned to either rapid cold shock or control treatments. Eggs were maintained at  $21^\circ\text{C}$  for 14 days to ensure diapause was initiated in all samples (Hawley et al. 1987; Pumpuni 1989; Urbanski et al. 2012). Next, temperature for the rapid cold shock samples was reduced from  $21^\circ\text{C}$  to  $-9^\circ\text{C}$  at a rate of  $2^\circ\text{C}/\text{h}$  and maintained at  $-9^\circ\text{C}$  for 24 h (see Figure S1), whereas control samples were maintained at a constant  $21^\circ\text{C}$ . Following treatment, all samples were held for 134 days to allow for diapause termination. Next, we assessed survival using the hatching assay described in the previous section (see SIMULATED WINTER TOLERANCE section), with 14 days between the first and second hatch attempts. Finally, rapid cold shock tolerance was calculated for each population as the difference between mean percent survival of control samples and mean percent survival of cold-shock-treated samples from each biological replicate.

We tested for differences in rapid cold shock tolerance using a linear mixed-effects model with the difference between survival in control and shock conditions as the response variable. The model included region as a fixed effect as well as block and population nested within region as random effects. We tested the significance of the region effect via ANOVA, using population within region as the error term.

### DIAPAUSE DURATION

We assessed diapause duration for all 12 populations (Table 1) using  $F_5$  diapause eggs generated as described above from three biological replicate adult cages for each population. Egg samples were maintained at  $21^\circ\text{C}$  under SD photoperiod until 56 dpov and then all eggs were transferred to LD photoperiod. Beginning at 56 dpov, diapause incidence was determined using at least one sample per biological replicate and population (i.e., at least three samples per population) as described above (see DIAPAUSE INCIDENCE section). This procedure was repeated with separate egg samples every two to three weeks until 175 dpov; in total, we assessed the percentage of individuals remaining in diapause at nine time points for each population.

Next, the diapause duration data were combined with the 14 dpov diapause incidence data described above and all data points were fit with dose-response models using diapause incidence as the response variable and age as the dose variable. We

used a four-parameter log-logistic model: the lower and upper limits were fixed at 0 and 1, respectively, whereas the slope and inflection point were allowed to vary. In these models, the inflection point indicates the age at which 50% of eggs have terminated diapause. We utilized populations as replicates in these models by averaging the measured diapause incidence at each time point across biological replicates. We constructed a full dose-response model including region as a fixed factor, then compared this to a reduced dose-response model excluding region as a factor via ANOVA. Finally, we performed *t*-tests to compare the relevant model parameters (slope and inflection point) between northern and southern regions using the *CompParm* and *EDcomp* commands in *drc* (Ritz et al. 2015).

### POSTDIAPAUSE LARVAL STARVATION TOLERANCE

To assess postdiapause energetic reserves, we measured postdiapause larval starvation tolerance of 10 populations (Table 1) after both a simulated southern and northern winter (see SIMULATED WINTER TOLERANCE section). After the simulated winter treatments, eggs were stimulated to hatch as described above. Within 4 h of hatching, first instar larvae were transferred individually to 3.4-mL wells of a sterile, untreated 24-well tissue-culture plate (Corning Inc., Corning, NY) with 1 mL sterilized water and checked daily for mortality. Starvation tolerance was recorded as the number of days to mortality, where larvae were identified as dead if they failed to swim in response to light exposure and mechanical probing with a pipette.

We analyzed starvation tolerance using a linear mixed effect model with age, region, and their interaction as fixed effects and population nested within region as a random effect. Including a random effect for plate did not significantly improve model fit, so we excluded this term from the final model. We tested the significance of the region effect via ANOVA using population within region as the error term.

### ADULT CHILL COMA RECOVERY TIME

To quantify adult chill coma recovery time (ACCRT), we adapted methods widely used for measuring chill coma recovery time in *Drosophila melanogaster* (David et al. 1998; Gibert et al. 2001; Hoffmann et al. 2002; Macdonald et al. 2004). F<sub>4</sub> larvae from each population were reared in 5.5-L Sterlite containers as described above and female pupae were transferred to adult cages at 21°C under LD photoperiod. Within one week of eclosion, adult females were placed on ice for 2.5 h to induce chill coma. Comatose females were transferred to a recovery arena made of plexiglass (30 cm × 30 cm × 55 cm) and lined with white paper to improve visibility. Females were placed in the arena in an inverted position (i.e., dorsal side down). A vortex mixer (Vortex-Genie 2, Scientific Industries Inc., Bohemia, NY) was set to run on top of the arena to agitate comatose mosquitoes and stimu-

late females to recover (i.e., reassume upright posture) as soon as they were capable. ACCRT was measured for each individual as the time required to reestablish an upright position and chill coma recovery time for a trial was calculated as the median of individual recovery times. No mortality was observed throughout the experiments.

The chill coma recovery experiments were performed on all 12 populations with four biological replicates per population. Populations were tested in three blocks staggered one to two weeks apart (Table S4) so that testing could be performed within a 4 h circadian time frame (see below). Each block consisted of two randomly chosen populations from each region tested on four consecutive days with one trial per population per day. On each trial day, one biological replicate was placed on ice at each of the four trial start times, alternating northern and southern populations. The trial start times were 9 am, 10 am, 11 am, and 12 pm corresponding to zeitgeber times (ZT): 0 h, 1 h, 2 h, and 3 h. Each population was tested once at each of the four trial start times.

We analyzed the adult chill coma recovery results using a linear mixed effect model. Median ACCRT was the response variable. Region, ZT, and the interaction between region and ZT were included as fixed effects; experimental block and population nested within region were included as random effects. We tested the significance of the region effect via ANOVA using population nested within region as the error term.

### LARVAL GROWTH TRAITS

We measured sex-specific development time, pupal mass, and larval growth rate in all 12 populations over three blocks (Tables 1 and S4). Larvae were reared individually in 3.4-mL wells of tissue-culture plates following methods described in Armbruster and Conn (2006). Briefly, F<sub>4</sub> eggs were stimulated to hatch by placing egg papers in a 5.5-L Sterlite container as described above at 26°C under LD photoperiod. Approximately 24 h later, hatched larvae were transferred to new 24-well culture plates; each well contained a single first instar larvae with 1 mL of DI water and 15 µL of larval food dispensed from a kitchen blender running at low speed to maintain even particle suspension (Armbruster and Conn 2006).

Every M-W-F, larvae were transferred to new plates with 1 mL DI water and 15 µL of larval food slurry as described above. Plates were checked daily for pupation and development time was measured as the number of days between larval hatch and pupation. Pupae were examined under a dissecting microscope to determine sex, gently transferred to an unbleached paper towel to remove excess water, and then weighed to the nearest 0.01 mg to determine pupal mass. Finally, we estimated larval growth rate for each individual as pupal mass divided by development time.

To assess regional differences in larval growth traits, we used separate, sex-specific linear mixed-effect models for

development time, pupal mass, and larval growth rate. We separated our models by sex because the larval growth traits examined are known to be sexually dimorphic (Armbruster and Conn 2006), but evaluation of sex differences was not a goal of our study. In each model, region was included as a fixed effect and both experimental block and population nested within region were included as random effects. For each model, we tested the significance of the region effect via ANOVA, using population within region as the error term.

## POPULATION GENETICS

Finally, to obtain molecular genetic-based estimates of genetic structure among populations, 30 field-collected adults from each population (Table 1; Fig. 1) were sequenced at 192 targeted genomic regions of ~120 bp (range: 92-137 bp), that is, “microhaplotypes” (sensu Kidd et al. 2014; Baetscher et al. 2018). Microhaplotype amplicon sequencing was performed using the Genotyping-in-Thousands by Sequencing approach outlined in Campbell et al. (2015) with the modifications described in Baetscher et al. (2018).

Briefly, we first selected microhaplotype candidate loci based on exome sequence data of 10 individual *Ae. albopictus* from Manassas, VA (Armbruster, unpubl. data). Microhaplotype candidate loci were identified from the exome data as 90-140 bp sequences containing three to eight third codon position SNPs supported by at least 10 reads (Table S5). Furthermore, all target loci were determined to be unique within the genome and located on separate genomic scaffolds. DNA was extracted from 360 individual field-collected adults using DNeasy 96 Blood and Tissue kits (Qiagen, Inc, Hilden, Germany) on a Qiagen BioRobot 3000 (Qiagen, Inc, Hilden, Germany) as described in Baetscher et al. (2018). Then, we performed two rounds of multiplex PCR: the first used locus-specific primers to amplify all 192 microhaplotype candidate loci, and the second added individual-specific DNA barcodes. Finally, indexed samples were combined in equal quantities and  $2 \times 75$  bp paired-end sequencing was performed on a MiSeq instrument (Illumina, San Diego, CA). Resulting reads were processed as described in Baetscher et al. (2018). We eliminated potentially spurious microhaplotypes by removing alleles with poor sequencing coverage ( $<10$  reads in an individual or read depth ratio  $<0.2$ ; see Baetscher et al, 2018). Additionally, we removed loci with (1) two alleles that deviated from Hardy-Weinberg equilibrium (HWE) in at least three populations ( $z$ -score  $> 3$ ); (2) three alleles that deviated from HWE in at least two populations; (3) more than two alleles per individual; or (4) no variation among individuals.

After bioinformatic processing, we retained genotypes of 360 individual mosquitoes genotyped at 145-154 microhaplotype loci. For each locus, we calculated expected heterozygosity and allelic richness using the *adegenet* package (Jombart and Ahmed

2011). We also performed a Ewens-Watterson test (Ewens 1972; Manly 1985) in PopGene (Yeh et al. 1997) to assess neutrality at each locus. Next, to determine if heterozygosity differed between regions, we performed a  $t$ -test on mean heterozygosity across loci following an arcsine square root transformation. Then we tested if the distribution of allelic richness differed between regions via a Kolmogorov-Smirnov test. We also calculated pairwise  $F_{ST}$  between all populations using the *adegenet* package and performed a Mantel test of isolation-by-distance in *Arlequin* (version 3.5; Excoffier and Lischer 2010). Finally, we performed an Analysis of Molecular Variation (AMOVA) in *Arlequin* to determine how genetic variation was partitioned among regions, among populations within regions, and within populations.

## STATISTICAL TESTS

All statistical tests were performed using R (R Core Team 2019). Linear mixed effect models were fit by maximum likelihood using the R package *lme4* (Bates et al. 2015). Denominator degrees of freedom were approximated by the Satterthwaite method (Gaylor 2006) via the *anova* function in the R package *lmerTest* (Kuznetsova et al. 2017). Diapause duration data were analyzed using the *drm*, *compParm*, and *EDcomp* functions in the *drc* package (Ritz et al. 2015). Maps were constructed using the R package *ggmap* (Kahle and Wickham 2013). All other plots were constructed using the package *ggplot2* (Wickham 2016).

## Results

### CLIMATE CONDITIONS

Climate data from collection sites between 1985 and 2017 indicated that, on average, winters at northern sites were longer and colder than those at southern sites (Fig. 1B). Additionally, northern and southern sites experienced a similar number of potentially lethal false spring days, but false spring days occurred later in the winter at northern sites than southern ones (Fig. 1B). The difference in mean daily temperature between regions was greater during the winter than other seasons (Fig. 1C). These results show that populations from the southern and northern regions experience distinct climatic conditions, particularly during winter.

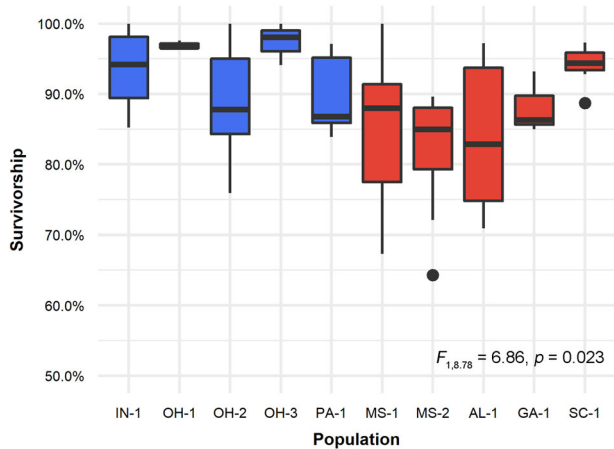
### DIAPAUSE INCIDENCE

All populations produced  $>90\%$  diapause eggs in response to SD photoperiod conditions (Table S6) and diapause incidence did not differ significantly between the northern and southern regions ( $F_{1,12} = 4.01$ ,  $P = 0.068$ ). Therefore, comparisons of diapause characteristics between regions are not confounded by regional differences in diapause incidence.

### SIMULATED WINTER TOLERANCE

After a simulated southern winter treatment (140 days), diapause egg survival was consistently high (northern mean: 95.8%;





**Figure 2.** Diapause eggs from northern populations have higher survival following simulated northern winter treatment compared to southern populations. Blue and red boxplots represent northern and southern populations, respectively. Populations are arranged west to east within each region. The results of ANOVA for the effect of region are displayed on the figure.

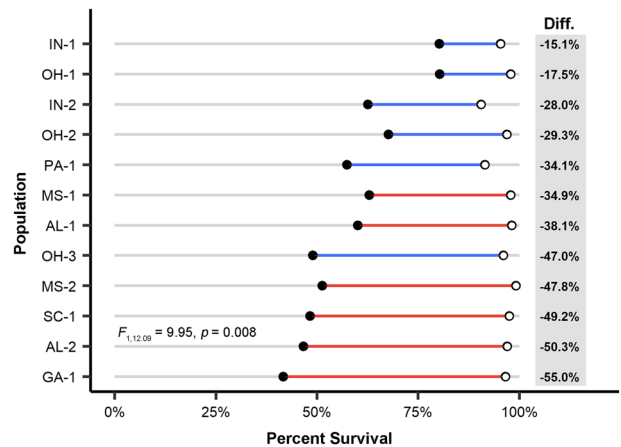
southern mean: 96.2%) and did not significantly differ between regions ( $F_{1,8.59} = 0.18$ ,  $P = 0.685$ ; Fig. S2). However, after a simulated northern winter treatment (185 days), survival of diapause eggs was significantly higher in northern populations (mean: 92.5%) compared to southern populations (mean: 86.2%; Fig. 2;  $F_{1,8.78} = 6.86$ ,  $P = 0.023$ ). These results show that survival decreases with extended exposure to winter conditions and northern populations have greater cold tolerance than southern populations when exposed to an extended simulated winter.

### RAPID COLD SHOCK TOLERANCE

Survivorship of diapause eggs exposed to rapid cold shock was higher for northern populations than southern populations (Fig. 3;  $F_{1,12.09} = 9.95$ ,  $P = 0.008$ ). In northern populations, 66.2% of eggs survived rapid cold shock compared to 94.7% of control eggs (28.5% reduction in survival). In contrast, among southern populations just 51.8% of eggs survived rapid cold shock relative to 97.7% of control eggs (45.9% reduction in survival). Thus, northern populations produce diapause eggs that are more resistant to acute cold exposure than southern populations.

### DIAPAUSE DURATION

At constant 21°C, diapause incidence remained high (>90%) in all populations until 56 dpov, but then declined until 175 dpov, at which point all populations except PA-1 reached <10% diapause incidence (PA-1 = 16.0% diapause incidence at 175 dpov; Fig. 4A). Comparison of full and reduced dose-response models indicates that region is a significant predictor of diapause duration ( $F_{2,114} = 11.79$ ,  $P < 0.001$ ). The time required for half of eggs to terminate diapause was significantly longer in northern (118.7



**Figure 3.** Diapause eggs for northern populations have greater survival following rapid cold shock compared to southern populations. Open points represent survival in control samples maintained at 21°C; filled points represent survival in eggs exposed to rapid cold shock (24 h at -9°C). The difference in percent survival between control and exposed is presented on the right of each plot. Blue and red lines represent populations from northern and southern regions, respectively. Lines are organized by difference between control and rapid cold shock survival. The results of ANOVA for the effect of region are displayed on the figure.

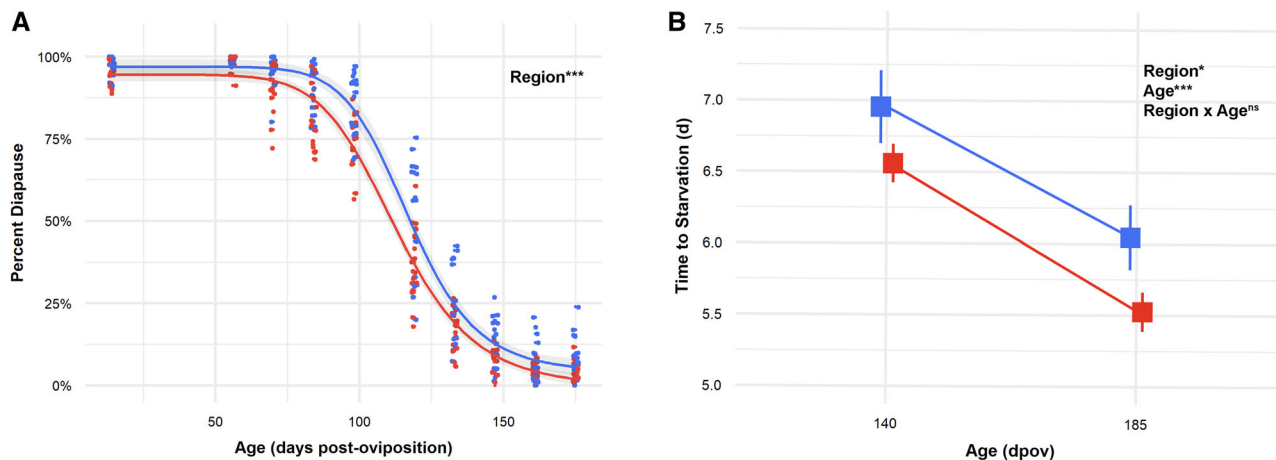
dpov) than southern (111.5 dpov) populations ( $t_{118} = 4.69$ ,  $P < 0.001$ ). In contrast, the slopes of the dose-response curves did not differ by region ( $t_{118} = 1.29$ ,  $P = 0.201$ ). Thus, southern populations exhibit a shorter diapause duration than northern population due to earlier onset of diapause termination rather than differences in the rate of diapause termination.

### POSTDIAPAUSE LARVAL STARVATION TOLERANCE

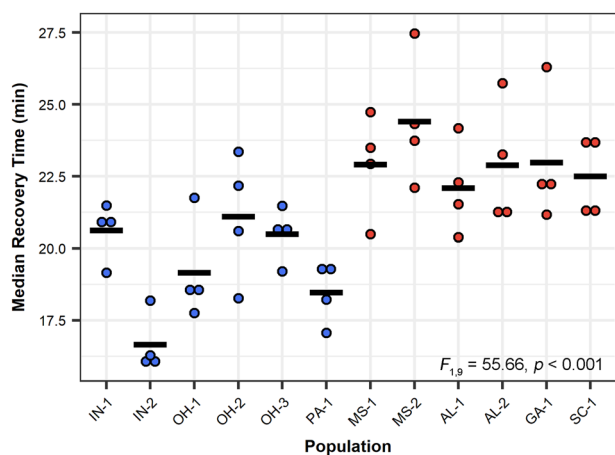
Postdiapause larval starvation tolerance decreased with the age of the eggs ( $F_{1,932.4} = 69.11$ ,  $P < 0.001$ ), was greater in northern than southern populations ( $F_{1,10.3} = 7.66$ ,  $P = 0.019$ ), and was not affected by an age-by-region interaction (Fig. 4B;  $F_{1,932.4} = 0.25$ ,  $P = 0.614$ ). This result shows that time to larval starvation is reduced in older eggs and that, regardless of age, northern populations have greater starvation tolerance than southern populations.

### ADULT CHILL COMA RECOVERY TIME

ACCRT was significantly shorter in northern populations than in southern populations (Fig. 5;  $F_{1,9} = 55.66$ ,  $P < 0.001$ ). On average, the median chill coma recovery time was 19.4 min for northern populations and 23.0 min for southern populations. ACCRT was significantly affected by circadian (ZT) time ( $F_{3,36} = 12.02$ ,  $P < 0.001$ ) and by a region-by-ZT time interaction (Fig. S3;  $F_{3,36} = 5.11$ ,  $P = 0.004$ ), primarily due to shorter recovery time during later trials in the southern populations. These results show adults



**Figure 4.** Northern populations exhibit longer diapause duration and have greater postdiapause larval starvation tolerance. (A) Percent of eggs remaining in diapause over time at constant 21°C. Points represent percent of eggs in a single replicate that fail to respond to two hatching attempts ( $n = 60\text{--}198$  eggs per sample). Blue and red lines represent four-parameter log-logistic dose-response curves for northern and southern regions, respectively. Shaded regions surrounding dose-response curves represent 95% confidence intervals. (B) Mean time to starvation for larvae hatched from ~140 dpov postdiapause eggs and ~185 dpov postdiapause eggs. Blue and red points correspond to northern and southern populations, respectively. Error bars represent 1 SE. Results of ANOVA shown in the insets indicate (A) the effect of region and (B) the effects of region, age, and their interaction; \*\*\* $P < 0.001$ ; \* $P < 0.05$ ;  $P > 0.05$  (ns).



**Figure 5.** Median adult chill coma recovery time is shorter in northern populations. Each point represents the median recovery time from a single trial. Blue and red points represent northern and southern populations, respectively. Black bars represent the mean of median recovery times for a single population across all four trials. The results of ANOVA for the effect of region are displayed on the figure.

from northern regions recover more quickly from chill-induced coma and their recovery time is less impacted by circadian time than southern populations.

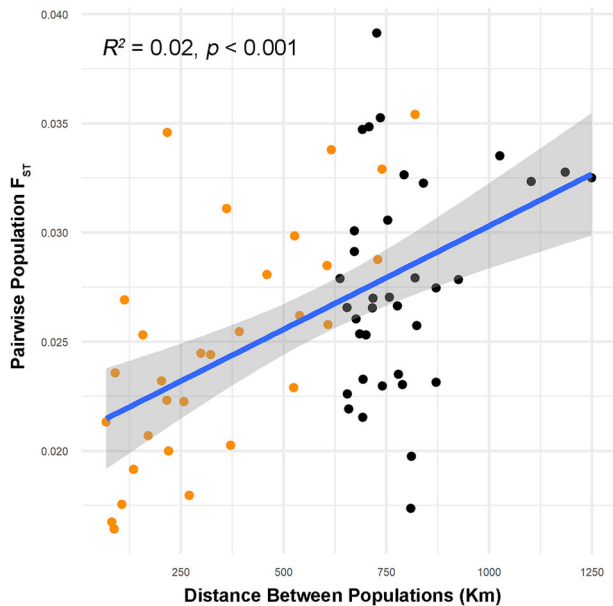
**LARVAL GROWTH TRAITS**

Mean larval development time did not differ by region for males (Fig. S4A;  $F_{1,8.78} = 0.68, P = 0.43$ ) or females (Fig. S4B;  $F_{1,8.68} = 0.10, P = 0.76$ ). Pupal mass also did not differ by region for

males (Fig. S5A;  $F_{1,11.84} = 0.96, P = 0.346$ ) but female pupal mass was significantly higher in the southern than the northern region (Fig. S5B;  $F_{1,9.41} = 7.43, P = 0.02$ ). Mean pupal mass was  $3.05 \pm 0.39$  mg for southern females and  $2.94 \pm 0.43$  mg for northern females (3.7% difference). Finally, larval growth rate did not differ between regions for either males (Fig. S6A;  $F_{1,8.86} = 0.08, P = 0.79$ ) or females (Fig. 6B;  $F_{1,8.01} = 4.47, P = 0.07$ ).

**POPULATION GENETICS**

There were between two and 19 alleles across 154 microhaplotype loci in our samples and allelic richness did not differ between the northern and southern regions (Fig. S8B;  $D_{154} = 0.06, P = 0.955$ ). Average expected heterozygosity across all loci was significantly higher in the southern than northern region ( $t_{10} = 2.52, P = 0.030$ ), but the difference was small (Fig. S8A; northern: 0.397, southern: 0.407). Most of the loci (141 of 154 loci; 91.6%) were consistent with neutral expectations from the Ewens-Watterson test (Fig. S7); because 5% of loci would be expected to deviate from neutral expectations by chance alone, we retained all loci for further analyses. A significant pattern of isolation-by-distance was indicated by an increase in genetic differentiation among populations ( $F_{ST}$ ) with increasing geographic distance between them ( $R^2 = 0.27, P < 0.001$ ), although all pairwise  $F_{ST}$  values were low ( $F_{ST} < 0.04$ ; Fig. 6). Similarly, individual locus  $F_{ST}$  values between populations were low and exhibited relatively little variation (Fig. S9; 95th percentile = 0.073, 99th percentile = 0.121). No individual locus had a significant  $F_{ST}$  after correcting for multiple comparisons. AMOVA showed that 0.55% of the total microhaplotype



**Figure 6.** Pairwise genetic differentiation increases with geographic distance between populations. Pairwise  $F_{ST}$  is averaged across all 154 microhaplotype loci. Orange and black dots represent pairs of populations within a region and between regions, respectively. The blue line displays a linear regression across all data points with the 95% confidence interval shaded.

variation was distributed among regions and 96.19% of total variation was distributed among individuals within populations (Table 2). As such, the finding of isolation-by-distance (Fig. 6) implies that gene flow among populations is limited by geographic distance, but there is minimal genetic differentiation among populations within regions and between regions, and the vast majority of genetic variation is partitioned within populations (Table 2).

## Discussion

The importance of diapause for local adaptation of insects to temperate winters is well documented (see summaries in Tauber et al. 1986; Danks 1987). However, few studies have examined how the rapid evolution of multiple traits expressed throughout the diapause program can contribute to climatic adaptation on a contemporary timescale (but see: Lehmann et al. 2015). We addressed

this fundamental gap by quantifying geographic divergence in a suite of traits expressed throughout the diapause program in replicate populations of the Asian tiger mosquito, *Ae. albopictus*, from two distinct climatic regions of eastern North America. We measured these traits under common-garden conditions, and because these populations were established after the 1985 invasion of North America by *Ae. albopictus*, the divergence we quantified between the southern and northern regions represents recently evolved genetic differences. Additionally, we characterized the divergence of nondiapause traits (chill coma recovery, and larval growth) and neutral molecular markers to evaluate if population structure, independent invasion histories, or other factors unrelated to diapause could contribute to divergence of diapause traits.

### RAPID DIVERGENCE OF THE DIAPAUSE PROGRAM

Despite the limited time for selection (<35 years or <70–105 generations; Sprenger and Wuithuranyagool 1986; Hawley et al. 1987), a small latitudinal range ( $\sim 6^\circ$ ), and low levels of divergence at molecular marker loci (see below), we identified distinct genetically based differences across a suite of traits expressed throughout the diapause program (Figs. 2, 3, 4A, and 4B). These differences are consistent with adaptation to regional variation in the length and severity of winter in eastern North America (Fig. 1). For example, we found that populations from both regions had similar survivorship after a 140-day winter treatment (i.e., a southern region winter length; Fig. S2), but northern populations had significantly greater survival after a 185-day winter treatment (i.e., a northern region winter length; Fig. 2). Regional differences in survival could be driven by evolution of cold tolerance during the developmental arrest stage and/or postdevelopmental arrest quiescence. Additionally, recently evolved differences in plasticity during cold acclimation could contribute to the regional divergence we observe in overwinter survivorship. However, when we restricted cold exposure to early in developmental arrest (rapid cold shock), diapause eggs from northern populations had significantly greater survival than southern populations (Fig. 3), suggesting that differences in cold tolerance during developmental arrest likely contribute to higher survivorship.

We also found that northern populations of *Ae. albopictus* undergo a significantly longer diapause than southern

**Table 2.** Analysis of molecular variation based on 154 microhaplotype loci.

Source of variation	Df	Sum of squares	Variance	Percent of total variation
Between regions	1	159	0.177	0.55%
Between populations within regions	10	949	1.070	3.26%
Between individuals within populations	718	22,260	31.004	96.19%
Total	729	23,368	32.231	

populations (Fig. 4A). Temperature affects the rate of diapause termination in many species, including *Ae. albopictus* (Table S3), but our samples were maintained at 21°C and thus we cannot directly translate this difference into a field context. Nevertheless, our experiment under common-garden conditions demonstrates clear genetic divergence among regions in diapause duration. One key fitness benefit of diapause is preventing resumption of development during intervals of permissive conditions subsequently followed by lethal conditions (i.e., “false springs”). On average, the last false spring event at northern sites occurs approximately 2.5 weeks later than at southern sites (Fig. 1B). Therefore, longer diapause duration in northern populations likely reflects selection favoring individuals that are refractory to premature hatching cues during these potentially lethal false springs. Additionally, northern populations had greater postdiapause larval starvation tolerance than southern populations after both 140 and 185 days in dormancy (Fig. 4B). We interpret postdiapause starvation tolerance as a reflection of larval energetic reserves upon resumption of development; consistent with this interpretation, larvae starved more quickly after 185 days in dormancy compared to 140 days. Several factors may contribute to these regional differences. For example, diapause termination may increase metabolic activity (Wipking et al. 1995; Singtripop et al. 2007; Ragland et al. 2009; Hahn and Denlinger 2011; but see Williams et al. 2015; Lehmann et al. 2016), so the earlier diapause termination in southern populations may lead to increased usage of energetic reserves (Fig. 4A). Alternatively, *Ae. albopictus* diapause eggs contain greater lipid stores than nondiapause eggs (Reynolds et al. 2012; Batz and Armbruster 2018), likely due to both increased maternal provisioning and decreased lipid mobilization by diapause embryos (Reynolds et al. 2012; Huang et al. 2015). Thus, regional divergence in energy utilization throughout the diapause program and/or changes in maternal nutrient allocation may contribute to regional differences in postdiapause starvation tolerance.

We hypothesize that the rapid regional divergence detected in these populations is adaptive for several reasons. First, the consistent differences we observed between replicate populations from the northern versus southern regions strongly imply divergence caused by a deterministic process such as natural selection, rather than a random process like genetic drift. Second, latitudinal temperature gradients are more pronounced in winter (i.e., nongrowing season) than in summer (i.e., growing season; Gaston and Chown 1999; Addo-Bediako et al. 2000; Bradshaw et al. 2004), presenting a clear selective force to act on traits expressed across the diapause program (Fig. 1). Third, minimal regional differentiation of nondiapause larval growth traits and neutral molecular markers implies that genetic structure caused by historical demographic events such as independent, secondary invasions cannot be invoked to explain the divergence of dia-

pause traits (see below). Finally, relative to southern populations (~32°N), *Ae. albopictus* diapause eggs from northern populations (~40°N) have higher survivorship after a two-month field winter trial at a northern site (Medley et al. 2019), strongly supporting the conclusion that the regional differences we detected have fitness consequences under ecologically relevant conditions.

### NONDIAPAUSE THERMAL PERFORMANCE

To evaluate regional divergence for a nondiapause trait potentially affected by temperature-mediated selection, we measured ACCRT. Chill coma recovery time is a widely used indicator of thermal performance, particularly in Diptera, and frequently varies with latitude (David et al. 1998; Hoffmann et al. 2002; Macdonald et al. 2004; Sinclair et al. 2012). We found that northern populations consistently recovered from chill coma faster than southern populations (Fig. 5). We cannot determine whether this divergence is due to direct selection on the adult stage or indirect selection on genetically correlated traits in nonadult stages (e.g., in diapause eggs, see: Ragland and Kingsolver 2008). However, previous studies have demonstrated genetic correlations between diapause and a wide range of life history traits, including many stress tolerance traits (Schmidt et al. 2005, Schmidt and Conde 2006, Schmidt and Paaby 2008). Regardless of the mechanism, our findings indicate among North American populations of *Ae. albopictus*, traits related to thermal performance in both diapause and nondiapause stages show consistent regional divergence. This finding contrasts with results of a wide range of non-thermal performance traits (see below) and emphasizes the importance of temperature as a selective factor across the invasive range of *Ae. albopictus* in North America.

### GENETIC POPULATION STRUCTURE

As noted above, a lack of regional differentiation at neutral molecular marker loci supports the conclusion that evolution of traits expressed throughout diapause and nondiapause thermal performance is due to natural selection. Analysis of variation at 154 microhaplotype loci indicated a significant pattern of isolation-by-distance among populations (Fig. 6), implying that the extent of gene flow between populations is dependent upon the geographic distance between them (Wright 1943; Slatkin 1993; Rousset 1997). However, despite the high genetic variation at these microhaplotype loci (expected heterozygosity ~0.4; Fig. S8), most of the variation was distributed within populations. Results of AMOVA (Table 2) indicate that variation both among regions (0.55%) and among populations within regions (3.26%) is very low relative to variation within populations (96.19%), and all pairwise  $F_{ST}$  values were <0.04 (Figs. 6 and S8; Table 2). These results clearly rule out the hypothesis of independent introductions for the northern and southern populations because the AMOVA indicates that <1% of the molecular variation is

partitioned between regions (Table 2) and the low  $F_{ST}$  values exhibit nearly complete overlap within and between regions (Fig. 6). In contrast, previous studies with both microsatellite markers (Manni et al. 2017) and ddRADseq (Kotsakiozi et al. 2017) have identified substantially greater levels of genetic differentiation among *Ae. albopictus* populations from different continents ( $F_{ST} = 0.05$ – $0.31$ ). These results are also consistent with other recent population genetic analyses of *Ae. albopictus* from North America. For example, low differentiation was detected among geographically disparate populations in eastern North America with ddRADseq data (Kotsakiozi et al. 2017) and landscape genetic models using microsatellite marker data found high gene flow among the regions investigated in our study, likely due to human-mediated transport (Medley et al. 2015).

### LARVAL GROWTH TRAITS

We detected no regional divergence in larval development time (Fig. S4) or larval growth rate (Fig. S6) suggesting that clinal selection acting on these traits is relatively weak or absent in the North America. There was also no significant regional difference in male pupal mass, but we observed that southern populations exhibited a slightly larger female pupal mass than northern populations (Fig. S5). The cause of this difference is unclear. No latitudinal cline for female pupal mass was observed among populations collected from the ancestral Japanese range in 2008 (Urbanski et al. 2012), suggesting that differences among southern and northern regions in the United States are not a general evolutionary response to latitudinally varying climatic factors. Similarly, previous work investigating variation in traits not directly related to diapause or thermal performance (e.g., development time, larval growth rate, adult survivorship, reproductive output, larval competitive ability, and egg volume) has found little or no divergence between geographic regions (Armbruster and Conn 2006; Leisnham et al. 2009; Urbanski et al. 2012). The minimal divergence observed in neutral genetic variation and in growth traits expressed during nondiapause life stages distinctly contrasts with the divergence observed in diapause-associated traits and supports the interpretation that the regional divergence of these traits is due to natural selection.

## Conclusions

We demonstrate rapid adaptive evolution of a suite of traits expressed across the diapause program between populations of the invasive mosquito, *Ae. albopictus*, over a limited geographic scale ( $\sim 6^\circ$  latitude). These findings emphasize that changes to traits expressed during multiple phases of the diapause program, including during the developmental arrest phase (i.e., cold tolerance), can contribute to adaptation across climatic gradients on contemporary timescales. Our results have important implica-

tions for both conceptual and applied topics in ecology and evolution. At the conceptual level, the consideration of diapause as a dynamic alternative developmental program, rather than simply a shutdown of growth and development, is well-appreciated in the physiological literature (Denlinger 2002; Košťál 2006; Košťál et al. 2017). However, this perspective is less widely recognized in the ecological literature, where diapause has been historically conceived of as a static “escape in time” or “ecological time out.” Our results support and extend the physiological perspective of diapause as a dynamic physiological state by demonstrating that traits expressed across the range of the diapause program can evolve rapidly in response to climatic selection pressures. From an applied perspective, many temperate insects overwinter in diapause and it is well appreciated that diapause plays a critical role in defining the upper latitudinal distribution (Addo-Bediako et al. 2000; Kimura 2004; Sunday et al. 2011). Thus, cold tolerance in diapause is a critical parameter for estimating future insect ranges after an invasion or in response to climate change. Although several recent reviews have highlighted the necessity of incorporating thermal adaptation into range projections (Hoffmann and Sgró 2011; Araújo and Peterson 2013; Bush et al. 2016; Diamond and Yilmaz 2018), almost no data are available regarding the adaptive potential of traits expressed during or after the developmental arrest phase of the diapause program. Our results highlight that rapid evolution of multiple components of the diapause program contributes to climatic adaptation across geographic gradients. We hypothesize that selection for regional divergence in traits expressed throughout diapause will be strongest for insects that, like *Ae. albopictus*, have limited capacity for mobility and behavioral thermoregulation during dormancy.

### AUTHOR CONTRIBUTIONS

ZB and PA conceived of and designed the study. ZB and TR performed the animal husbandry. ZB and JF carried out the phenotypic experiments. ZB identified the microhaplotype targets. AC and JCG performed the genotyping. ZB and AC performed the data analyses. ZB and PA drafted the initial manuscript. All authors contributed to manuscript revision and approved of the submitted version.

### ACKNOWLEDGMENTS

We thank L. Ries, A. Rosenwald, S. Cook, A. Mushegian, J. Boyle, and four anonymous reviewers for helpful comments on previous version of this manuscript. We also thank E. Campbell, C. Columbus, and E. Correa for assistance with genetic data generation and E. Anderson for assistance with genetic data analyses. This work was supported by funds from the Cosmos Club Foundation of Washington D.C. to ZB and National Institute of Health grant 1R01AI132409-01A1 and the Davis Family Endowment to PAA.

### DATA ARCHIVING

Upon acceptance, data associated with this project will be available in the Dryad Data Repository at the following URL: <https://doi.org/10.5061/dryad.Orxwdbwt>.

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Associate Editor: P. Schmidt  
Handling Editor: T. Chapman

### Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**Figure S1** Schematic overview of cold treatments for (A) short simulated winter, (B) long simulated winter, and (C) rapid cold shock.

**Table S1** Samples per biological replicate (independent adult cages) for short and long simulated winter experiments.

**Table S2** Overview of samples in simulated winter experiments.

**Table S3** Percent hatch at ~115 dpov under simulated winter and control (constant 21°C) conditions.

**Table S4** Blocking design for larval growth rate, adult chill coma recovery, and rapid cold shock experiments.

**Table S5** Summary of microhaplotype target loci

**Table S6** Diapause incidence at 14 dpov under SD photoperiod by population.