Contrasting effects of an extended fall period and winter heatwaves on the overwintering fitness of diapausing disease vector, *Aedes albopictus*

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Running head: Impacts of fall duration and winter heatwaves on overwintering fitness

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Abstract

Climate change is expected to dramatically alter autumnal and winter conditions in many temperate regions. However, limited data is available to accurately predict how these changes will impact species' overwinter survival and post-winter fitness. Here, we determine how a longer, warmer fall period and winter heatwaves affect overwintering fitness and post-winter performance of the invasive mosquito vector, *Aedes albopictus.* We found that a longer, warmer fall period representative of early entry into diapause did not affect overwinter survival but did lead to reduced post-winter performance for multiple traits. Specifically, larvae that experienced longer, warmer fall conditions as diapause embryos exhibited reduced post-diapause larval starvation tolerance, increased post-diapause larval mortality, and longer post-diapause larval development compared to individuals from the short-fall treatments. These negative post-diapause fitness effects likely resulted from the greater energetic demands and/or damage incurred during the warmer, longer fall period. In contrast, exposure to winter heatwaves increased overwinter survival, possibly by allowing diapausing embryos to escape or repair cold injury. Finally, fall treatment and winter heatwaves had an interactive effect on male development time, while neither treatment impacted pupal mass in either sex. Overall, our results highlight that experiments that fail to measure post-diapause fitness are likely to substantially under-estimate the impacts of climate change on post-winter performance. Additionally, our results emphasize that it is crucial to consider the potentially conflicting effects of different aspects of climate change on a species' overall overwintering success.

1. Introduction

Predicting biological responses to climate change is a major challenge with important implications for biodiversity conservation (Scheffers et al. 2016), agriculture (Luedeling et al. 2009; Lehmann et al. 2020), and disease ecology (Carlson et al. 2022). A substantial body of research investigating the biological impacts of climate change has emerged over the last 20 years, but many studies focus exclusively on the effects of warmer spring and summer (i.e., growing season) conditions (Gallinat et al. 2015; Williams et al. 2015; Lehmann et al. 2020). However, climate change will also affect other seasons, generally causing extended growing seasons (i.e., later onset of winter and earlier start to spring), increases in average winter temperatures, and a greater frequency of extreme events such as winter heatwaves (Kunkel et al. 2004; Linderholm 2006; Liu et al. 2006; Hansen et al. 2012; Liu et al. 2018; Marshall et al. 2020). There is increasing evidence across taxa that conditions during winter can cause significant carry-over effects on reproduction, life history, and population growth the following growing season (Bradshaw et al. 1998; Norris and Marra 2007; Harrison et al. 2011; Boggs and Inouye 2012; O'Connor et al. 2014). Thus, the paucity of studies that investigate how autumnal and winter thermal conditions impact overwinter and post-winter fitness presents a major limitation to accurately predicting overall biological responses to climate change.

Many temperate arthropods survive winter by entering diapause, a pre-programmed developmental arrest triggered by a token cue (e.g., photoperiod) that forecasts environmental degradation (Koštál 2006). Because diapause is an anticipatory response, individuals typically spend several months or more in developmental arrest while conditions are still suitable for growth (Denlinger 2022). Diapause often confers protection from environmental stressors through enhanced nutrient storage, metabolic suppression, increased cold tolerance, and greater desiccation resistance compared to non-diapause individuals (Denlinger 2022). Nevertheless, diapausing arthropods are still sensitive to changes in thermal conditions. For example, some diapausing species exhibit increased energy expenditure (i.e., energy drain) at high temperatures (Han and Bauce 1998; Hahn and Denlinger 2007; Sinclair 2015; Nielsen et al. 2022). This mechanism likely underlies observations of reduced mass and survival of diapausing arthropods following longer and/or warmer fall or winter conditions (Han and Bauce 1998; Gomi 2000; Irwin and Lee 2000; Williams et al. 2003; Bosch and Kemp 2004; Sgolastra et al. 2010; Sgolastra et al. 2011; Abarca et al. 2019; Nielsen et al. 2022). Additionally, winter heatwaves can deleteriously reduce cold tolerance levels during diapause, causing cold injury once temperatures drop (Šlachta et al. 2002; Sobek-Swant et al. 2012; Williams et al. 2014). These results suggest that, despite their dormant state, diapausing arthropods may still be vulnerable to changes in fall and winter climate.

It remains difficult to predict the net impact of a warming climate on winter-diapausing arthropods for several reasons. First, limited studies exist across species (Bale and Hayward 2010), and not all species show similar fitness effects in response to warming. While some species exhibit reduced fitness following warmer overwintering periods, other species show little to no negative effects (Williams et al. 2003; Mercader and Scriber 2008; Williams et al. 2012).

For some overwintering arthropods, warmer winter temperatures have even facilitated northern range expansions (Jepsen et al. 2008). Second, diapause represents an alternative developmental trajectory with distinct physiological characteristics that can modify post-arrest growth and reproduction (Denlinger 2002; Koštál 2006; Batz et al. 2020; Denlinger 2022), but not all studies consider the post-diapause phase of this trajectory in assessments of overwintering fitness (Han and Bauce 1998; Sgolastra et al. 2011; Abarca et al. 2019). As a result, these studies cannot detect carry-over effects at later life stages that may impact individual fitness and population dynamics in the growing season (O'Connor et al. 2014). Finally, few studies have simultaneously manipulated both fall and winter temperatures when investigating the impacts of thermal conditions during diapause (but see (Bosch and Kemp 2004)). Diapause is a dynamic physiological process (Koštál 2006), meaning that the impacts of heat exposure may depend on the timing during the overwintering period as well as prior thermal conditions. Thus, it remains difficult to predict how species will respond to the cross-seasonal warming expected to occur in nature.

Here, we examine the fitness consequences of ecologically realistic heat exposure during diapause in an important vector species, the Asian tiger mosquito, *Aedes albopictus*. We exposed diapausing *Ae. albopictus* embryos to a full-factorial combination of thermal treatments that differed in: (1) fall duration and temperatures to simulate early versus late entry into diapause; and (2) exposure to winter heatwaves (zero or three heatwaves). Fall treatments were designed to simulate the extremes of the natural range of *Ae. albopictus'* diapause entry dates while winter treatments were designed to simulate average to above-average winter heatwave challenge based on recent temperature records. This design allowed us to examine the consequences of ecologically realistic conditions that will likely become more extreme as climate change continues to progress. We then measured overwinter survival, and post-diapause larval mortality, larval development time, larval starvation tolerance, and pupal mass to assess the cumulative fitness consequences of these cross-seasonal treatments. We predicted that heat exposure resulting from earlier entry into diapause (i.e., a longer, warmer fall period) and winter heatwaves would decrease overwinter survival and post-diapause fitness by increasing energy drain in diapausing embryos. We also predicted that winter heatwaves would decrease overwinter survival by deleteriously reducing cold tolerance.

2. Materials and Methods

2.1. Study system

Temperate populations of *Aedes albopictus* undergo a photoperiodically-mediated, maternally-induced embryonic diapause at the pharate larvae stage (Armbruster 2016). This species is a highly invasive pest which has rapidly expanded from its native range in Southeast Asia to occupy all continents except Antarctica (Benedict et al. 2007; Kraemer et al. 2019). Most research on the response of diapause *Ae. albopictus* embryos to temperature focuses on lower thermal limits due to interest in understanding the role of cold temperatures in determining the northern distribution limit of this species (Hanson and Craig 1994; Thomas et al. 2012).

However, diapausing embryos also routinely encounter warm temperatures in nature, but the fitness consequences of this exposure remain unknown. For example, field collections performed in Washington D.C. show that female *Ae. albopictus* begin to lay diapause embryos in August when temperatures often reach as high as 20-30°C (Mushegian et al. 2021). Moreover, exposure to high temperatures is likely to increase as climate change causes growing seasons to lengthen (Kunkel et al. 2004; Linderholm 2006; Liu et al. 2006; Liu et al. 2018) and increases the frequency of winter heatwaves (Hansen et al. 2012; Abarca et al. 2019). Because *Ae. albopictus* is a competent vector for several human diseases such as those caused by Zika, dengue, and chikungunya arboviruses, understanding the potential impact of climate change on its overwintering fitness has direct public health implications (Gratz 2004; Lwande et al. 2020).

2.2. Population collection, rearing, and embryo counting

The stock population used for this experiment was originally collected as larvae during June 2018 in Manassas, Virginia (Mushegian et al. 2021). The population was propagated for 10 generations in the lab with previously described rearing procedures (Batz et al. 2020). Briefly, all rearing was done in a temperature-controlled walk-in incubator held at 21°C, 80% relative humidity, and long-day (LD) photoperiod (16:8 Light:Dark) unless otherwise noted. For the parental generation of the experimental animals, embryos were hatched in an 8.5-L rearing pan with approximately 3-mL of food slurry. The food slurry was made by blending 120-g of 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) in 1-L of deionized (DI) water. Three days after hatching, larvae were split into groups of approximately 200 and each group was put in a separate 5.5-L Sterlite container with 2-L of DI water placed in the walk-in incubator. Every Monday, Wednesday, and Friday (M-W-F), larvae were transferred to fresh water with approximately 2-mL of food slurry. Pupae were collected three times weekly from larval bins and transferred to six replicate adult cages consisting of 2.5-gallon plastic buckets. Each adult cage contained approximately 430 adults. Adult cages were provisioned with organic raisins (Newman's Own, Westport, CT) ad libitum for sugar feeding. To stimulate females to produce diapausing embryos, adult cages were maintained under an unambiguous short-day (SD) photoperiod (8:16 Light:Dark) inside photoperiod-cabinets located in the walk-in incubator as previously described (Mushegian et al. 2021).

One week after the last pupae was collected, all cages were blood-fed to repletion on a human host. 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. Three to four days after blood feeding, each adult cage was provisioned with three oviposition cups consisting of plastic cups half filled with DI water and lined with a strip of unbleached paper towel (i.e., oviposition paper). Three oviposition papers per cage were collected for three days (M-W-F) from the first gonadotrophic cycle, resulting in a total of 54 oviposition papers (six cages × three papers per cage × three collections).

All oviposition papers were left moist for 48 hours and then gently dried. Each oviposition paper was then cut and split into replicates of 118 embryos per paper on average (range: 58-265). For each replicate oviposition paper, desiccated embryos (i.e., not viable) were removed and thus not included in pre-treatment embryo counts. Each oviposition paper replicate was placed in an individual petri dish and haphazardly assigned one of four temperature treatments (Figure 1; Supplementary Table S1). Before and during treatments, replicate dishes were stored inside large plastic containers containing a mesh-covered cup of water to maintain constant relative humidity of approximately 80%. All embryos were kept on LD at 21°C in the walk-in incubator until treatments started on August 12, 2021. Thus, the embryos were 6-10 days post-collection at the start of treatments. Additional details regarding the organization of embryos in growth chambers throughout fall and winter treatments are available in the Supplementary Text section of the Supplementary Information.

2.3. Fall treatments

In nature, earlier fall entry into diapause entails both a longer diapause duration and exposure to warmer temperatures relative to embryos that enter into diapause later in the fall. Therefore, to most accurately approximate natural conditions, our fall treatments differed in both duration and temperature (see Figure 1). Populations of *Ae. albopictus* in the Virginia-Maryland-DC (VA-MD-DC) area first begin to oviposit diapause embryos in mid-August, with 50% diapause incidence observed September 7th, and close to 100% diapause incidence of embryos by mid-November (Mushegian et al. 2021). Therefore, to compare survival and post-diapause fitness of diapause embryos laid early and late in the fall, we chose fall treatment lengths of 122 days (corresponding to a diapause start date of August 1st) for "long-fall" and 30 days (corresponding to a diapause start date of November 1st) for "short-fall" (Figure 1; Supplementary Table S1). The start of winter was defined as December 1st. Thus, our fall treatments simulate the extremes of the natural range of diapause entry dates. The daily temperature range for each month of fall treatments (August-November for the long fall treatment and November for the short fall treatment) was set by taking the average daily maximum and the average daily minimum temperatures for each month from historical temperature data from the years 1990-2020 for the VA-MD-DC area (DayMet TileID 11572, latitude 38 to 40, longitude -78 to -76). To best approximate natural conditions, temperatures during fall treatments oscillated daily between these minimum and maximum temperatures at a rate that approximated a sinusoidal function (Abarca et al. 2019). Embryos were subjected to a decrease in photoperiod throughout the fall treatment to best match natural conditions (see Supplementary Table S1).

2.4. Winter heatwave treatments

Both winter treatments lasted 109 days and consisted of daily fluctuations between -5°C and 5°C, which approximates winter length and temperatures for the VA-MD-DC area (Batz et al. 2020) (Figure 1). We defined a heatwave as three or more consecutive days above the 90th percentile temperature of a reference period of 1990-2010 (Abarca et al. 2019). To determine an appropriate heatwave treatment, we first computed the 90th percentile temperature for each individual winter month using maximum daily temperature data from 1990-2010, with 1990 used as the earlier boundary due to *Ae. albopictus'* arrival to the United States in the late 1980s (Armbruster 2016). We then used a custom Python script (see Supplementary File 2) to record the frequency, timing (i.e., month of occurrence), duration, and maximum daily temperature of heatwaves for each winter month using maximum daily temperature data from 2011-2020 (see Supplementary Information for details). We used 2011-2020 to estimate heatwave parameters that were most relevant to current climate conditions. To simulate a season of average to above-average winter heatwave challenge based on this analysis, we presented heatwave treatment embryos with three heatwaves (two in simulated December and one in simulated February)

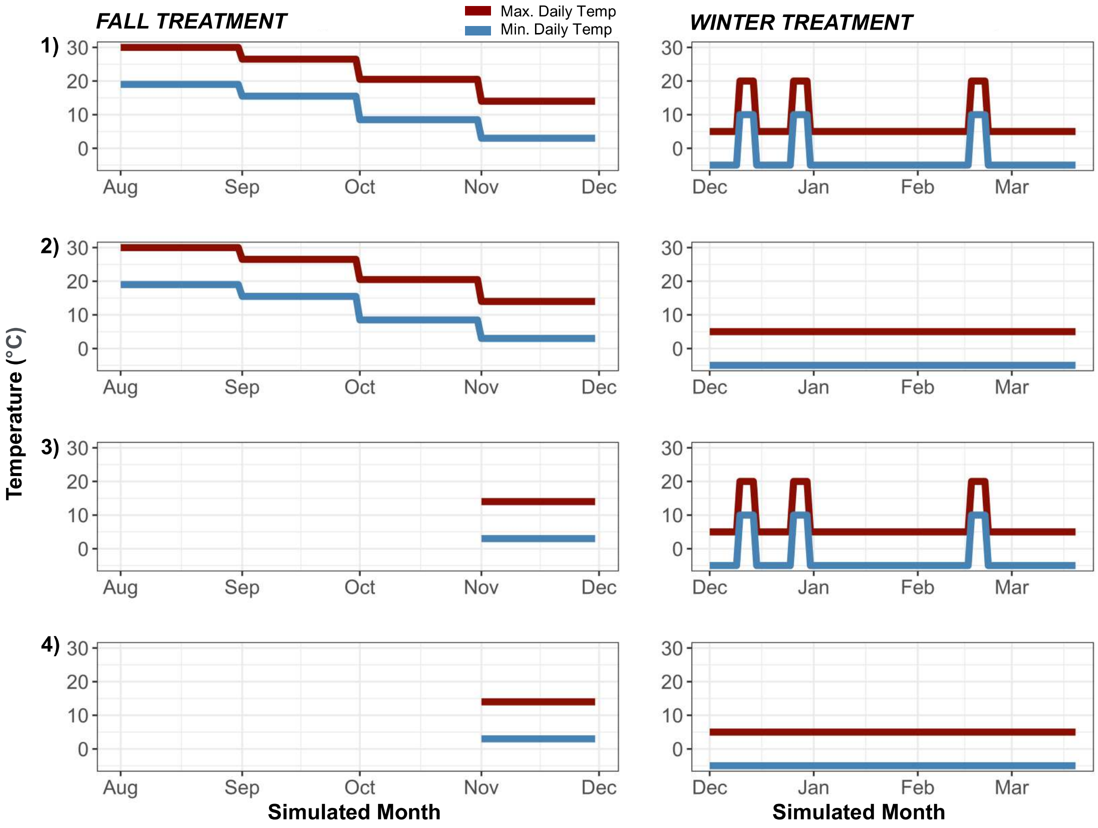

Figure 1. Four temperature treatments used in the experiment: 1) long-fall, winter heatwaves; 2) long-fall, no winter heatwaves; 3) short-fall, winter heatwaves; 4) short-fall, no winter heatwaves. The start of winter treatments immediately followed the end of fall treatments. Red and blue lines represent maximum and minimum daily thermal limits; temperatures oscillated daily between the limits.

lasting four days each with daily fluctuations between 10°C and 20°C (Figure 1; Supplementary Figures S1). For both winter treatments, we used a short photoperiod representative of winter in the VA-MD-DC area (9:15 L:D).

2.5. Overwinter survival

The day that winter treatments ended, the plastic containers containing replicate papers with embryos were moved to the walk-in incubator and maintained at 21°C, 80% relative humidity, and LD photoperiod for one week. After one week at 21°C, we immersed embryos in water to initiate hatching; all long-fall treatment embryos were 248 days post-collection and all short-fall treatment embryos were 156 days post-collection. To hatch out embryos, we moved each oviposition paper to its own medium sized (10-cm in diameter) petri dish with 50-mL of DI water and four drops of larval food slurry. Larvae were counted for each oviposition paper replicate three days after hatching. Each paper was then gently dried, left for one week in the walk-in incubator at 21°C, then hatched and counted again (Batz et al. 2020). We hatched out each oviposition paper replicate a total of four times. Overwinter survival was calculated for each replicate by dividing the total number of hatched larvae collected by the number of viable embryos counted at the beginning of treatments. Hatched larvae were then either discarded, set aside for the larval starvation tolerance assay, or set aside for the larval growth assay.

2.6. Post-diapause larval starvation tolerance

To measure larval energetic reserves after overwintering, first instar larvae from the overwinter survival assay described above were subjected to a starvation tolerance assay described previously (Batz et al. 2020). On the first hatching stimulus of each oviposition paper replicate described above for overwinter survival, we checked for larvae two to three hours after stimulating hatch. If no hatch occurred, dishes were checked hourly until at least 200 larvae had been collected from each treatment. We made sure to collect larvae within three hours of hatching to minimize the amount of food they ingested before isolation. We also took care to transfer as little water as possible with the larvae. Collected larvae came from multiple replicates from multiple original parental cages for each treatment to minimize bias in representation (see Supplementary Tables S2,S3). Each larva was transferred gently from the petri dish to a well in a sterile 3.4-mL 24-well tissue culture plate (Corning Inc., Corning, NY) containing 1-mL of autoclaved DI water. Larvae were checked daily for death between 1-3pm each day. Death was identified as a failure to move when touched gently with a pipette tip several times. Starvation tolerance was measured as the number of days the larvae was recorded as alive from hatch to death.

2.7. Post-diapause larval growth

Twenty-four hours after the first hatching stimulus described above, larvae were collected from replicate oviposition papers for a larval growth assay conducted as previously described (Armbruster and Conn 2006). Care was taken to spread the selected larvae across multiple replicates from multiple original parental cages for each treatment (see Supplementary Tables S2,S3). Larvae were placed individually in clean 3.4-mL 24-well tissue culture plates (Corning

Inc., Corning, NY), with each well containing a near-optimal amount of diluted and homogenized larval food mixture. The diluted food was made by mixing 5-mL of the original larval food slurry with 1000-mL of DI water. Beginning four days after hatch, larvae were moved to new culture plates containing fresh food/water mixture every M-W-F. Larvae were checked daily for death and pupation. Pupae were sexed and weighed to the nearest 0.01-mg on a microbalance (Mettler Toledo AX105 DeltaRange). We recorded larval developmental time, larval mortality, as well as the sex and mass of each pupa.

2.8. Statistical analysis

Sample sizes for each experiment are given in Table 1. Because overwinter survival data was in the form of proportions of discrete counts (i.e., number of successes and failures per replicate), we compared overwinter survival across the four temperature treatments using a generalized linear mixed effects model (glmmTMB function of *glmmTMB* R package) with a beta-binomial distribution with logit link to account for overdispersion (Demétrio et al. 2014). In this model, fall treatment and winter treatment were included as fixed effects, and date of embryo collection, replicate parental cage, and plastic container number (nested within fall treatment) were included as random effects.

To analyze starvation tolerance, we used a linear mixed effects regression (lmer) model (*lme4* R package), with fall treatment and winter treatment as fixed effects, and date of embryo collection, replicate parental cage, plate number, and plastic container number (nested within fall treatment) as random effects.

To analyze data on proportion of larvae that died during larval growth assays, we used a generalized linear mixed effects model (glmer) (*car* R package) with a binomial distribution, with fall treatment and winter treatment as fixed effects, and date of embryo collection, replicate parental cage, plate number, and plastic container number (nested within fall treatment) as random effects.

To analyze data on larval development time and pupal mass, we used lmer models, with fall treatment and winter treatment as fixed effects, and date of embryo collection, replicate parental cage, plate number, and plastic container number (nested within fall treatment) as random effects. Separate models were run for each sex because it is already well established that larval development time and pupal mass are sexually dimorphic in this species (Armbruster and Conn 2006); this approach simplifies the interpretation of the model results by avoiding a potential three-way interaction between fall treatment, winter treatment, and sex. Furthermore, because larval development time and pupal mass have different impacts on male versus female fitness (Bedhomme et al. 2003), using a separate model for each sex allowed us to more directly evaluate the impact of our experimental treatments on sex-specific fitness. Larval development time was log-transformed to improve the normality of residuals for both female and male models.

For all models, a type II Anova (*car* R package) was performed to detect significance of fixed effects. For all analyses, all interactions between fixed effects were included in initial models but removed if found to be non-significant ($P > 0.2$). All statistical analyses were conducted in R (version R-4.1.3) (R Core Team 2021). All plots were generated using the *ggplot2* package in R. We ensured that models met the assumptions of normality of residuals and homogeneity of variances by visually inspecting a quantile-quantile plot (Q-Q plot) and a residual plot, respectively (residplot function in *predictmeans* R package).

If an interaction between fall and winter treatments was found to be significant, we performed a Tukey's HSD test (*TukeyHSD* function in R) on a one-way ANOVA with treatment (the four possible combinations of fall and winter treatments) as the explanatory variable (*aov* function in R) to determine which of the four groups differed significantly from the others.

Table 1 Sample sizes used for each experiment per treatment combination.

	Long Fall, Winter Heatwaves	Long Fall, No Winter Heatwaves	Short Fall, Winter Heatwaves	Short Fall, No Winter Heatwaves	Total
Overwinter Survival	58[†] (6810)*	58 (6745)	59 (6873)	59 (6967)	234 (27,395)
Starvation Tolerance	215	215	219	233	882
Larval Growth	165	166	167	168	666

[†] The number of experimental units used for each treatment group (oviposition paper replicates for overwinter survival and individual larvae for starvation tolerance and larval growth assays).

*The number in parentheses indicates the total number of embryos used for each treatment group (summed across oviposition paper replicates).

3. Results

3.1. Overwinter survival

Overwinter survival was not affected by fall treatment ($\chi^2_{1,224} = 0.043$, $P = 0.836$; Figure 2), but was significantly affected by winter heatwave treatment, with approximately 10% greater survival observed for embryos that experienced winter heatwaves compared to embryos that did not experience winter heatwaves ($\chi^2_{1,224} = 70.746$, $P < 0.001$; Figure 2). The interaction between fall and winter treatment was not significant ($P > 0.2$) and was thus removed from the final model. Table S5 provides summary statistics for data on overwinter survival and all other measured traits.

Figure 2. Diapause embryos that experienced winter heatwaves exhibited greater overwinter survival compared to embryos that experienced no winter heatwaves. Color indicates long-fall groups (green) or short-fall groups (orange). Filled circles represent winter with heatwaves (HWs) and unfilled circles representing winter without heatwaves (No HWs). Plots illustrate treatment means ± 2 standard errors. Significance is shown in the top right corner of the plot. *** indicates $P < 0.001$, ^{ns} indicates $P > 0.05$

3.2. Post-diapause larval starvation tolerance

Larval starvation tolerance was greater for larvae from the short-fall treatments compared to larvae from long-fall treatments ($\chi^2_{1,874} = 152.529$, $P < 0.001$; Figure 3A). Winter heatwaves did not affect larval starvation tolerance ($\chi^2_{1,874} = 0.394$, $P = 0.530$; Figure 3A). The interaction between fall and winter treatment was non-significant ($P > 0.2$) and was thus removed from the final model.

3.3. Post-diapause larval growth

Larvae from the long-fall treatments exhibited higher post-diapause mortality than larvae from the short-fall treatments ($\chi^2_{1,659} = 59.305$, $P < 0.001$; Figure 3B). Winter heatwaves did not affect larval mortality ($\chi^2_{1,659} = 0.049$, $P = 0.825$; Figure 3B). The interaction between fall and winter treatment was not significant ($P > 0.2$) and was thus removed from the final model.

Of the 666 larvae used for the larval growth assay, 498 survived to pupation (254 females and 244 males) and were thus included in comparisons of post-diapause larval development time and pupal mass. Larvae from the long-fall treatments had a significantly longer post-diapause larval development time for both females ($\chi^2_{1,246} = 17.409$, $P < 0.001$; Figure 3C) and males ($\chi^2_{1,235} = 8.338$, $P = 0.004$; Figure 3D). There was no effect of winter heatwaves in either sex ($\chi^2_{1,246} = 2.019$, $P = 0.155$ for females; $\chi^2_{1,235} = 0.628$, $P = 0.428$ for males). However, for males, the interaction between winter and fall treatments was significant ($\chi^2_{1,235} = 7.283$, $P = 0.007$;

Figure 3D) with winter heatwaves resulting in shorter larval development only in male larvae from the long fall treatment. Based on a Tukey's HSD test with a 95% family-wise confidence level, males that experienced a long fall and no winter heatwaves had significantly greater development time than those that experienced a long fall and winter heatwaves (*P-adj* = 0.004, 95% C.I. = 0.019 - 0.139), those that experienced a short fall and winter heatwaves (*P-adj* < 0.001, C.I. = -0.139 - -0.035), and those experienced a short fall and no winter heatwaves *(P-adj* < 0.001, C.I. = -0.165 - -0.061). For females, the interaction between fall and winter treatment was non-significant ($p > 0.2$) and was thus removed from the final model.

For post-diapause pupal mass, we did not detect a significant effect of fall treatment on either sex ($\chi^2_{1,246} < 0.001$, $P = 0.989$ for females; $\chi^2_{1,236} = 1.257$, $P = 0.262$ for males; Figure 3E,3F). We also did not detect an effect of winter heatwave treatment on either sex ($\chi^2_{1,246} = 0.475$, $P = 0.491$ for females; $\chi^2_{1,236} = 1.877$, $P = 0.171$ for males; Figure 3E,3F). In both sexes, the interaction between fall and winter treatment was non-significant ($P > 0.2$) and was thus removed from the final models. Full model results for all traits can be found in Supplementary Table S4.

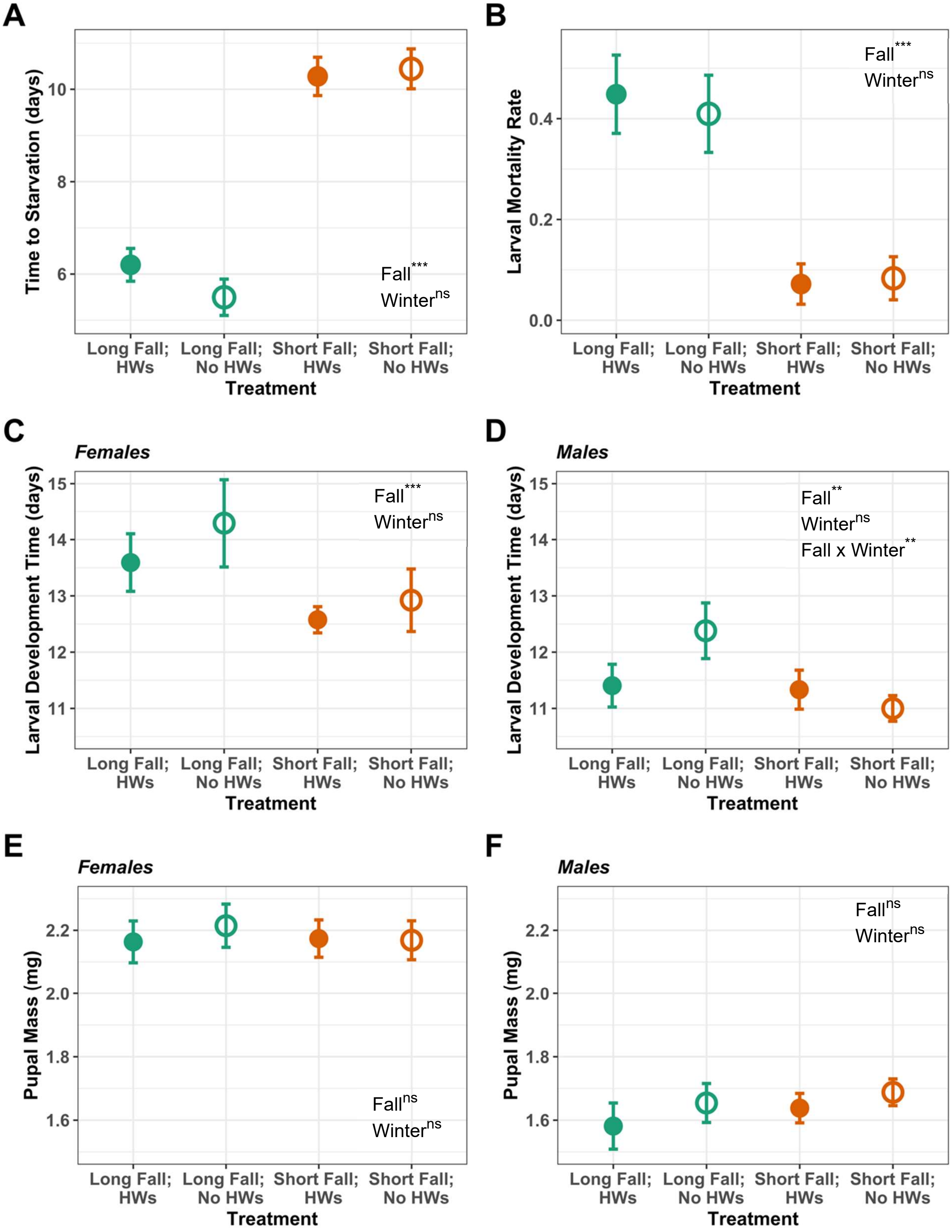

Figure 3 Longer, warmer falls (i.e., "Long Fall") resulted in negative post-diapause fitness effects, while the inclusion of winter heatwaves (i.e., "HWs") had minimal effects. Traits measured include: (A) time to starvation; (B) larval mortality rates; (C) female larval development time; (D) male larval development time; (E) female pupal mass; and (F) male pupal mass. Symbols and conventions as in Figure 2. * indicates $P < 0.05$, ** indicates $P < 0.01$

4. Discussion

Many important arthropod pests and pollinators rely on diapause to successfully overwinter in temperate environments (Denlinger 2022). However, it remains difficult to predict the impact of warmer fall and winter conditions resulting from climate change on the overwintering success of these arthropods. Here, we investigated how heat exposure affects the overwinter survival and post-diapause fitness of diapausing arthropods by exposing embryos of *Ae. albopictus* to treatments differing in (1) fall duration and temperatures and (2) winter heatwaves.

4.1. Overwinter survival was unaffected by fall treatment but improved in response to winter heatwaves

We did not find any effect of fall treatment on overwinter survival (Figure 2). This result was counter to our expectation that a longer, warmer fall would cause greater overwinter mortality due to energy drain when compared to the short-fall treatment. Survival at the embryonic stage during diapause likely requires relatively little energy expenditure compared to larval or adult stage diapausing arthropods. Thus, any effect of fall treatment on energetic reserves might not have been discernable based on hatching rates alone.

In contrast, three simulated four-day winter heatwaves during a 109-day winter period resulted in approximately 10% higher overwinter survival, regardless of whether diapause embryos had been exposed to a long or short-fall (Figure 2). Only a handful of studies have investigated the impact of winter heatwaves on diapausing arthropods. These studies found that winter heatwaves resulted in decreased survival, either through possible thermally induced energy drain (Abarca et al. 2019) or through loss of cold tolerance (Šlachta et al. 2002; Sobek-Swant et al. 2012; Williams et al. 2014).

It is possible that in the current study we did not observe heatwave-induced mortality because *Ae. albopictus* undergoes diapause at an immobile stage and is therefore potentially less vulnerable to energy drain than mobile larval or adult diapausing arthropods (Williams et al. 2012; Abarca et al. 2019). Arthropods that diapause at mobile stages may be more prone to energy drain either due to the occurrence of incidental movement during warm temperatures (Abarca et al. 2019) or because mobile stages exhibit an inherently less severe metabolic suppression than immobile stage diapausing arthropods (Williams et al. 2012). Our results also contrast with the expectation that winter heatwaves increase overwinter mortality through loss of cold tolerance, though it is possible that our heatwave exposure or cold shock was not severe enough to induce such an effect.

There are several possible reasons that winter heatwaves increased overwinter survival in *Ae. albopictus*. Prolonged exposure to cold temperatures can cause injury in arthropods, but these injuries can be repaired during brief, periodic exposure to warmer temperatures (on the scale of hours), leading to improved survival of individuals exposed to fluctuating cold temperature regimes compared to those at constant cold regimes (Renault et al. 2004; Colinet et al. 2006; Koštál et al. 2007; Lalouette et al. 2011). Thus, it is possible that winter heatwaves may provide an opportunity for *Ae. albopictus* to process metabolic waste and undergo repair of damage accumulated during cold exposure. An alternative explanation is that winter heatwaves increased overwinter survival simply by shifting overwintering temperatures closer to the thermal optimum of this species. *Aedes albopictus* it is thought to have originated in tropical Southeast Asia and then expanded to temperate areas in both its native and invasive ranges (Porretta et al. 2012). Although few studies have simultaneously assessed multiple insect species' sensitivities to winter warming, those which have suggest more southernly- or widely-distributed species may be less vulnerable to thermally-induced energy drain during overwintering when compared to species with more restricted, northern distributions (Williams et al. 2003; Mercader and Scriber 2008; Williams et al. 2012). Thus, it is possible that *Ae. albopictus* may be inherently more vulnerable to cold than warm temperatures because it is an ancestrally tropical species that has expanded into temperate ranges.

4.2. Post-diapause fitness was reduced following exposure to longer, warmer fall, with only minimal effects of winter heatwaves

While the longer, warmer fall treatment did not affect overwinter survival, it did have several carry-over effects on post-diapause fitness. Specifically, embryos exposed to a longer, warmer fall exhibited lower larval starvation tolerance (Figure 3A), greater larval mortality (Figure 3B), and longer larval developmental times (Figure 3C,3D). One possible interpretation of these results is that a longer, warmer fall caused energy drain that did not result in observable fitness consequences until after hatching into a more active larval state. Lower larval starvation tolerance observed following longer, warmer fall (Figure 3A) is likely indicative of energy drain because larvae in sterile water are solely reliant on post-diapause energy reserves to survive. Moreover, larvae of the long-fall treatment might have been forced to extend their development time (Figure 3C,3D) to compensate for lower post-diapause energy reserves. The interpretation that a warmer, longer fall reduced energy stores in diapausing *Ae. albopictus* embryos is consistent with studies in other diapausing species. Greater fall temperature and duration led to reduced energy reserves in diapausing larvae of *Choristoneura fumiferana* (Han and Bauce 1998) and greater weight loss in diapausing pupae of *Pieris napi* (Nielsen et al. 2022), diapausing adult *Osmia cornuta* (Bosch and Kemp 2004), and diapausing pupae of *Hyphantria cunea* (Gomi 2000).

An additional, but not necessarily mutually exclusive explanation for these post-diapause negative fitness effects is that a longer, warmer fall led to an accumulation of cellular damage (e.g., oxidative stress, aggregation of misfolding proteins) that did not impede successful hatching but did impact post-diapause survival and development. Some species exhibit slower developmental rates following cold stress during diapause (Turnock et al. 1985) or following acute heat shock outside of diapause (Yocum et al. 1994), likely to repair thermally-induced cellular damage. Additionally, delayed mortality has been observed following cold shock of insects in diapause (Turnock et al. 1983; Turnock et al. 1985; Bale et al. 1989; Marshall and Sinclair 2015; Štětina et al. 2018) and prolonged heat stress of insects in diapause (Nielsen et al. 2022). This delayed mortality is generally thought to result from an inability to repair damage incurred during thermal stress (Koštál et al. 2019). Thus, it is possible that in this experiment, stress associated with a warmer, longer fall led to delayed mortality, and in surviving individuals, necessitated a longer post-diapause development period to accommodate repairs. Regardless of the mechanism underlying these carry-over effects, our finding that fall conditions did not impact overwinter survival (Figure 2) but did cause delayed effects on post-diapause larval mortality (Figure 3B) and larval development time (Figure 3C,3D) emphasizes that consideration of post-diapause performance is necessary to accurately assess overwintering fitness following thermal stress.

We did not detect an effect of fall treatment on post-diapause pupal mass (Figure 3E,3F). This result suggests that under near-optimal post-diapause rearing conditions, it is possible for surviving larvae to compensate for the effects of a longer, warmer fall. Pupal mass is an accurate predictor of adult female fecundity in this species (Armbruster and Hutchinson 2002); thus, it is likely that surviving females from all treatments would have achieved comparable adult fecundity. Some authors have suggested that species which can feed after diapause are less likely to suffer post-diapause fitness consequences following warmer fall or winter conditions because they can compensate for thermally-induced energy drain (Tauber and Tauber 1986; Irwin and Lee 2000; Williams et al. 2015). Thus, post-diapause feeding may have allowed surviving *Ae. albopictus* larvae from the long-fall treatment to ultimately achieve equivalent potential fecundity as those exposed to short fall conditions.

Our fall treatments were designed to approximate natural conditions for our study species, where earlier diapause induction leads to both longer fall duration and warmer fall temperatures. As a result, we cannot determine which of these factors (i.e., fall duration or fall temperatures) caused the observed negative carry-over effects on post-diapause fitness. Only one study has simultaneously investigated the effects of pre-winter temperature versus duration on fitness of diapausing insects (Nielsen et al. 2022). Nielsen et al. (2022) found that longer pre-winter duration led to greater weight loss and post-winter mortality in diapausing *P. napi* pupae, and this effect was exaggerated at higher temperatures due to thermally-induced metabolic upregulation. These results suggests that both factors can play an important role during diapause. Regardless, climate change is expected to increase both fall temperatures and length (Kunkel et al. 2004; Linderholm 2006; Liu et al. 2006; Liu et al. 2018), so it is important to investigate the simultaneous effects of these two factors.

Despite the strong effect of winter heatwaves on overwinter survival, winter treatment had only a slight impact on post-diapause fitness traits. We observed a significant interaction between fall and winter treatment on male development time (Figure 3D). Males that had experienced a long-fall with no winter heatwaves exhibited significantly longer larval development time compared to the other three treatment groups (Figure 3D). As described above, stressful thermal conditions during diapause can result in slower post-diapause development, likely to allow time for repairs (Turnock et al. 1985). Thus, the increased cold exposure associated with the no-heatwave treatment could have caused additional damage which only had observable effects on development time if it was preceded by a more stressful long fall treatment.

4.5. Implications for the response of Ae. albopictus to climate change

Our results indicate that winter warming caused by climate change will likely lead to expansion of *Ae. albopictus* populations on both spatial and temporal scales. Our finding that winter heatwaves increased overwinter survival of diapausing *Ae. albopictus* embryos suggests that this species may be able to expand further northwards as climate change increases the frequency of such extreme events (Liu et al. 2006; Hansen et al. 2012). Many models of *Ae. albopictus* abundance and habitat suitability do not consider thermal variability created by winter heatwaves when selecting their climate variables, instead focusing on more broad-scale variables such as annual mean minimum temperature or mean temperature of the coldest quarter (Benedict et al. 2007; Medley 2010; Rochlin et al. 2013). Our results stress that this may cause model predictions of habitat suitability to underestimate the ability for this species to expand to higher latitudes. Even if average or minimum winter temperatures remain low, an increased frequency of short periodic warm spells could reduce cold-induced mortality if they permit repair of cold injury. Climate change has also led to increases in the length of the growing season in many areas (Kunkel et al. 2004; Linderholm 2006; Liu et al. 2006; Liu et al. 2018). As this trend continues, it will likely create strong selective pressure on *Ae. albopictus* to evolve a delayed seasonal entry into diapause due to the negative post-diapause fitness impacts associated with a longer, warmer pre-winter period. Such an evolutionary shift has already been observed in another mosquito species, *Wyeomyia smithii* (Bradshaw and Holzapfel 2001), and other multivoltine diapausing species (Gallinat et al. 2015). Epidemiological modeling of chikungunya virus (CHIKV) dynamics in the United States shows that, in locations with high thermal seasonality, the time of year with the greatest epidemic risk following CHIKV introduction aligned closely with the timing of active *Ae. albopictus* growth and reproduction (Ruiz-Moreno et al. 2012). Thus, temporal expansion of the mosquito's active season is likely to result in prolonged epidemic risk for CHIKV and other vector-transmitted viruses.

4.6. Conclusions

It remains challenging to predict the response of diapausing species to warmer overwintering temperatures due to the limited number of existing studies across species as well as the observed variability in responses. Here, we found that the important vector species, *Ae. albopictus*, exhibited a complex response to heat exposure during fall and winter. Exposure to winter heatwaves increased overwinter survival of diapausing embryos, likely because winter heatwaves allow diapausing embryos to escape and/or repair cold stress. In contrast, exposure to longer, warmer fall conditions led to lower post-diapause starvation tolerance, longer larval development, and increased larval mortality, which could have been caused by energy drain or the accumulation of cellular damage. Ultimately, the development of a more generalizable predictive framework for assessing winter climate change vulnerability may be possible through further studies on the relationship between key life history traits (e.g., metabolic thermal sensitivity, stage at which diapause occurs, the capacity for post-diapause feeding, range breadth) and vulnerability to winter warming.

Our finding that fall conditions did not impact immediate hatching success but did cause carry-over effects on post-diapause starvation tolerance, larval mortality, and larval development time emphasizes that consideration of post-diapause fitness effects can radically change assessments of overwintering success following thermal stress. It is crucial that studies consider these carry-over effects to avoid underestimating species' sensitivity to thermal stress. For example, delayed life history effects can lead to changes in population dynamics (Beckerman et al. 2002) and disease transmission (Evans et al. 2021), meaning that failure to consider these effects may result in inaccurate modeling of population-level responses to climate change and shifts in vector-borne disease risk.

Finally, while both the long-fall treatment and winter heatwaves treatment involved increasing exposure to warmer temperatures, they had contrasting effects on overwintering fitness. This result challenges the assumption that warmer conditions will be uniformly good or bad for a given species. Thus, to determine the net fitness impact of warmer overwintering conditions, it is critical to simultaneously assess the effects of different aspects of climate change.

Declaration of Competing Interests: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CRediT Authorship Contribution Statement: **Samantha L. Sturiale**: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Roles/Writing – original draft; Writing - review & editing. **Peter A. Armbruster**: Conceptualization, Funding acquisition, Methodology; Project administration, Resources, Supervision, Visualization, Writing - review & editing.

Data Availability: All data on overwinter survival, post-diapause starvation tolerance, post-diapause larval mortality, post-diapause larval development, pupal mass, and heatwave occurrence from 2011-2020 in the VA-MD-DC area are available in a single Excel file (see Supplementary File 1). The python script used to compute heatwave data with which we parameterized our winter heatwave treatments is available as Supplementary File 2. The R code used to conduct statistical analyses and produce plots is available as Supplementary File 3.

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