Evidence of thermal adaptation in a widely distributed mosquito species

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Abstract

Whether mosquitoes can adapt apace with rapid climate warming will have a large impact on their future distributions, and consequently those of mosquito-borne diseases, but remains unknown for most species. We investigated the adaptive potential of a wide-ranging mosquito species, *Aedes sierrensis*, by conducting a common garden experiment measuring mosquito fitness and its component life history traits. Although field-collected populations originated from vastly different thermal environments that spanned over 1,200 km, we found that populations varied in maximum fitness, but not in the thermal performance of fitness, with upper thermal limits varying by <1°C across the species range. However, for one life history trait – pupal development rate – we found clear evidence of local thermal adaptation. The upper thermal limits of pupal development rate varied between populations by 1.6°C – five times greater than the average variation in ectotherm upper limits across the same latitudinal extent – and was significantly associated with source temperatures. Despite this evidence of local thermal adaptation, we found that for all populations, temperatures in the source environment already frequently exceed their estimated upper thermal limits, suggesting high vulnerability to additional warming. This was particularly true at the adult life stage, which had the lowest upper thermal limits across traits (31.6°C), the largest impact on mosquito fitness, and occurs during the warmest part of the year. Our results suggest that evolutionary adaptation alone may be insufficient to sustain mosquito populations, and that behavioral thermoregulation and temporary coping strategies are likely important for mosquito persistence under ongoing climate warming.

Introduction

How mosquitoes respond in the face of rapid anthropogenic climate warming is a key open question of ecological and public health concern. Mosquito-borne diseases currently threaten over half the global population, causing over 500 million cases and one million deaths each year[1]. As temperature impacts nearly all aspects of mosquito life cycles, climate warming may cause large shifts in their distributions and dynamics[2,3]. Correspondingly, current predictions suggest that mosquito-borne disease distributions may shift higher in latitude and elevation, expanding into temperate regions as they become newly suitable for transmission, and contracting in some tropical regions as they become too warm[4–7]. However, these predictions have not typically incorporated the potential for adaptive mosquito responses, and thus may overestimate declines in disease at current warm edges.

Temperature sets fundamental limits on mosquito-borne disease distributions as mosquito survival and reproduction is inhibited beyond critical thermal limits. As temperatures exceed those limits under warming, mosquito populations could persist through a variety of mechanisms including range shifts to track suitable temperatures, behavioral changes to temporarily avoid or cope with high temperatures, and increased heat tolerance through evolutionary adaptation[8]. Of these responses, evolutionary adaptation may be particularly important for enabling long-term

persistence, but the potential for mosquito thermal adaptation remains poorly understood, owing to several empirical knowledge gaps[9,10].

A key component of whether a given mosquito species can evolutionarily adapt to warming is the presence of standing variation in thermal tolerance. Several studies have investigated variation in mosquito thermal tolerance for species including *Culex pipiens*[11], *Cx. tarsalis*[12,13], *Nyssorhynchus* (*Anopheles*) *darlingi*[14], and *Wyeomyia smithii*[15]. Using common garden lab experiments, these studies have generally found some evidence of standing variation in thermal tolerance (*i.e.*, differing rates of survival, reproduction, or development among populations at high temperatures), but little evidence of local *thermal adaptation* (*i.e.*, higher heat tolerance observed in populations from warmer environments than those from cooler environments). However, these studies typically investigated relatively few mosquito populations from a limited portion of the species range, owing to logistical challenges of collecting, rearing, and experimenting on many wide-ranging populations. Further, mosquito thermal tolerance was typically measured on select life history traits or metabolic rates as proxies for fitness—the ultimate target of natural selection—potentially obscuring patterns of thermal adaptation[10,16,17]. Thus, the extent of variation in thermal tolerance within a species and the evidence for thermal adaptation is still unknown.

We set out to rigorously investigate the evidence for mosquito thermal adaptation by using *Aedes sierrensis*, the western tree hole mosquito, as a novel model system. *Ae. sierrensis* makes an ideal model species for this investigation because its distribution (ranging from Southern California to British Columbia and coastal to montane environments[18,19]) covers a large range of thermal environments, presenting varying selection pressures and opportunities for local thermal adaptation. This species is also highly amenable to field collection and lab manipulation because it is commonly occurring, breeds in a discrete, easy-to-sample habitat, and is not a known vector of human disease. Further, *Ae. sierrensis* is congeneric to major human disease vectors, making results likely informative for understanding these vector species. Leveraging this model system, we set out to answer the following specific research questions: (i) How much does mosquito thermal performance vary across the species range? (ii) How does thermal performance vary between life history traits and which life history traits have the largest impact on mosquito fitness? (iii) How does variation in thermal tolerance, if observed, relate to aspects of the source thermal environment (*i.e.*, is there evidence of local thermal adaptation, and how close are species to their thermal limits)?

To answer these questions, we conducted a common garden experiment using ten *Ae. sierrensis* populations spanning nearly the entire species range (1,200 km; Figure 1). The thermal environments of collected populations varied widely, with annual mean temperatures varying by >7°C, and daily maxima in the spring and summer varying by >5°C. We reared these field-collected populations in the lab for one generation at common temperatures, then separated F1 individuals into one of six temperature treatments ranging from 5-32□. We tracked individuals daily to estimate individual-level fitness, as well as its component life history traits—larval and pupal survival and development rates, adult lifespan, and fecundity. We then fit thermal

performance curves to this experimental data to estimate upper and lower critical thermal limits, thermal optima and breadth, and maximum performance for each population and trait.

We found minimal variation between populations in the thermal performance of fitness, but substantial variation in maximum fitness, with the population from the warmest environment achieving the highest fitness at nearly all temperatures. Further, we found between-population variation in the thermal performance of pupal development rates that was associated with the source thermal environment, providing some of the clearest evidence to date of mosquito thermal adaptation. Despite this, temperatures at all source environments frequently exceeded our highest estimated upper thermal limits, suggesting that evolutionary adaptation alone may be insufficient for mosquito thermal tolerance to keep pace with rapid climate warming. Temporarily coping with and/or behaviorally avoiding high temperatures are likely important mechanisms for mosquito persistence.

Methods

Field collection

Ae. sierrensis typically completes one life cycle per year, with adults laying eggs in naturally occurring tree holes. Eggs hatch when the tree holes fill with water beginning in the late fall and advance through four larval instars and one pupal life stage throughout the winter before eclosing as adults in the spring and summer[18]. We collected larval *Ae. sierrensis* from 346 tree holes spanning over 1,200 km across the Western U.S. between October 2021 and April 2022 (Figure 1, Supplemental Table S1 for collection metadata). We collected *Ae. sierrensis* and tree hole water in plastic cups and maintained these at cold temperatures (< 10°C) during transportation to the lab, then at 4□ until processing. We visually inspected individuals from each sampled tree hole for the presence of *Lambornella clarki*—a ciliate parasite that can infect larval *Ae. sierrensis*. Only larvae from tree holes without the parasite were used in this experiment. Further, to maintain sufficient genetic variation and avoid excessive inbreeding, we reared only larvae from tree holes with at least 30 collected individuals.

Lab rearing

After processing, we maintained select populations (*i.e.*, those from tree holes with ≥30 individuals and no *L. clarki*) under shared lab conditions of 21.5□, and a 13 h: 11 h light:dark cycle. We periodically fed larvae a finely-ground mix of Tetramin fish flakes (48% by weight), guinea pig chow (48%), and liver powder (2%). Once reaching the adult stage, we housed populations in 8 x 8 x 8 cm aluminum collapsible cages (BioQuip, Rancho Dominguez, CA, USA) with continuous access to a 10% sugar solution. We offered each population a blood meal of defibrinated sheep's blood approximately once per week and placed an oviposition cup, consisting of a paper cup lined with water-soaked coffee filter paper, inside each cage within four days of the first blood-feeding. We collected eggs and held these at room temperature for two weeks, then in the refrigerator at 4□ and near 24 h darkness until hatching (pers. comm. Bret

Barner, Solano County vector control), which occurred 1-3 months later. To ensure sufficient sample sizes for each treatment of the experiment, we only used populations that produced >300 eggs in total. This resulted in 10 populations for use in the experiment (Figure 1), wherein 'population' refers to a group of individuals originating from the same tree hole. We note that a more precise definition of a population would incorporate dispersal capabilities and/or genetic structuring, but this has not yet been investigated for *Ae. sierrensis*.

To hatch eggs, we prepared a separate tray for each population, which consisted of 500 mL Arrowhead distilled water, 300 mL autoclaved tree hole water (combined from all sampled tree holes), and ¾ tsp Brewers' yeast. We submerged egg papers from each population in trays between July 4 - 6, 2022, 24 h after the respective hatching tray was prepared.

Experimental design

The experiment consisted of tracking life histories for individual *Ae. sierrensis* from one of ten populations, held at one of six temperature treatments (Figure 1; see Supplemental Table S2 for sample sizes). The temperature treatments–5, 13, 17, 24, 28, and 32□–were chosen based on the range of temperatures realistically experienced by *Ae. sierrensis* in the field and based on survival rates assessed during pilot experiments conducted in the lab. These constant temperatures were maintained using Fisher Scientific Isotemp incubators (for the 13, 24, 28, and 32□ treatments) and climate-controlled rooms (for the 5 and 17□ treatments). Although fluctuating temperatures could have more closely mimicked natural conditions, we chose to use constant temperatures here as it provides a baseline for characterizing thermal responses and because measuring all possible combinations of temperature mean and variability would have been intractable.

The experiment began with larvae emerging 48 h after egg paper submersion (*i.e.*, approximately 1-day old larvae). For each individual, we measured the following traits: larval survival, larval development rate, pupal survival, pupal development rate, adult lifespan, and estimated fecundity (see *Methods: Fecundity estimation*). We visually inspected each individual on a daily basis, recording life stage transitions and deaths, and moving individuals into the appropriate housing for the given life stage. We maintained larvae in plastic containers in groups of five with approximately 100 mL of water and 4 mg of larval food, in accordance with *Aedes* rearing protocols that promote high larval survivorship in the absence of other factors[20,21]. We maintained pupae individually in glass vials with approximately 5 mL deionized water. Upon eclosion, we transferred adults to individual 4 oz plastic specimen cups with one 10% sugar-soaked cotton ball and observed each individual until death. To estimate fecundity of individuals that died as adults, we removed and measured the length of their left wing, a commonly used proxy[22,23] (see *Methods: Fecundity estimation*). Any larva that was alive but had not pupated by September 28, 2022 (*i.e.*, 82-84 days after larval emergence) was counted as survived for the larval survival trait and considered to be in diapause.

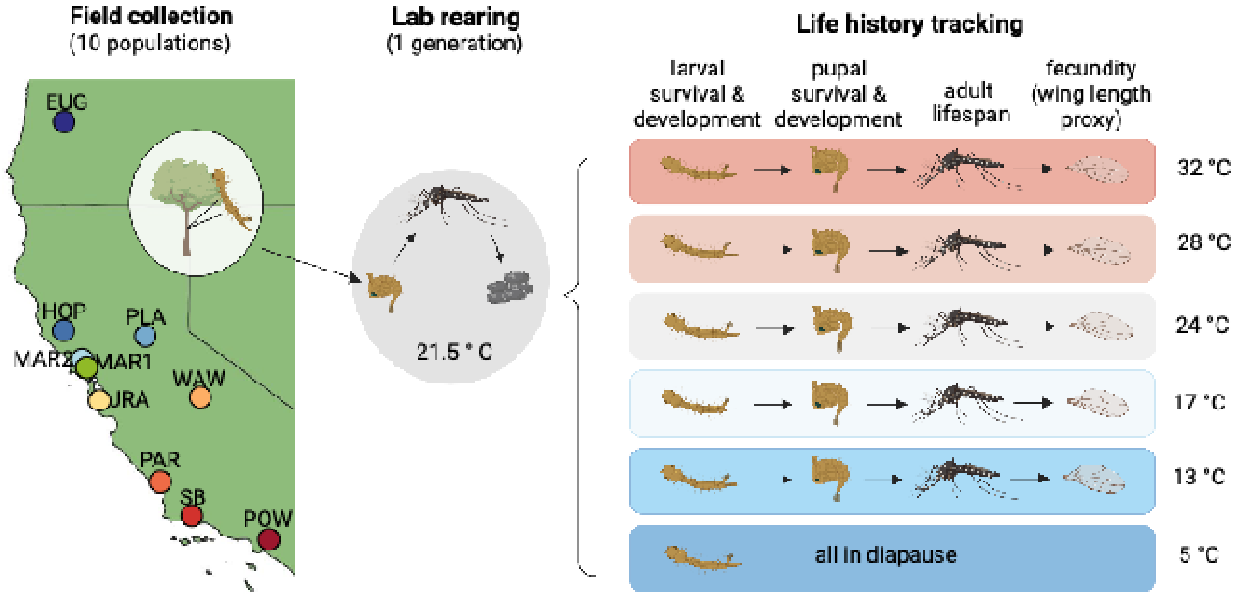

Figure 1. Sample collection locations and experimental design used to measure mosquito thermal performance. Ten populations were collected as larvae from tree holes across the Western U.S. and reared in the lab under common conditions for one generation. The resulting larvae from each population were randomly designated into one of six temperature treatments. Individuals were checked daily for life stage transitions (*e.g.*, larvae to pupae, pupae to adult) or death. Population metadata, including full site names, latitude, longitude, and elevation are provided in Supplemental Table S1.

Fecundity estimation

To estimate individual fecundity, we measured the wing length of each individual used in the experiment. We removed and photographed the left wing mounted on a microscope slide with a 1 mm graticule. We then used ImageJ to measure the wing length as the distance from the alular notch to the tip of the wing margin excluding the fringe scales, using the 1 mm graticule for calibration[24] (Supplemental Figure S1). We then used the relationship between *Ae. sierrensis* female wing length and the number of eggs laid in the first clutch established in Washburn et al. 1989[25] (*i.e.*, 51.33 x female wing length (mm) - 87.96). We validated this relationship in the lab using a separate, smaller number of individuals from our experimental populations (see Supplemental Methods). While the approximation is not perfect, it enabled us to obtain both a lifespan and fecundity estimate for each individual used in the experiment, whereas individually blood-feeding hundreds of mosquitoes held inside incubators would have been intractable.

Fitness estimation

We estimated individual-level mosquito fitness—here defined as individual reproductive output—as survival to reproductive maturity multiplied by estimated fecundity. For survival to reproductive maturity, we considered whether an individual survived to adulthood and achieved

an adult lifespan of 10 days at 24 or 28□, 11 days at 17□, or 17 days at 13□. These lifespans represent the minimum number of days from adult eclosion to egg-laying at a given temperature, as observed in the validation experiment (*Methods: Fecundity estimation*). As no individuals eclosed at 5□ and no individuals survived longer than one day at 32□, all individuals at these two temperature treatments were estimated to have zero fitness. For estimated fecundity, we used the wing length approximation described above (*Methods: Fecundity estimation*). As these estimates were made for both males and females, we multiplied the estimated fecundity of a given adult by the proportion of females from that population and temperature treatment.

Characterizing the source thermal environment

We characterized the source thermal environment of each population using climate data from PRISM, which we accessed and analyzed using Google Earth Engine[26]. PRISM provides gridded climate data at a 4 km resolution by downscaling data from a network of monitoring stations[27]. We used either daily or monthly temperature data from 2000 – 2020 to calculate key variables capturing temperature means, variations, and extremes. We specifically sought to include only biologically meaningful temperature variables, such as those previously associated with thermal tolerance in ectotherms[28], rather than many possible characterizations of climate (*e.g.*, all 19 WorldClim bioclimatic variables). These variables included annual mean temperature, mean temperature in January – March (the period when eggs typically exit diapause and hatch as larvae), seasonal variation in temperature (defined as the difference between the mean warmest month temperature and the mean coolest month temperature), average warm-season maximum (defined as the mean daily maxima in the Spring and Summer), and the number of days exceeding 35□ (the highest upper thermal limit for any trait estimated from our experimental data). Variables were calculated at a 1 km buffer around the sampled tree hole for each population. We investigated correlations between these temperature variables and select thermal performance parameters and traits (*i.e.*, those with significant between-population variation). To qualitatively understand how the thermal maximum for adult mosquitoes compares to environmental conditions experienced, we also calculated the number of days exceeding 31.6□ during the adult activity period (roughly March – September) as this was the estimated upper thermal limit for adult lifespan, and the lowest limit for any trait. However, we did not investigate correlations between this environment variable and thermal performance characteristics to minimize multiple testing.

Analysis: Fitting thermal response curves

To estimate the thermal limits and performance characteristics of each trait and population, we fit thermal response curves to the experimental data using a Bayesian approach following methods described in detail in Shocket et al. 2020[29]. We first visually inspected the temperature-performance data to determine the most appropriate functional form of the thermal response for each trait. Consistent with prior work, we used quadratic fits truncated to a maximum of 1 for

larval and pupal survival, quadratic fits for adult lifespan, and Brière fits for larval and pupal development rate and fitness[29,30] (Supplemental Table S3).

We fit a first set of Bayesian models for each combination of trait and population across temperatures using uniform priors for the thermal limit parameters bounded by biologically plausible temperature cut-offs (*i.e.*, trait performance was set to zero below 0□ and above 40-45□ depending on the trait; Supplemental Table S3). For larval and pupal development rate, adult lifespan, and fitness, we modeled the observed data as normally distributed with the mean predicted by the thermal response function at that temperature and the standard deviation, σ, as a gamma distributed parameter, $\frac{1}{\sigma^2}$, with shape parameter $\alpha = 0$ and rate parameter $\beta = 1000$. For larval and pupal survival probabilities, we modeled the observed data as binomially distributed with the probability and number of trials based on the proportional survival and sample size for that temperature – population combination. We truncated thermal response functions at zero for all traits, as well as at one for survival probability traits. We fit models using Markov Chain Monte Carlo (MCMC) sampling, which uses simulation to approximate the posterior distribution, using the 'R2jags' package[31]. For each thermal response, we ran three independent chains with a 5,000-iteration burn-in, and thinned the chains by saving every eighth iteration. This fitting process produced 7,500 values in the posterior distribution for each parameter of the thermal response function (*i.e.*, $\mathrm{CT_{min}}$, $\mathrm{CT_{max}}$ and q) and enabled us to calculate additional derived quantities for each trait and population including the maximum trait performance value ($\mathrm{P_{max}}$), the temperature at maximum performance ($\mathrm{T_{opt}}$), and the temperature range where performance is at least 50% of the maximum ($\mathrm{T_{breadth}}$; See Supplemental Figure S3 for theoretical thermal performance curve). We refer to the above fitting process as our 'low information' model specification.

To reduce the uncertainty in our parameter estimates, we then fit a second set of models—the main models presented in the text—using informative priors generated using a two-step process. In the first step, we specified low information priors as described above for each population and trait but using only the temperature-performance data from the other nine populations (*i.e.*, a 'leave-one-out' approach[29]). We fit a Gamma probability distribution to the posterior distributions of each thermal response parameter using the 'MASS' package[32]. We then used these hyperparameters as informative priors in a second round of model fitting. To ensure the hyperparameters did not have an outsized influence on the resulting posterior distributions, we increased the variance of the priors through multiplication by a constant k, set at 0.1 or 0.01, depending on the trait (Supplemental Table S3). The parameter estimates from this 'informative' model specification are presented as the main results in the text but did not differ qualitatively from those made through the 'low information' model specification presented in the supplement. When investigating variation in thermal performance parameters, we interpreted non-overlapping credible intervals as biologically meaningful and statistically supported differences between populations and/or traits. It is worth noting that the leave-one-out informative prior approach biases our thermal performance curve fits to be more similar across populations, making the resulting estimates of differences among populations conservative. On the other hand, this

approach has the advantage of realistically constraining uncertainty, for example in cases where a trait was poorly quantified at a given temperature (*i.e.*, few individuals in a given population survived to the relevant life stage).

Analysis: Impact of life history traits on fitness

To determine which life history trait(s) had the largest impact on mosquito fitness, we used a linear mixed-effects model implemented using the 'lme4' package in R[33]. We included life history traits (*i.e.*, larval survival and development rate, pupal survival and developmental rate, adult lifespan, and estimated fecundity) as fixed effects and temperature treatment as a dummy variable (*i.e.*, a categorical fixed effect). Variation between populations was modeled using a random intercept. We considered two metrics of fitness as our outcome variable: (i) the average fitness per individual, and (ii) the proportion of individuals with positive fitness. In both models, variables were included as population averages for each temperature treatment, rather than as individual-level values to reduce multicollinearity between traits and given the zero-inflated fitness data. Life history trait values were converted to z-scores prior to modeling to make coefficient estimates between traits directly comparable. Coefficient estimates thus reflect the effect of a one standard deviation change in a given life history trait on the standard deviational change in fitness, after controlling for the direct effect of temperature on fitness.

Results

How much does mosquito thermal performance vary across the species range?

To investigate patterns of mosquito thermal adaptation, we assessed the thermal performance of fitness across 10 populations. We characterized the thermal performance of fitness for each population by fitting thermal response curves (Figure 2) to our experimental data and estimating critical thermal limits, thermal optima and breadth, and maximum performance. We found that populations collected across the species range did not vary in their upper or lower critical thermal limits, thermal optima, or thermal breadth of fitness (Figure 3). Both upper thermal limits and thermal optima varied by <1□ across all populations, ranging from 27.8 – 28.4□ and 22.4 – 23.1□, respectively. Lower thermal limits displayed the greatest variation, ranging from 0.3 – 4.6□, and thermal breadth varied from 12.9 – 14.8□. These results were highly similar when using the low information model specification (Supplemental Figures S5-6).

While there was minimal between-population variation in the thermal tolerance of fitness, populations varied substantially in maximum fitness performance (Figures 2-3; Supplemental Figures S5-6), with population-average maximum fitness ranging from 2.2 – 10.1 offspring per individual. We found that populations with higher maximum fitness were typically those from lower latitudes, with the population from the lowest latitude ('POW') achieving the highest maximum fitness across all temperatures (Figure 2). In particular, this population had the highest proportion of individuals reaching adulthood and surviving to the date of potential oviposition (*i.e.*, achieving non-zero fitness, Supplemental Figure S7), which was the major bottleneck in this experiment: only 141 out of the 1,736 total individuals (8%) used in the experiment achieved

non-zero fitness. This low-latitude population also had the highest estimated fecundity of the surviving adults at nearly all temperatures (Supplemental Figure S7), suggesting the absence of trade-offs in survival and reproduction. We did not detect consistent correlations between fitness thermal performance characteristics (*i.e.*, between P_{max} and $T_{breadth}$ or between P_{max} and T_{opt}); this investigation and results are discussed further in the Supplemental Results.

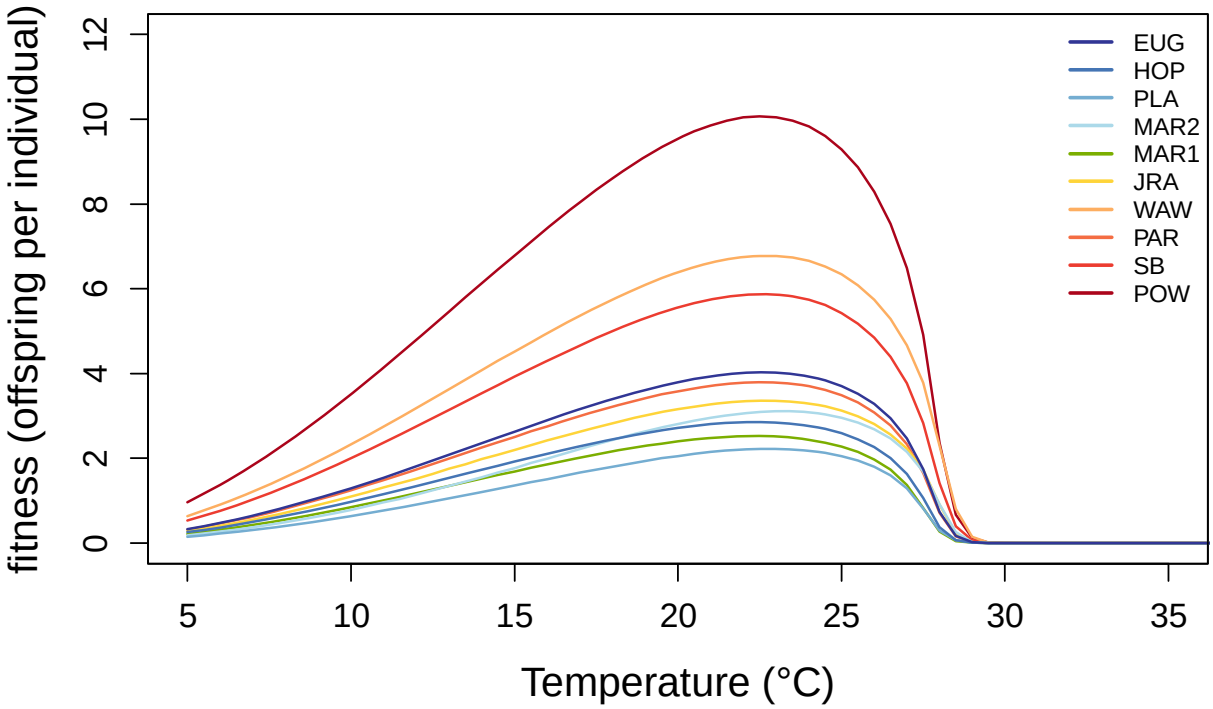

Figure 2. Populations vary in absolute fitness, but not in the thermal performance of fitness. Each line denotes the thermal performance of fitness for one population. Populations are colored and ordered in the legend by their latitude of collection from north (blue) to south (red); this color scheme and ordering is consistent across all figures in the paper.

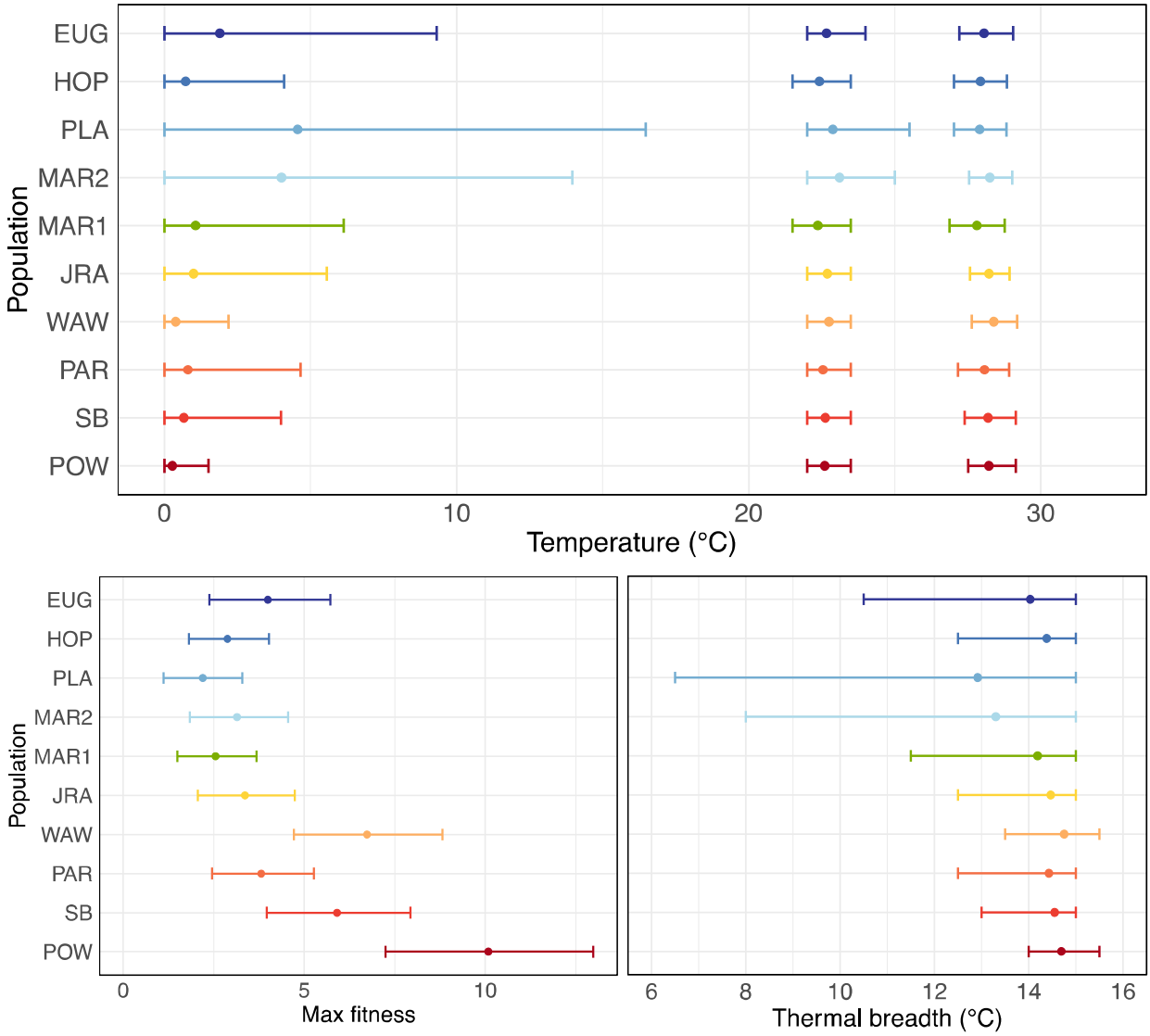

Figure 3. Populations vary in maximum performance (bottom left), but not in the thermal limits and optima (top), or breadth (bottom right) of fitness. Points denote estimated fitness thermal performance parameters for each population, including critical thermal minima, thermal optima, and critical thermal maxima (top), maximum fitness (bottom left), and thermal breadth (bottom right). Error bars denote the 95% credible intervals for each parameter. Populations (listed on the left) are colored and ordered by their latitude of collection.

How does thermal performance vary between life history traits?

To examine the impact of individual life history traits on mosquito fitness, we first investigated variation in the thermal performance of traits measured in this experiment (*i.e.*, larval and pupal survival and development rates, adult lifespan, and fecundity). As with fitness, we characterized thermal performance for each population by fitting thermal response curves for each life history trait, and estimating critical thermal limits, thermal optima and breadth, and maximum

performance (Figure 4, Supplemental Figures S8-13). We found substantial variation in the critical thermal limits and optima between traits (Figure 5, Supplemental Figure S14; Supplemental Tables S4-5): averaging across populations, the highest upper thermal limits occurred for fecundity (34.7°C) and larval development rate (34.0°C) and the lowest upper thermal limits occurred for adult lifespan (31.6°C). Larval development rate also had the highest thermal optimum (27.2°C), while larval survival had the lowest (14.3°C). Averaging across all populations, we found that thermal optima and lower thermal limits varied more between traits than did upper thermal limits. Specifically, the range in population-averaged thermal optima between traits (13.0°C) or thermal minima (7.0°C) was more than twice as large as that of thermal maxima (3.1°C; see Supplemental Table S4-5 for the specific values and traits for which these ranges correspond). This finding is consistent with patterns of thermotolerance in ectotherms[34,35] and may reflect the presence of shared physiological constraints on upper heat tolerance across traits.

We found significant variation between populations in a few specific life history traits and thermal performance characteristics: the upper thermal limits (CT_{max}) of larval and pupal development rates, and the thermal optima (T_{opt}) of pupal development rates. Upper thermal limits for larval and pupal development rates each varied by 1.6°C across populations (33.3 – 34.9°C and 32.1 – 33.7°C, respectively), while the thermal optima of pupal development rate varied by 1.4°C (26.3 – 27.7°C). For all other life history traits and thermal performance characteristics, variation between populations was not significant (*i.e.*, credible intervals overlapped for all populations).

Which life history traits have the largest impact on mosquito fitness?

When investigating the relative impacts of distinct life history traits on mosquito fitness across populations, we found that adult lifespan had the largest effect on average fitness (Table 1). This was true both when considering the average fitness per individual and the proportion of individuals achieving non-zero fitness (Table 1). Specifically, a one standard deviation increase in adult lifespan was associated with an approximately 1.3 standard deviation increase in average fitness per individual, or a 1.2 standard deviation increase in the proportion of individuals with non-zero fitness, indicating that adult lifespan had an outsize impact on fitness.

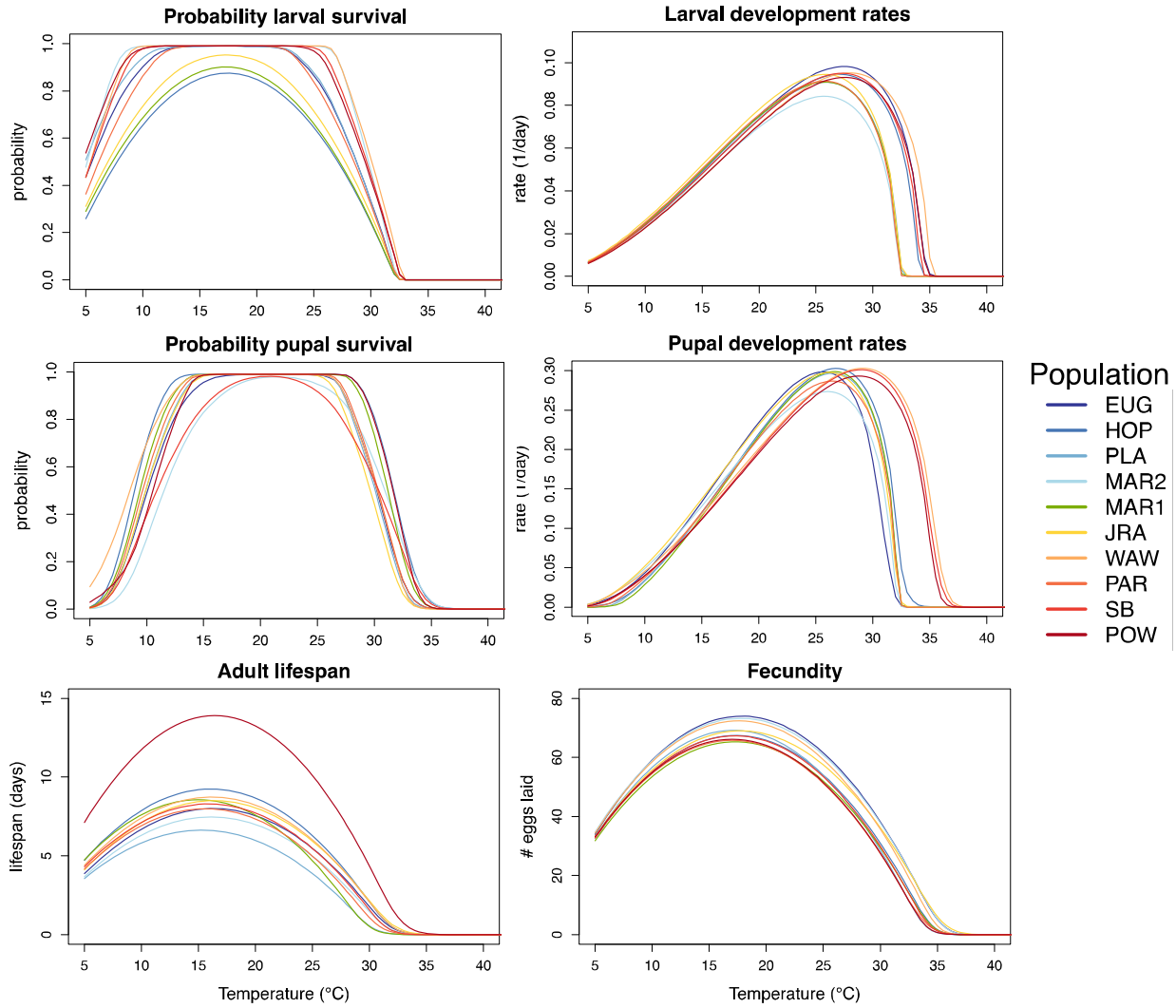

Figure 4. Thermal performance varies between life history traits, but minimally between populations for most traits. Each curve denotes the average thermal performance for one population for a given trait. Populations are colored and ordered in the legend by their latitude of collection.

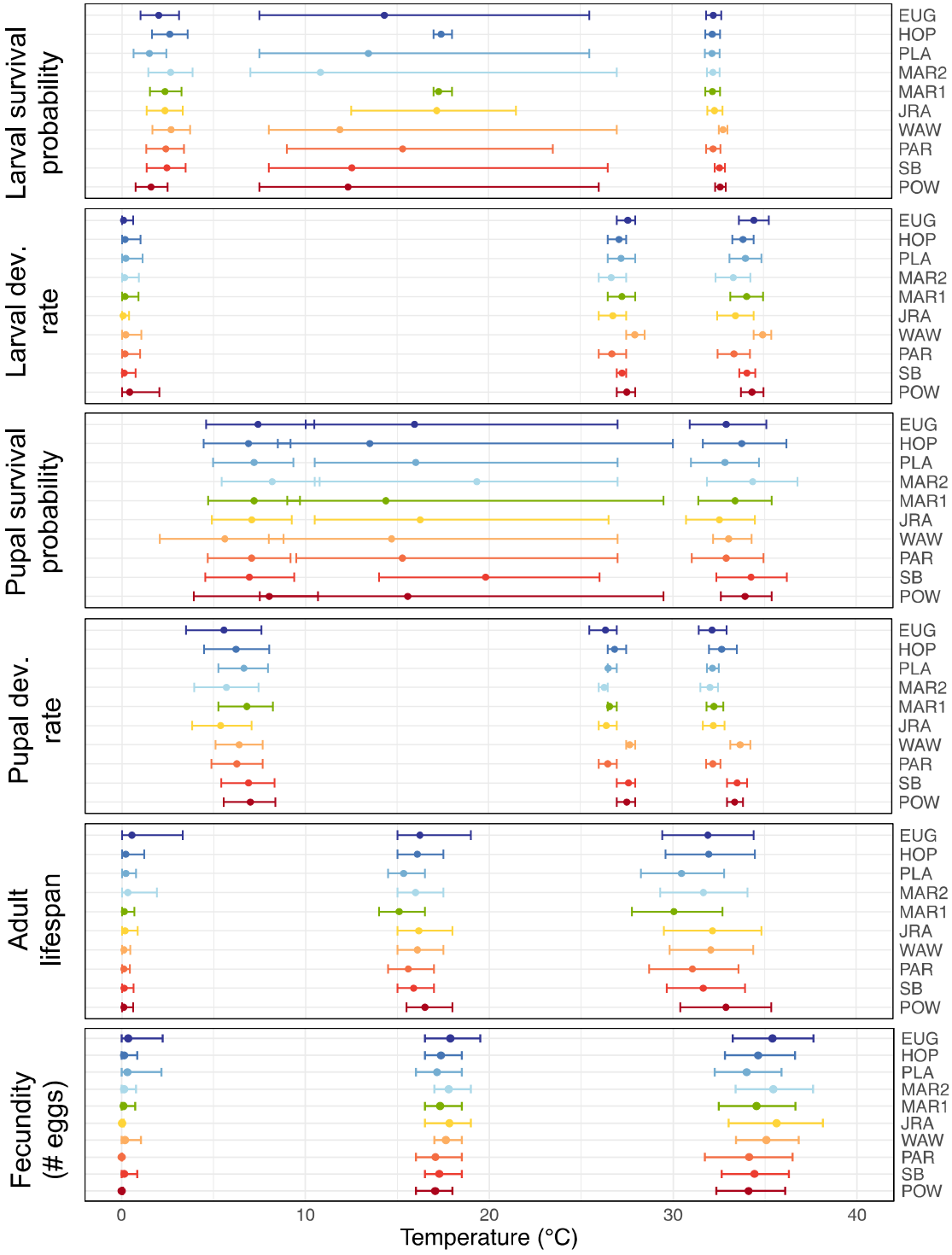

Figure 5. Thermal limits and optima vary between life history traits, but minimally between populations. Critical thermal minima, thermal optima, and critical thermal maxima for each life history trait and population (left, middle, and right points and error bars in each panel, respectively). Points and error bars denote the mean and 95% credible intervals for each parameter, respectively. Populations (listed on the right) are colored and ordered by latitude of collection. Units of development rates and lifespan are 1/days and days, respectively.

Table 1. Adult lifespan has the largest estimated impact on mosquito fitness. The scaled coefficient estimates ('Coef. estimate') shown here reflect the standard deviation change in fitness for a one standard deviation change in the life history trait. The coefficients are scaled so that the effects of different life history traits are directly comparable. Models are shown with average fitness per individual across all individuals (2nd and 3rd columns) and for the proportion of individuals with non-zero fitness (4th and 5th columns) as response variables. Statistically significant coefficients ($p < 0.05$, < 0.001), are denoted with (*) and (**), respectively.

	Fitness per individual		Proportion with non-zero fitness	
	Coef. estimate	Std. error	Coef. estimate	Std. error
Larval survival	0.777*	0.318	0.618*	0.247
Larval development rate	0.029	0.435	0.279	0.342
Pupal survival	0.090	0.302	0.539*	0.028
Pupal development rate	-0.030	0.486	-0.247	0.515
Adult lifespan	1.324**	0.140	1.240**	0.113
Fecundity	0.178	0.190	-0.277	0.147

Table 2. Thermal characteristics of the source environment for each population listed in order of decreasing latitude (*i.e.*, north to south). Values represent averages from 2000 – 2020, calculated from PRISM climate data at a 1 km buffer around the sampled tree hole. Seasonal temperature variation is defined as the difference between the mean warmest month temperature and the mean coolest month temperature. Warm-season maximum is defined as the mean daily maxima in the Spring and Summer.

Pop.	Annual mean temp. (°C)	Jan – March mean temp. (°C)	Seasonal temp. variation (°C)	Warm-season maximum (°C)	# days > 35°C	# days > 31.6°C
EUG	11.48	6.33	16.01	22.69	4.29	18.33
HOP	14.57	9.41	15.38	26.77	24.90	57.67
PLA	16.08	9.91	18.08	28.04	29.95	76.24
MAR2	14.24	10.31	11.62	24.77	8.71	25.95
MAR1	14.04	10.18	11.29	24.41	6.33	20.86
JRA	15.45	11.49	11.61	25.29	10.43	27.14
WAW	15.83	8.73	20.51	28.19	41.57	87.76
PAR	14.40	9.88	13.26	26.52	14.71	46.67
SB	16.44	11.82	13.51	27.86	26.81	59.95
POW	18.75	15.08	11.65	27.77	18.14	50.62

Is variation in thermal performance associated with the source thermal environment?

Field-collected mosquito populations originated from a wide range of thermal environments, with average annual mean temperatures ranging from 11.5 – 18.7 °C, seasonal temperature variation ranging from 11.3 – 20.5°C, and daily maxima in the Spring and Summer ranging from

22.7 – 28.2°C (Table 2). In general, mean temperatures were positively correlated with high temperature extremes and negatively correlated with seasonal temperature variation, but the strength of the correlations varied between 0.06 – 0.87 (Supplemental Figure S15), indicating that no single temperature variable holistically characterizes the source thermal environment. Further, we found that temperatures at all source environments frequently exceeded our estimated upper thermal limits. In particular, the number of days per year with temperatures exceeding 35°C—above the highest upper thermal limit we estimated for any life history trait—ranged from 4 to 42 days, while the number of days exceeding 31.6°C—the lowest estimated upper thermal limit (adult lifespan)—ranged from 18 to 88 days.

To assess evidence of local thermal adaptation, we investigated the relationship between the source thermal environment and experimentally measured thermal performance parameters, using only the parameters with biologically significant between-population variation (*i.e.*, some populations had non-overlapping 95% credible intervals). This included the upper thermal limits (CT_{max}) of larval and pupal development rates, and the thermal optima (T_{opt}) of pupal development rates. We found several correlations that reflected patterns of local thermal adaptation. In particular, we found that CT_{max} and T_{opt} of pupal development were positively correlated with annual mean temperature, maximum daily temperatures in the Spring and Summer, and the number of days exceeding 35°C (r: 0.64 – 0.71; Figure 6, Table 3). CT_{max} of larval development rate was not strongly correlated with any temperature variable. We note that these reported correlations are only statistically significant ($p < 0.05$) prior to adjustment for multiple comparisons, the necessity of which is debated when making only specific, biologically meaningful comparisons (as we have done here) rather than all possible comparisons[36,37].

We also found that maximum fitness (P_{max}), which varied significantly between populations, was positively correlated with annual mean temperature (r = 0.66; no correlations with other temperature variables were statistically significant). This result that warm-adapted populations have higher maximum fitness has frequently been found in other ectotherms[38–40], but does not necessarily reflect local thermal adaptation, in which peak fitness is expected to occur at conditions most similar to the source environment.

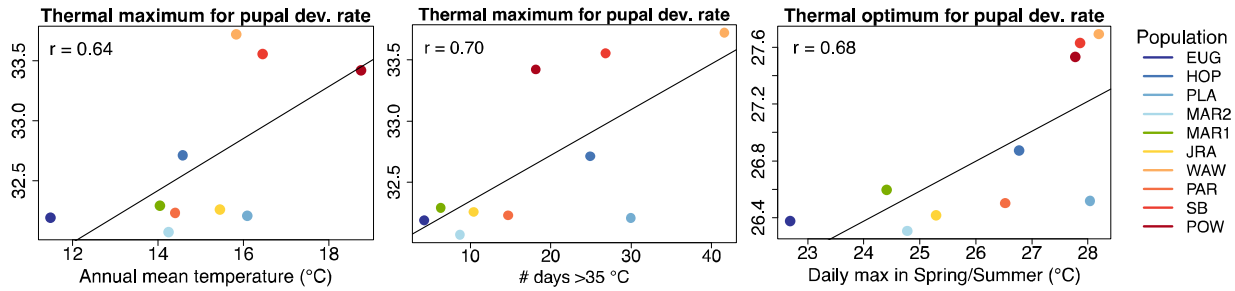

Figure 6. Local thermal adaptation is evident for pupal development rate. Thermal performance characteristics are: CT_{max} (left and center) and T_{opt} (right) of pupal development rate (units are °C). Thermal characteristics in the source environment are average annual mean

temperature (left), average number of days with temperatures exceeding 35°C (center), and average daily maxima in the Spring and Summer (right). Correlations (r) are shown as inset text on each plot. Not all moderate correlations are visualized here; see Table 3 for the full list.

Table 3. Correlations between traits with significant between-population variation and aspects of the source environment reflect local thermal adaptation. Statistically significant correlations (r; $p < 0.05$) are denoted with (*).

	Annual mean temp.	Jan – March mean temp.	Seasonal temp. variation	Warm-season maximum	# days > 35°C
CT_{max} of larval development rate	0.14	-0.17	0.63	0.23	0.49
CT_{max} of pupal development rate	0.64*	0.39	0.31	0.68*	0.70*
T_{opt} of pupal development rate	0.67*	0.42	0.29	0.71*	0.70*

Discussion

Standing variation in thermal tolerance may enable mosquito species to persist under climate warming through evolutionary adaptation. Our common garden experiment using ten *Aedes sierrensis* populations collected across a large climate gradient and spanning over 1,200 km found that this standing variation is minimal for most traits. In particular, the upper thermal limits and thermal optima for fitness each varied by <1□ across all populations (27.8 – 28.4□ and 22.4 – 23.1□, respectively; Figure 3). This level of variation in upper thermal limits across latitude (*i.e.*, 0.6°C across populations spanning 10° of latitude) is roughly double the estimate for terrestrial ectotherms broadly (0.3°C per 10° latitude[35]) but is considerably less than the level of variation in environmental temperature across this range. We did, however, find substantial differences in absolute fitness performance between populations. Average maximum fitness varied from 2.2 – 10.1 offspring per individual, with the population from the lowest collection latitude having the highest maximum fitness at nearly all experimental temperatures (Figure 2).

Our finding that geographical variation in thermal performance curves was primarily evident in vertical shifts (*i.e.*, absolute trait values), rather than horizontal shifts (*i.e.*, thermal dependence) is consistent with prior findings across a broad range of ectotherm species[41]. Although the exact mechanism underlying this pattern remains unclear, it may be that horizontal shifts require larger physiological changes, such as in enzyme structure and stability, while vertical shifts can be achieved through up- or down-regulation of enzymes, requiring less evolutionary change[41,42]. Along these lines, upper thermal limits of ectotherms including insects, arachnids, reptiles, and amphibians, have consistently been found to vary minimally across broad climatic and geographic space[35,43–45]. This minimal variation, as was found in our study, has been suggested to reflect hard evolutionary constraints on heat tolerance, potentially imposed by an

inability to meet increased oxygen demand at high temperatures[46–48]. Alternatively, this observation may be driven by behavioral strategies enabling populations to experience and adapt to similar thermal regimes across their range[49], and/or trade-offs in adapting to temperature versus other abiotic or biotic selection pressures[50].

Despite generally limited variation in thermal tolerance between populations, we did observe meaningful variation in the thermal responses of larval and pupal development rates (Figure 3). For both traits, upper thermal limits varied by approximately 1.6°C across populations—over twice as large as the variation estimated in fitness upper thermal limits in our study, and five times that of terrestrial ectotherms spanning a similar latitudinal extent[35] (Supplemental Table S4). When investigating associations between these thermal performance characteristics (CT_{max} for larval development rate, and T_{opt} and CT_{max} for pupal development rate) and the source environment, we found evidence for local thermal adaptation. Specifically, higher thermal optima and limits for pupal development rate were associated with higher mean and extreme temperatures at the source environment (Figure 6, Table 3). Prior investigations of mosquito thermal tolerance have generally found limited or inconclusive signatures of thermal adaptation[11–14]. However, these studies typically measured a subset of mosquito life history traits or metabolic rates rather than fitness itself, thus potentially missing the extent of adaptation[51]. Further, prior studies typically compared thermal tolerance in representative 'warm' and 'cool' populations, potentially missing broader patterns in adaptation across the species range. By measuring the thermal performance of fitness, as well as individual life history traits, on many populations from across a wide thermal gradient, we believe our results provide the clearest evidence yet of mosquito thermal adaptation.

Despite this evidence of local thermal adaptation, the potential for further evolutionary adaptation to warming may be limited. In addition to the minimal variation observed in upper thermal limits, we found that temperatures at all source environments already frequently exceed our experimentally measured upper thermal limits (Table 2). In particular, all sites experienced at least four days out of the year (and up to 42 days) with temperatures at or above 35°C—exceeding the highest upper thermal limit we estimated for any trait. Given this, *Ae. sierrensis* appears to have substantially lower warming tolerance (the difference between CT_{max} and habitat temperature) than related mosquito species[52] and other mid-latitude ectotherms[3], suggesting it may be particularly vulnerable to additional warming. However, juvenile *Ae. sierrensis* may be buffered from these extremes in air temperatures as they complete egg, larval, and pupal life stages in tree holes, which are typically shaded and/or water filled. Better characterizing the realized thermal environment remains an important next step for linking experimental results to estimates of mosquito life histories and disease transmission in the field.

We found that the upper thermal limits for adult lifespan—31.6°C—was the lowest for all measured life history traits (Figure 5), suggesting that *Ae. sierrensis* may be particularly vulnerable to thermal extremes in the adult life stage, which also occurs during the warmest part of the year. In other *Aedes* and *Culex* vector species, adult lifespan was similarly found to have the lowest upper thermal limits across traits[2], and our estimate closely matches a prior estimate

for *Ae. albopictus* (31.51°C[30])—a vector of several arboviral diseases including chikungunya, dengue, and Zika. For *Ae. sierrensis*, this estimated upper thermal limit was frequently exceeded at all collection sites, with temperatures above 31.6°C occurring between 18 to 88 days of the year (Table 2), reinforcing the potentially low warming tolerance of this species. Further, we found that adult lifespan had the largest impact on mosquito fitness, with nearly twice the effect on fitness as the next most important life history trait (larval survival; Table 3). In our experiment, no adults survived more than one day at 32°C and, across all temperatures, only 8% survived long enough to potentially lay eggs (*i.e.*, 10 - 17 days), suggesting that adult longevity was the major bottleneck to fitness. This is consistent with prior work finding that, at high temperatures, *Aedes*-borne disease transmission is most strongly constrained by adult lifespan[30,53].

The relatively low warming tolerance at the adult life stage, and the large impact of adult lifespan on fitness indicate that behavioral strategies to avoid high temperatures may be critical for mosquito persistence under ongoing warming[49,54]. In laboratory settings, behavioral avoidance of high temperatures (typically >30°C) has been documented in adult *Aedes*, *Anopheles*, and *Culex* spp[55–57]. In field settings, the biting activity of several mosquito species was found to shift during warmer months from dusk to late at night, but this was not conclusively linked to temperature[58]. These behavioral strategies to avoid high temperatures, while temporarily buffering individuals from unfavorable conditions, may dampen selection for greater thermal tolerance, decreasing the likelihood of evolutionary adaptation (termed the 'Bogert effect'[59]). Identifying the extent of behavioral thermoregulation in natural settings, and its impacts on disease transmission and adaptive potential are important directions for future research. In addition to behavioral thermoregulation, short-term, plastic responses could enable mosquitoes to temporarily cope with thermal extremes. Studies in other ectotherms have identified highly conserved heat shock responses, wherein extreme temperatures activate protective heat shock proteins that typically return to baseline levels after about four hours[60–62]. This response allows ectotherms to tolerate short-term adverse high temperatures, and recover or repair themselves during cooler periods[63–65], but has not been well studied in mosquitoes. As our experiment was conducted at constant temperatures—an important first step in characterizing thermal performance for a given species—we were not able to assess the impact of temperature fluctuations or short-term extremes on mosquito thermal tolerance. Incorporating realistic diurnal temperature fluctuations into measurements of mosquito thermal performance across species ranges is an important future direction[66–68].

Conclusion

Our investigation of mosquito thermal adaptation highlighted three major findings. First, populations varied substantially in maximum fitness, but not in their thermal performance of fitness, displaying <1°C variation in upper thermal limits across the species range. Second, we found clear evidence of local thermal adaptation in pupal development rates: upper thermal limits between populations varied by 1.6°C—five times greater than the average variation in

ectotherm upper thermal limits across a similar latitudinal gradient—and was associated with temperatures at the source environment. Lastly, across the entire species range, populations frequently experience temperatures exceeding their estimated upper thermal limits, indicating high vulnerability to further warming, particularly at the adult life stage, which had the lowest estimated thermal limits and the largest impact on mosquito fitness. Collectively, our results suggest that evolutionary adaptation alone may be insufficient for mosquito persistence in the face of ongoing climate warming, and other strategies such as behavioral thermoregulation and temporary coping responses will likely necessary.

Authors' Contributions

L.I.C. and E.A.M. conceived of and designed the project. L.I.C., J.E.F, and K.P.L. performed the field collection and laboratory rearing. L.I.C., J.E.F., K.P.L., and A.S.L. conducted the experiment. L.I.C. conducted the analyses and drafted the manuscript. All authors revised the manuscript and read and approved the final version.

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Data accessibility

All data and code used in this project are publicly available on GitHub: https://github.com/lcouper/MosquitoThermalAdaptation

References

1. World Health Organization. *Vector-borne diseases*. (2022).
2. Mordecai, E. A. *et al.* Thermal biology of mosquito-borne disease. *Ecol. Lett.* **22**, 1690–1708 (2019).

3. Deutsch, C. A. *et al.* Impacts of climate warming on terrestrial ectotherms across latitude. *Proc. Natl. Acad. Sci.* **105**, 6668–6672 (2008).
4. Ryan, S. J., Carlson, C. J., Mordecai, E. A. & Johnson, L. R. Global expansion and redistribution of Aedes-borne virus transmission risk with climate change. *PLoS Negl. Trop. Dis.* **13**, e0007213 (2019).
5. Servadio, J. L., Rosenthal, S. R., Carlson, L. & Bauer, C. Climate patterns and mosquito-borne disease outbreaks in South and Southeast Asia. *J. Infect. Public Health* **11**, 566–571 (2018).
6. Lafferty, K. D. The ecology of climate change and infectious diseases. *Ecology* **90**, 888–900 (2009).
7. Gething, P. W. *et al.* Climate change and the global malaria recession. *Nature* **465**, 342–345 (2010).
8. Aitken, S. N., Yeaman, S., Holliday, J. A., Wang, T. & Curtis-McLane, S. Adaptation, migration or extirpation: climate change outcomes for tree populations. *Evol. Appl.* **1**, 95–111 (2008).
9. Couper, L. I. *et al.* How will mosquitoes adapt to climate warming? *eLife* **10**, e69630 (2021).
10. Pawar, S. *et al.* Variation in temperature of peak trait performance will constrain adaptation of arthropod populations to climatic warming. *bioRxiv* (2023) doi:10.1101/2023.01.18.524448.
11. Ruybal, J. E., Kramer, L. D. & Kilpatrick, A. M. Geographic variation in the response of Culex pipiens life history traits to temperature. *Parasit. Vectors* **9**, 116 (2016).
12. Reisen, W. Effect of Temperature on Culex tarsalis (Diptera: Culicidae) from the Coachella and San Joaquin Valleys of California. *J. Med. Entomol.* **32**, 636–645 (1995).
13. Vorhees, A. S., Gray, E. M. & Bradley, T. J. Thermal Resistance and Performance Correlate with Climate in Populations of a Widespread Mosquito. *Physiol. Biochem. Zool.* **86**, 73–81 (2013).
14. Chu, V. M. *et al.* Regional variation in life history traits and plastic responses to temperature of the major malaria vector Nyssorhynchus darlingi in Brazil. *Sci. Rep.* **9**, 5356 (2019).
15. Zani, P. A. *et al.* Geographic Variation in Tolerance of Transient Thermal Stress in the Mosquito Wyeomyia Smithii. *Ecology* **86**, 1206–1211 (2005).
16. Orr, H. A. Fitness and its role in evolutionary genetics. *Nat. Rev. Genet.* **10**, 531–539 (2009).
17. Dobson, F. S., Murie, J. O. & Viblanc, V. A. Fitness Estimation for Ecological Studies: An Evaluation in Columbian Ground Squirrels. *Front. Ecol. Evol.* **8**, (2020).
18. Hawley, W. A. The Effect of Larval Density on Adult Longevity of a Mosquito, Aedes sierrensis: Epidemiological Consequences. *J. Anim. Ecol.* **54**, 955 (1985).
19. Garcia, R. & Pontig, G. Studies on the ecology of the treehole mosquito Aedes sierrensis (Ludlow). *Calif. Mosq. Control Assoc. Proc.* (1972).
20. Puggioli, A. *et al.* Development of Aedes albopictus (Diptera: Culicidae) Larvae Under Different Laboratory Conditions. *J. Med. Entomol.* **54**, 142–149 (2017).
21. International Atomic Energy Agency. Guidlines for routine colony maintenance of Aedes mosquito species. (2017).
22. Ameneshewa, B. & Service, M. W. The relationship between female body size and survival rate of the malaria vector Anopheles arabiensis in Ethiopia. *Med. Vet. Entomol.* **10**, 170–172 (1996).
23. Lyimo, E. O. & Takken, W. Effects of adult body size on fecundity and the pre-gravid rate of Anopheles gambiae females in Tanzania. *Med. Vet. Entomol.* **7**, 328–332 (1993).
24. Abramoff, M. D., Magalhães, P. J. & Ram, S. J. Image processing with ImageJ. *Biophotonics Int.* **11**, 36–42 (2004).
25. Washburn, J. O., Anderson, J. R. & Mercer, D. R. Emergence Characteristics of Aedes sierrensis (Diptera: Culicidae) from California Treeholes with Particular Reference to Parasite Loads. *J. Med. Entomol.* **26**, 173–182 (1989).
26. Gorelick, N. *et al.* Google Earth Engine: Planetary-scale geospatial analysis for everyone. *Remote Sens. Environ.* **202**, 18–27 (2017).
27. Daly, C. *et al.* Physiographically sensitive mapping of climatological temperature and precipitation across the conterminous United States. *Int. J. Climatol.* **28**, 2031–2064 (2008).
28. Sunday, J. *et al.* Thermal tolerance patterns across latitude and elevation. *Philos. Trans. R. Soc. B Biol. Sci.* **374**, 20190036 (2019).

29. Shocket, M. S. *et al.* Transmission of West Nile and five other temperate mosquito-borne viruses peaks at temperatures between 23°C and 26°C. *eLife* **9**, e58511 (2020).
30. Mordecai, E. A. *et al.* Detecting the impact of temperature on transmission of Zika, dengue, and chikungunya using mechanistic models. *PLoS Negl. Trop. Dis.* **11**, e0005568 (2017).
31. Su, Y. & Yajima, M. R2jags: A Package for Running jags from R. (2009).
32. Venables, W. & Ripley, B. *Modern Applied Statistics with S*. (Springer, 2002).
33. Bates, D., Mächler, M., Bolker, B. & Walker, S. Fitting Linear Mixed-Effects Models Using lme4. *J. Stat. Softw.* **67**, 1–48 (2015).
34. Kellermann, V. & van Heerwaarden, B. Terrestrial insects and climate change: adaptive responses in key traits. *Physiol. Entomol.* **44**, 99–115 (2019).
35. Sunday, J. M., Bates, A. E. & Dulvy, N. K. Global analysis of thermal tolerance and latitude in ectotherms. *Proc. R. Soc. B Biol. Sci.* **278**, 1823–1830 (2011).
36. Rothman, K. J. No Adjustments Are Needed for Multiple Comparisons. *Epidemiology* **1**, 43–46 (1990).
37. Frane, A. V. Planned Hypothesis Tests Are Not Necessarily Exempt From Multiplicity Adjustment. *J. Res. Pract.* (2015).
38. Knies, J. L., Kingsolver, J. G. & Burch, C. L. Hotter Is Better and Broader: Thermal Sensitivity of Fitness in a Population of Bacteriophages. *Am. Nat.* **173**, 419–430 (2009).
39. Angilletta, M. J. *Thermal Adaptation: A Theoretical and Empirical Synthesis*. (OUP Oxford, 2009).
40. Kontopoulos, D.-G. *et al.* Phytoplankton thermal responses adapt in the absence of hard thermodynamic constraints. *Evolution* **74**, 775–790 (2020).
41. Tüzün, N. & Stoks, R. Evolution of geographic variation in thermal performance curves in the face of climate change and implications for biotic interactions. *Curr. Opin. Insect Sci.* **29**, 78–84 (2018).
42. Angilletta, M. J., Wilson, R. S., Navas, C. A. & James, R. S. Tradeoffs and the evolution of thermal reaction norms. *Trends Ecol. Evol.* **18**, 234–240 (2003).
43. Addo-Bediako, A., Chown, S. L. & Gaston, K. J. Thermal tolerance, climatic variability and latitude. *Proc. R. Soc. Lond. B Biol. Sci.* **267**, 739–745 (2000).
44. MacLean, H. J. *et al.* Evolution and plasticity of thermal performance: an analysis of variation in thermal tolerance and fitness in 22 Drosophila species. *Philos. Trans. R. Soc. B Biol. Sci.* **374**, 20180548 (2019).
45. Klepsatel, P. *et al.* Variation in Thermal Performance and Reaction Norms Among Populations of Drosophila Melanogaster. *Evolution* **67**, 3573–3587 (2013).
46. Somero, G. N. Adaptation of enzymes to temperature: searching for basic “strategies”. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **139**, 321–333 (2004).
47. González-Tokman, D. *et al.* Insect responses to heat: physiological mechanisms, evolution and ecological implications in a warming world. *Biol. Rev.* **95**, 802–821 (2020).
48. Pörtner, H.-O. Oxygen- and capacity-limitation of thermal tolerance: a matrix for integrating climate-related stressor effects in marine ecosystems. *J. Exp. Biol.* **213**, 881–893 (2010).
49. Sunday, J. M. *et al.* Thermal-safety margins and the necessity of thermoregulatory behavior across latitude and elevation. *Proc. Natl. Acad. Sci.* **111**, 5610–5615 (2014).
50. Pörtner, H. O. *et al.* Trade□Offs in Thermal Adaptation: The Need for a Molecular to Ecological Integration. *Physiol. Biochem. Zool.* **79**, 295–313 (2006).
51. Blanquart, F., Kaltz, O., Nuismer, S. L. & Gandon, S. A practical guide to measuring local adaptation. *Ecol. Lett.* **16**, 1195–1205 (2013).
52. Oliveira, B. F., Yogo, W. I. G., Hahn, D. A., Yongxing, J. & Scheffers, B. R. Community-wide seasonal shifts in thermal tolerances of mosquitoes. *Ecology* **102**, e03368 (2021).
53. Mordecai, E. A. *et al.* Optimal temperature for malaria transmission is dramatically lower than previously predicted. *Ecol. Lett.* **16**, 22–30 (2013).
54. Kearney, M., Shine, R. & Porter, W. P. The potential for behavioral thermoregulation to buffer “cold-blooded” animals against climate warming. *Proc. Natl. Acad. Sci.* **106**, 3835–3840 (2009).

55. Ziegler, R., Blanckenhorn, W. U., Mathis, A. & Verhulst, N. O. Video analysis of the locomotory behaviour of Aedes aegypti and Ae. japonicus mosquitoes under different temperature regimes in a laboratory setting. *J. Therm. Biol.* **105**, 103205 (2022).
56. Verhulst, N. O., Brendle, A., Blanckenhorn, W. U. & Mathis, A. Thermal preferences of subtropical Aedes aegypti and temperate Ae. japonicus mosquitoes. *J. Therm. Biol.* **91**, 102637 (2020).
57. Blanford, S., Read, A. F. & Thomas, M. B. Thermal behaviour of Anopheles stephensi in response to infection with malaria and fungal entomopathogens. *Malar. J.* **8**, (2009).
58. Reisen, W. K. & Aslamkhan, M. Biting rhythms of some Pakistan mosquitoes (Diptera: Culicidae). *Bull. Entomol. Res.* **68**, 313–330 (1978).
59. Huey, R. B., Hertz, P. E. & Sinervo, B. Behavioral Drive versus Behavioral Inertia in Evolution: A Null Model Approach. *Am. Nat.* **161**, 357–366 (2003).
60. Loeschcke, V. & Hoffmann, A. Consequences of Heat Hardening on a Field Fitness Component in Drosophila Depend on Environmental Temperature. *Am. Nat.* **169**, 175–183 (2007).
61. Moghadam, N. N., Ketola, T., Pertoldi, C., Bahrndorff, S. & Kristensen, T. N. Heat hardening capacity in *Drosophila melanogaster* is life stage-specific and juveniles show the highest plasticity. *Biol. Lett.* **15**, 20180628 (2019).
62. Sørensen, J. G., Kristensen, T. N. & Loeschcke, V. The evolutionary and ecological role of heat shock proteins. *Ecol. Lett.* **6**, 1025–1037 (2003).
63. Ma, G., Hoffmann, A. A. & Ma, C.-S. Daily temperature extremes play an important role in predicting thermal effects. *J. Exp. Biol.* jeb.122127 (2015) doi:10.1242/jeb.122127.
64. Colinet, H., Sinclair, B. J., Vernon, P. & Renault, D. Insects in Fluctuating Thermal Environments. *Annu. Rev. Entomol.* **60**, 123–140 (2015).
65. Bai, C.-M., Ma, G., Cai, W.-Z. & Ma, C.-S. Independent and combined effects of daytime heat stress and night-time recovery determine thermal performance. *Biol. Open* **8**, (2019).
66. Paaijmans, K. P. *et al.* Temperature variation makes ectotherms more sensitive to climate change. *Glob. Change Biol.* **19**, 2373–2380 (2013).
67. Lambrechts, L. *et al.* Impact of daily temperature fluctuations on dengue virus transmission by Aedes aegypti. *Proc. Natl. Acad. Sci.* **108**, 7460–7465 (2011).
68. Alto, B. W. *et al.* Diurnal Temperature Range and Chikungunya Virus Infection in Invasive Mosquito Vectors. *J. Med. Entomol.* **55**, 217–224 (2018).