

ARTICLE

Anticipating change: The impact of simulated seasonal heterogeneity on heat tolerances along a latitudinal cline

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Abstract

An understanding of thermal limits and variation across geographic regions is central to predicting how any population may respond to global change. Latitudinal clines, in particular, have been used to demonstrate that populations can be locally adapted to their own thermal environment and, as a result, not all populations will be equally impacted by an increase in temperature. But how robust are these signals of thermal adaptation to the other ecological challenges that animals commonly face in the wild? Seasonal changes in population density, food availability, or photoperiod are common ecological challenges that could disrupt patterns of thermal tolerance along a cline if each population differentially used these signals to anticipate future temperatures and adjust their thermal tolerances accordingly. In this study, we aimed to test the robustness of a cline in thermal tolerance to simulated signals of seasonal heterogeneity. Experimental animals were derived from clones of the Australian water flea, *Daphnia carinata*, sampled from nine distinct populations along a latitudinal transect in Eastern Australia. We then factorially combined summer (18 h light, 6 h dark) and winter (6 h light, 18 h dark) photoperiods with high (5 million algal cells individual⁻¹ day⁻¹) and low (1 million algal cells individual⁻¹ day⁻¹) food availabilities, before performing static heat shock assays to measure thermal tolerance. We found that the thermal tolerances of the clonal populations were sensitive to both measures of seasonal change. In general, higher food availability led to an increase in thermal tolerances, with the magnitude of the increase varying by clone. In contrast, a switch in photoperiod led to rank-order changes in thermal tolerances, with heat resistance increasing for some clones, and decreasing for others. Heat resistance, however, still declined with increasing latitude, irrespective of the manipulation of seasonal signals, with clones from northern populations always showing greater thermal resistance, most likely driven by adaptation to winter thermal conditions. While photoperiod and food availability can clearly shape thermal tolerances for specific populations, they are unlikely to overwhelm overarching signals of thermal adaptation, and

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thus, observed clines in heat resistance will likely have remained robust to these forms of seasonal heterogeneity.

KEYWORDS

food availability, knockdown times, latitudinal clines, local adaptation, photoperiod, seasonal heterogeneity, thermal resistance

INTRODUCTION

Global change has led to a gradual increase in both mean temperatures and extreme climatic events and is forecast to continue into the future (Buckley & Huey, 2016a; Rahmouf & Coumou, 2011; Schär et al., 2004). An understanding of thermal limits and variation amongst geographic regions is central to predicting how any population may respond to global change (Hoffmann et al., 2002; Hoffmann & Sgrò, 2011; Sgrò et al., 2010; Somero, 2010). Studies of populations sampled from along latitudinal clines have been widely used to detect local adaptation in response to the thermal environment (Hoffmann et al., 2002; Kelly et al., 2012; Yampolsky et al., 2013). Such studies show that populations often vary in their sensitivity to thermal stress, with populations from lower latitudes having higher tolerance of elevated temperatures, and the rate at which tolerance changes with latitude indicating the strength of local adaptation (e.g., Arthur et al., 2008 vs. Sgrò et al., 2010). However, accurate predictions of population responses to global change based on clinal studies depend on how robust observed clinal patterns in thermal limits are to other sources of environmental variation that also shape the life history and physiology of populations (Kellermann et al., 2012; Manenti et al., 2018).

Populations will be exposed to a range of environmental conditions throughout the year that can modify their tolerance to heat stress (Noer et al., 2022; Oliveira et al., 2021; Turko et al., 2020), including stochastic variation in food supply (Cheng et al., 2018), sudden heat waves (Buckley & Huey, 2016b) or the presence of competitors or parasites (Hector et al., 2019; Thawley et al., 2019). These biotic and abiotic factors can disrupt the predictable change in thermal limits that is the hallmark of latitudinal clines, by either shifting the average thermal tolerance of all populations equally, or by inducing population-specific responses that can reduce or even completely erode the previously established clines (Hector et al., 2019; Thawley et al., 2019). Exposure to biotic stressors, such as invasive species (Thawley et al., 2019) or pathogens (Hector et al., 2019) for example, has been shown to negate the increase in upper thermal limits that is commonly observed in low-latitude, tropical populations.

In contrast, both gradual (acclimation) and abrupt (hardening) increases in temperature are well-established mechanisms by which individuals can increase their thermal resistance (Hoffmann & Parsons, 1994; Noer et al., 2022; Pereira et al., 2017; Sgrò et al., 2016; van Heerwaarden et al., 2016). The realized thermal tolerances of individuals and populations thus depend on the combined effects of multiple co-occurring sources of environmental variation.

Seasonal changes in environmental conditions also offer the opportunity for organisms to anticipate future temperatures and plastically adjust their thermal tolerances (Machado et al., 2021; Moghadam et al., 2019; Oliveira et al., 2021). The warmer conditions experienced in the peak of summer, for example, commonly follow predictable changes in population density (Bent & Goulder, 1981; McGuire et al., 1993), food availability (Cross et al., 2015), or photoperiod (as reviewed in Bradshaw & Holzapfel, 2007; Erickson et al., 2020). Some signals, such as changes in food supply, are linked to thermal tolerances in complex ways that might limit their use as a signal of future thermal conditions. Owing to the likely condition-dependent nature of thermal limits and their sensitivity to energy supply or expenditure (e.g., Bujan & Kaspari, 2017; Chown et al., 2009; Terblanche et al., 2011), for example, even small changes in food supply will impact an organism's thermal tolerances, with a reduction in food leading to lower thermal limits, even if correlated with warmer conditions. Photoperiod, however, is a simple and highly reliable cue that signals the coming of warmer conditions (Bradshaw & Holzapfel, 2007). Many bird species, for example, use photoperiods for timing long migrations or breeding periods with peak resources (Cohen et al., 2020; Dawson et al., 2001). Indeed, studies have begun to quantify the impact of seasonal variation on thermal resistance by manipulating photoperiod as a proxy for season. For example, reductions in knockdown times during summer light conditions have been found for *Drosophila melanogaster* (Bauerfeind et al., 2014) and population-dependent increases in CT_{max} due to increasing photoperiod in *Drosophila subobscura* (Moghadam et al., 2019). While these studies highlight the potential for signals of seasonality, such as photoperiod, to modulate a population's thermal tolerance, the extent to which locally adapted populations

along a cline might vary in their response to seasonality signals remains to be tested.

In this study, we aimed to detect the signal of a latitudinal cline for thermal tolerance in the Australian water flea, *Daphnia carinata*, and then determine how robust any cline may be to signals of seasonal heterogeneity. *Daphnia* are ideal for testing both the robustness of clinal variation in thermal limits to seasonal change, and the capacity of individuals to anticipate future conditions using the same signals. Temperature is predicted to be a strong driver of evolution in *Daphnia*, with populations shown to be locally adapted to their own thermal environment (Williams et al., 2012; Yampolsky et al., 2013) and rapid evolution occurring in response to only a few degrees change in temperature (Geerts et al., 2015). Seasonal changes in environmental conditions also trigger the adaptive switch to sexual reproduction and the production of sexual resting eggs (i.e., ephippia) that can withstand harsh environmental conditions (Gust et al., 2019; Jiang et al., 2014; Lever et al., 2021; Roulin et al., 2013). For Australian *Daphnia*, for example, longer photoperiods are congruent with harsh summer conditions that lead to seasonal population crashes (Barry, 1997; Lund & Davis, 2000). Coinciding with this change is an increase in food availability during spring, due to increases in the abundance of algal species (Moss et al., 2003; Winder & Schindler, 2004; Wojtal-Frankiewicz, 2012), followed by an increase in population density and consequently food limitation during early summer (Barry, 1997; Lund & Davis, 2000). *Daphnia* recognize these signals and invest strategically into sexual reproduction, with longer day lengths, higher population density, and reduced food availability increasing the production of sexual eggs and relative investment in males (Gust et al., 2019; Lever et al., 2021).

Here, we integrated manipulation of environmental cues associated with seasonality into the study of thermal limits along a latitudinal cline, using clonal genotypes of *D. carinata* derived from latitudinally distinct populations from Eastern Australia. The seasonal change was simulated by manipulating two key variables that vary significantly with the season in the Australian system, photoperiod and food availability (Lever et al., 2021). We then measured the upper thermal limits of each clone using static heat shock assays (Hector et al., 2019; Sgrò et al., 2010; Yampolsky et al., 2013), and used microclimate data available for each population to explore the environmental factors potentially underlying local adaptation of thermal tolerance along a cline in *D. carinata* (as per Yampolsky et al., 2013; Roast et al., 2019). Based on the strong link between local thermal conditions and thermal tolerances documented in other *Daphnia* species (Seefeldt & Ebert, 2019; Yampolsky et al., 2013), we predicted that a latitudinal cline for thermal

tolerance would be evident, with clones from populations closer to the equator exhibiting higher resistance to heat stress (Hoffmann et al., 2002; Yampolsky et al., 2013). We also expected that, if the thermal resistance of clones was sensitive to seasonal signals, populations would have the greatest thermal tolerance during conditions that predict warmer conditions and trigger known life-history shifts, long photoperiods and high food availability.

The consistency with which each clone responds to the signals of seasonal change, however, will determine the robustness of any observed cline, with three broad scenarios potentially occurring: (i) if all clones respond in the same direction and magnitude to the seasonal signals, the relative change in thermal limits along the cline remains robust, even if it is shifted upward as expected, or otherwise; (ii) if clones from the southern regions, which naturally experience the largest changes in photoperiod throughout the year (Bradshaw & Holzapfel, 2007), have the strongest response to photoperiod changes, then we might observe a weakening of the cline as the southern clones responded by increasing their thermal limits the most; and (iii) finally, if each clone responds uniquely to the seasonal signals, then a complete breakdown of the latitudinal cline in thermal limits may be possible (e.g., see Thawley et al., 2019), but only if the changes induced by the seasonal signals was strong enough to overwhelm the differences observed amongst clones in general.

METHODS

Study system

We used the aquatic crustacean *D. carinata*, a cyclical parthenogen found throughout Australia, New Zealand, and India within freshwater bodies, varying in size from small puddles to large lakes (Barry, 1997; Lund & Davis, 2000; Venkataraman, 1981). *Daphnia* graze on green algal cells through filtration, with food availability peaking during warmer spring months and can reach levels double that of cooler periods (Miiller-Navarra & Lampert, 1996). Reproduction is asexual during most periods of the year but switches to sexual reproduction in response to environmental cues that indicate stressful conditions, such as longer photoperiods, low food availability, and higher population density (Gerber et al., 2018; Kleiven et al., 1992; Lever et al., 2021). Sexual reproduction facilitates the production of resting eggs, called ephippia, that lay dormant during inhospitable conditions and is vital for both metapopulation survival as well as dispersal (Altermatt & Ebert, 2010). *D. carinata* commonly exhibits seasonal

boom/bust population dynamics, with population cycles correlating with algal food availability and thermal environment (Barry, 1997; Lund & Davis, 2000; Venkataraman, 1981). Active season length varies with population, but *Daphnia* populations typically experience severe decline during early summer (Barry, 1997; Lund & Davis, 2000; Venkataraman, 1981).

Experimental animals were derived from nine clones (PLD, BWD, BLD3, WWD2, YYD, OZD, DC28, CMD, and LBD, see Figure 1 and Appendix S1: Table S1) of *D. carinata*, each sampled from and representing one of nine populations collected along a latitudinal gradient from Tamworth (30° S) in northern New South Wales to

Geelong (38° S) in southern Victoria (Drapes et al., 2021; Lever et al., 2021). This approach assumes that a single clone per population represents the broader patterns of adaptation to local temperatures (as per Yampolsky et al., 2013). As a clonal organism, *Daphnia* populations are expected to be dominated by a few founder clones that have a numerical advantage in both the existing population and the diapausing egg bank (Hebert & Crease, 1983). Previous studies have leveraged this feature to study local adaptation across continents and latitudes (Seefeldt & Ebert, 2019; Williams et al., 2012; Yampolsky et al., 2013), uncovering patterns that are similar to that observed in other model systems like

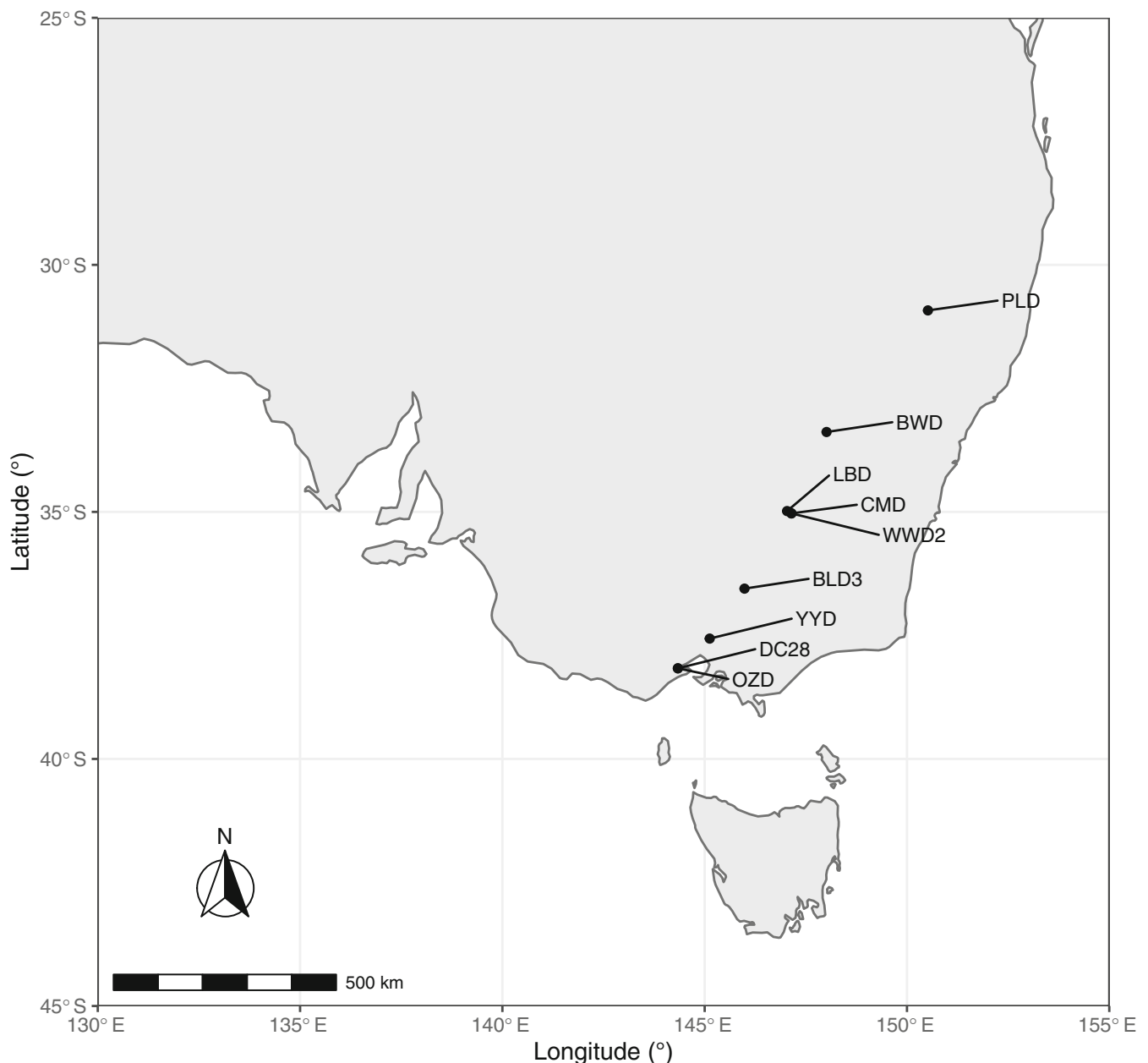


FIGURE 1 Map of South-eastern Australia showing the locations of the populations from which each of the clonal populations of *Daphnia carinata* was sampled.

Drosophila (Hoffmann et al., 2002; Sgrò et al., 2010), where differences between populations are greater than the variation within.

All lines were collected from late 2016 to mid 2017 (Drapes et al., 2021). Experimental stocks were maintained under standard laboratory conditions, stored at 20°C and under a 16:8 L:D light regime, and maintained at low densities. Stock populations were maintained in 400-mL jars and fed ad libitum with up to 80 million green algal cells three times weekly. These conditions ensure asexual reproduction, and minimize recombination, ensuring that experimental animals were representative of their initial genotypes. From the stock animals, individual females presenting eggs were first isolated in 70-mL glass jars containing 40 mL of Artificial *Daphnia* Medium (ADaM, Klüttgen et al., 1994; modified in Ebert et al., 1998). *Daphnia* were maintained individually for three generations to minimize any maternal effects, before isolating experimental animals from clutches three to seven, correlating with experimental block numbers one to four. Parental clutches were divided evenly across treatment groups.

Basic experimental design

Seasonal treatments were created by manipulating photoperiod and the availability of *Scenedesmus* sp. green algal cells (Moss et al., 2003; Winder & Schindler, 2004; Wojtal-Frankiewicz, 2012). To mimic seasonal conditions, we factorially combined summer (18 h light, 6 h dark) and winter (6 h light, 18 h dark) photoperiods that were typical of extreme latitudes (Lever et al., 2021), with high (5 million algal cells individual⁻¹ day⁻¹) and low (1 million algal cells individual⁻¹ day⁻¹) food availabilities (Lever et al., 2021), resulting in four treatment groups. Levels of food availability were chosen to relate to large deviations in seasonal food observed in wild populations (Barry, 1997; Miiller-Navarra & Lampert, 1996), with the choice of food levels based on previous *Daphnia* studies (Hall & Ebert, 2012; Lever et al., 2021). Each treatment contained 40 mature experimental animals that were partitioned into four temporal blocks of 10 replicates ([9 clonal populations × 2 food qualities × 2 photoperiods = 36 treatments] × 4 blocks × 10 individuals = 1440 total animals). Animals were maintained in controlled climate chambers from collection at day 0 ± 1, until assay days at 20 ± 1 days old. Climate chambers maintained a thermal environment of 20°C with either summer or winter photoperiod and high or low food availability. The animals were fed daily with green algae (*Scenedesmus* sp.). The *Daphnia* medium did not contain the nutrients required for algal growth, and the culture medium was changed twice weekly

to remove any excess food until static heat shock assays were performed (described below). Within the climate chambers, the location of animals was shuffled daily to minimize any positional effects.

Static heat shock assays

Static heat shock assays were performed to determine the time to immobilization (T_{imm}) as a measure of thermal tolerance, herein referred to as knockdown time (Yampolsky et al., 2013). Knockdown times are commonly used to measure the ability of individuals to persist through temperature extremes. We selected a temperature of 37°C, following previous clinal studies analyzing thermal resistance in *Daphnia magna* (Yampolsky et al., 2013), as it is an acute heat stress that is able to detect measurable differences in thermal resistance within a few hours (see Hector et al., 2019; Laidlaw et al., 2020). Additionally, this temperature was comparable with the 39°C that is commonly used in clinal studies focusing on *D. melanogaster* (Hoffmann et al., 2002; Sgrò et al., 2010).

Animals were assayed at 20 ± 1 days old. Assays were performed in 70-L glass aquariums, using a thermoregulator to maintain temperatures at 37°C with constant agitation and bubblers to maintain oxygen saturation throughout the assay. Animals were kept inside 5-mL glass vials, covered by a mesh lid to prevent oxygen depletion within the vial (as per Hector et al., 2019). Knockdown times were scored beginning from when the vials entered the assay until individuals ceased all visible movement, including filtering (Hector et al., 2019; Yampolsky et al., 2013). All assays were observed in person, with only one researcher scoring knockdowns to minimize bias in reaction times. Individuals from each clonal line were randomly selected for each assay. Each assay could contain 48 animals, and as such as four or five animals from each clonal population were present in each assay. The experiment contained four experimental blocks. Each experimental block was assayed over 2 days, with eight total assays being performed during that time period. Immediately following static heat shock assays, the body size of each individual was measured using a stereomicroscope from the individual's eye to the base of the tail (Laidlaw et al., 2020).

Statistical analyses

All analyses were performed using R (v.4.0.5, 2021; www.R-project.com). Following common approaches to studying latitudinal clines in *Drosophila* (Cockerell et al., 2014; Sgrò et al., 2010), we performed a two-step

analysis to explore the response of the different *Daphnia* clones to the seasonality manipulations, and then how the latitudinal sampling location of each clone related to our observed patterns. With only a single representative clone per sampling location, it was not possible to explore both clone-specific and latitudinal effects in combination (see also Seefeldt & Ebert, 2019). We first assessed the impact of seasonal change on each clone's thermal response via a linear mixed-effects model as implemented via the *lme4* package (Bates et al., 2015). This model included knockdown time as the response variable, and photoperiod (summer and winter), food availability (high and low), and *Daphnia* genotype (nine levels), as interacting fixed effects. Body size was included as a covariate while experimental block was included as a random effect. This analysis determines the effects of each treatment on the thermal resistance of populations, regardless of any spatial orientation, and allowed us to determine the consistency with which clones could improve thermal resistance in conjunction with seasonal cues.

We then modeled knockdown times across the latitudes of our populations from which the clones were derived. This analysis would determine if there was evidence of local adaptation in the thermal tolerance of *Daphnia* along a cline, how the seasonality treatments might modify the average thermal tolerance at each location, or if the impact of these seasonal signals could change the strength of any clinal relationship (for similar analyses see: Cockerell et al., 2014; Sgrò et al., 2010). To do so we used a two-factor analysis of covariance with photoperiod and food availability as fixed effects and latitude ($^{\circ}$ S) as the interacting covariate, implemented as a linear mixed-effects model via the *lme4* package with experimental block as a random effect again (Bates et al., 2015). Analyses of this model would provide evidence for which scenario might be occurring, as outlined in hypotheses (i), (ii), and (iii).

Finally, we used microclimate data available for each location (population) to explore the environmental factors potentially underlying adaptation to thermal tolerance along a cline in *D. carinata* (as per Bramer et al., 2018). Microclimate data were obtained for 10 km² areas at the source GPS locations for each population (New et al., 2002), and included average monthly min and max temperatures, precipitation data, wind speed, altitude, cloud cover, and humidity (see Appendix S1: Table S1). These parameters were chosen as we believed that they were capable of altering the thermal environment experienced by *Daphnia* populations. Data were summarized to obtain average values of each variable for the winter and summer months, as populations do not persist year-round. Variables were then mean scaled and centered before analyses.

To fit the environmental data to knockdown times, we used the dredge function from the *MuMin* package (Barton, 2022). The package initially created linear models with knockdown times as a response and containing all combinations of up to four explanatory variables from the 19-total available (Appendix S1: Table S2), including the experimental treatments (food availability by photoperiod) as a required fixed effect. Parameters were added to potential model selection based on availability of data, and on their ability to manipulate the thermal regime of water bodies. Model selection compared all model permutations using their Akaike information criterion (AIC). Models within AIC < 2 from the best fitting model would be averaged to obtain effect size estimates of each parameter (as seen in Roast et al., 2019; top 10 models available in Appendix S1: Table S3).

RESULTS

We first considered how seasonal variation affected upper thermal limits by manipulating food availability and photoperiod and then performing static heat shock assays. We found that knockdown times varied significantly with both food availability and photoperiod, but that any observed effect was dependent on the *Daphnia* genotype (i.e., two-way interaction terms between genotypes and treatment groups, Table 1). The nature of the clone-specific responses, however, differed between the two environmental manipulations. Reducing the availability of food, for example, led to a 6-min, on average, decrease in knockdown times, with clones

TABLE 1 Linear mixed-effects models for the effects of photoperiod (winter light length and summer light length), food availability (high food, low food), and body size on knockdown times during a 37°C static heat shock assay.

Term	df	χ^2	p-value
Food availability	1	7.23	0.007*
Photoperiod	1	0.01	0.154
<i>Daphnia</i> clone	8	269.46	<0.001*
Photoperiod $\times$ <i>Daphnia</i> clone	8	22.55	0.004*
Food availability $\times$ <i>Daphnia</i> clone	8	17.28	0.027*
Photoperiod $\times$ food availability $\times$ <i>Daphnia</i> clone	8	4.75	0.784
Photoperiod $\times$ food availability	1	0.96	0.664
Size	1	1.33	0.249

Note: Analyses in which the identity of the *Daphnia* clone was included as an interacting fixed effect are shown. Block was a random effect, $\sigma = 2.36$.

*Denotes significant terms.

differing in the magnitude of this reduction. For example, for DC28, knockdown times decreased by ~12 min with diet, while LBD decreased by less than 3 min (Figure 2). In contrast, in response to changes in the photoperiod, the *Daphnia* clones varied in both the direction and magnitude of change in their knockdown times, leading to rank-order changes in the performance of populations in any photoperiod. Notably, populations did not all exhibit greater thermal resistance leading into the summer conditions, as initially predicted. For some genotypes, such as BWD, knockdown times increased with longer photoperiods, but for others, like PLD, knockdown times instead decreased with rising photoperiods (Figure 2).

We tested if the variation in observed clonal knockdown times (within treatment groups) correlated with variation in latitude, which we would expect to see if clonal populations were adapted to their local thermal environment. We found a negative relationship between latitude (°S) and knockdown times (Table 2; Figure 3). Lower latitude populations tended to have higher knockdown times than populations from higher latitudes, confirming a latitudinal cline in thermal resistance for the populations studied. The relationship between latitude and knockdown times was affected by the manipulation of seasonal signals, as captured by the significant food availability by latitude interaction (Table 2). In response to reduced food availability, populations from higher latitudes were especially intolerant to heat, with differences between populations increased by 16% (slope change of $3.78 \pm \text{SE}$ min per degree south to $4.40 \pm \text{SE}$ min per degree south).

To understand the potential environmental drivers of the cline in knockdown times, we obtained fine-scale microclimate data and modeled predictors against knockdown times. The best fitting model contained the variables wind speed, average high temperature during winter months (AHT winter) and average monthly min temperature (ALT), as well as the fixed treatment effects (Figure 4). Both predictors pertaining to temperature were positively correlated with thermal tolerance, with clones from populations that experienced higher winter AHT and higher ALT associated with elevated knockdown times (Figure 4). Conversely, the average wind speed of each location was negatively associated with knockdown times (Figure 4). Importantly, only the temperature predictors that were conducive to winter were present in the significant model, not temperature predictors from summer months, nor changes in photoperiod throughout the year. This suggests that seasonal variation in temperature, in combination with wind speeds, may be mostly responsible for local adaptation of thermal resistance, with warmer temperatures and lower wind speeds during the winter months, potentially leading to the evolution of greater thermal tolerance.

DISCUSSION

The capacity of individuals to anticipate future environmental conditions and adjust their thermal tolerance accordingly is an important mechanism for population

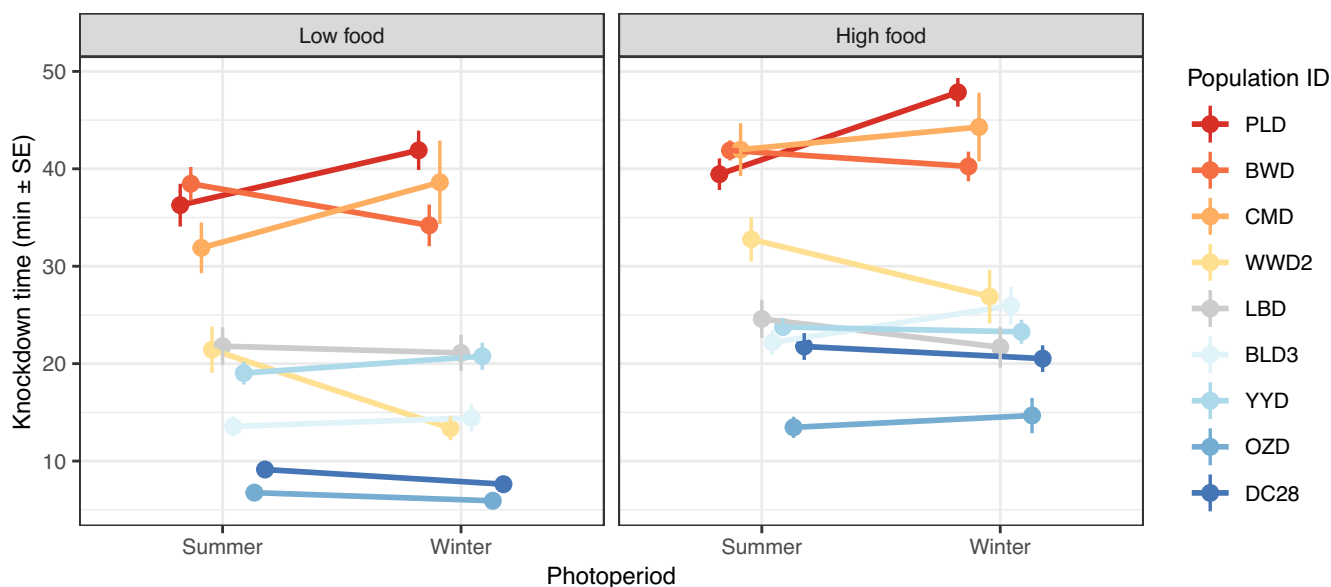


FIGURE 2 Reaction norm for the effect of photoperiod and food availability on knockdown time for each clone of *Daphnia carinata*. High food treatment consisted of 5 million green algal cells daily, low food was 1 million green algal cells daily. Summer photoperiods consisted of an 18 h:6 h photoperiod, while winter photoperiods consisted of a 6 h:18 h photoperiod. Points displayed are mean values ($\pm \text{SE}$) for knockdown times at 37°C. Clone IDs are displayed in the legend and are ordered by latitude, with clones from lower latitudes (°S) at the top (red) and higher latitude clones at the bottom (blue).

persistence (as reviewed in Scheiner, 1993), but populations may not all respond to seasonal signals in the same way. Here we analyzed the thermal resistance of *D. carinata* sampled along a latitudinal cline in response to two common seasonal cues. Initially, we proposed that animals may be able to use these signals of seasonal change, herein the increase in food availability and increase in photoperiod which signal the coming of summer, to anticipate future thermal conditions. Our results showed that both food availability and

photoperiod did indeed appear to influence the thermal tolerance of an individual, but their effects, not surprisingly, depended entirely on the clone of *Daphnia* used and its origin of sampling along the latitudinal cline. While populations did respond to both signals, there was no clear directional effect that would signify populations were able to increase their thermal performance in response to these specific signals of seasonal change.

For photoperiod, we observed rank-order changes in the reaction norms, with some clones performing better under summer light conditions and others performing better under winter light conditions. In contrast to scenario (ii) of our hypotheses, higher latitude populations from the southern end of the cline did not respond comparatively more to seasonal change. Rather, populations from the lower latitudes had relatively greater changes to thermal resistance, albeit with inconsistent direction of effect. Additionally, low food availability led to a relatively consistent decrease in knockdown times across all clones, with only the magnitude of the effect differing, which is similar to that observed in other species where we see prolonged dietary restriction reducing thermal resistance with length of starvation (Manenti et al., 2018; Mulaudzi et al., 2022; Nguyen et al., 2017). The presence of these clone-specific responses ruled out the possibility that clones from all locations are uniformly using photoperiod and food availability signals to modify their thermal tolerances but still highlights the importance of non-thermal seasonal cues in determining thermal resistance (Alford et al., 2012; Leclair et al., 2020).

TABLE 2 Linear mixed-effects models for the effects of photoperiod (winter light length and summer light length), food availability (high food, low food), and body size on knockdown times during a 37°C static heat shock assay.

Term	df	χ^2	p-value
Food availability	1	2.11	0.146
Photoperiod	1	2.73	0.098
Latitude	1	148.53	<0.001*
Photoperiod × latitude	1	2.64	0.104
Food availability × latitude	1	4.07	0.044*
Food availability × photoperiod	1	0.52	0.471
Food availability × photoperiod × latitude	1	0.46	0.499
Size	1	3.77	0.052

Note: Analysis in which latitude of population from which the *Daphnia* clones were sampled was included as an interacting fixed effect is shown. Block was a random effect, $\sigma = 2.48$.

*Denotes significant terms.

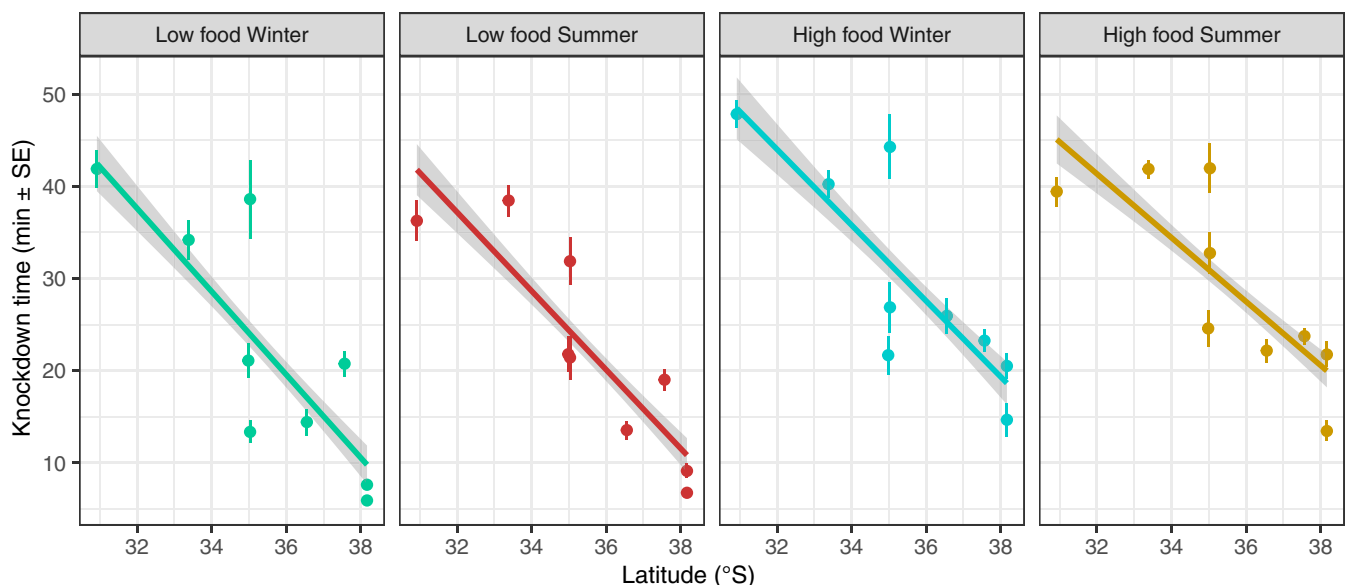


FIGURE 3 Thermal tolerance along a latitudinal cline (° S) and the effects of the food availability and photoperiod treatments of *Daphnia carinata*. Shown are the mean ($\pm$ SE) knockdown times at 37°C for the locations at which each *Daphnia* clone was sampled and the predicted trend across latitude ($\pm$ CI). High food availability treatment consisted of 5 million green algal cells daily, low food availability 1 million green algal cells daily. Summer photoperiod treatments consisted of an 18:6-h photoperiod, while winter photoperiod was 6 h:18 h.

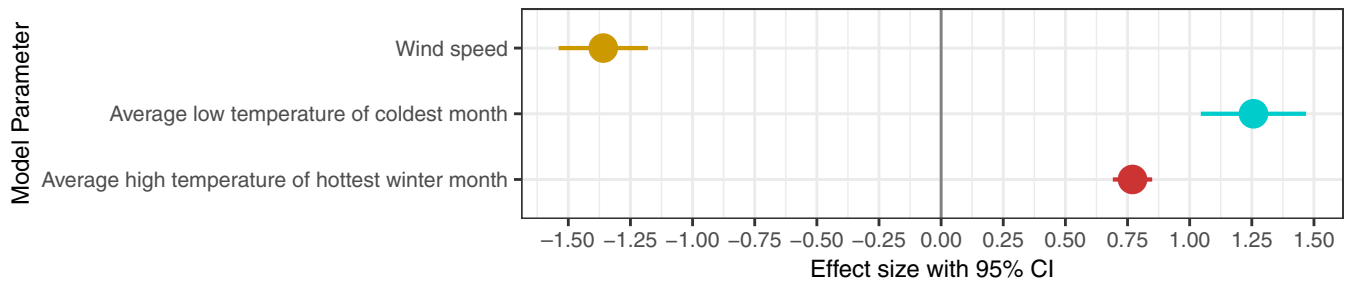


FIGURE 4 Effects of the micro-climatic variables of each sampling location on the knockdown times of *Daphnia carinata*.

The standardized effect size estimates from the best fitting model (lowest Akaike information criterion value) derived from eight different starting micro-climatic variables, covering high and low monthly temperatures, rainfall and altitude, are shown. Error bars indicate 95% confidence intervals. Parameters missing from the figure were amongst variables that were omitted from the best fitting model, and thus do not contain estimates. Photoperiod and food availability treatments were included in all models but omitted from the figure.

To explore how the geographic origin of each clone may shape its thermal tolerance in light of seasonal change, we modeled knockdown times against the latitude of the sampled populations. We found clear evidence of a latitudinal cline in thermal knockdown times, with clones from low-latitude populations exhibiting greater thermal resistance. The clinal relationship was similar in direction, with similar if not larger effect sizes, to reported for terrestrial ectotherm species such as *D. melanogaster* (Hoffmann et al., 2002; Sgrò et al., 2010), other *Daphnia* (Seefeldt & Ebert, 2019; Williams et al., 2012; Yampolsky et al., 2013) and interspecific trends (Sunday et al., 2011). For example, in *D. melanogaster* knockdown times decline at a rate of 0.21 min per degree south for heat shock assays at 39°C (Hoffmann et al., 2002) or 0.15 min per degree south at 37°C (Sgrò et al., 2010). Here, we observed a decrease of 3.78 min per degree south in high diet treatments or 4.40 min per degree south in low diet treatments, which corresponded to the relatively greater latitudinal variation in thermal resistance that is known to be a property of aquatic species when compared with terrestrial species (Sunday et al., 2011).

Driving these results is likely to be a local adaptation to the thermal conditions experienced at each location along the cline during winter (Figure 4). Across a wide collection of fine-scale microclimate data, both high and low winter temperatures remained some of the best predictors of variation in knockdown times amongst the clones. This was consistent with what we know about *D. carinata*, with populations expanding during cooler autumn and winter conditions, before declining during the extreme heat of summer (Burns, 2013; Lund & Davis, 2000; Vijayaraghavan, 1970). By comparison, European populations of *Daphnia* (*D. magna*) that are active in their planktonic state during summer are predicted to be locally adapted to the average high temperature during the hottest month of the year (Yampolsky et al., 2013). Follow-up studies involving a subset of “winter-active”

D. magna populations that survive summer in a dormant phase, found that thermal resistance did not correlate with the summer temperatures, similar to that observed in our analyses (Seefeldt & Ebert, 2019). Wind speed was also negatively correlated with knockdown times. Higher wind speeds are capable of decreasing surface temperatures of both marine (Qu et al., 2012) and freshwater water bodies (Ptak et al., 2019), particularly in smaller pools of water (Lamoureux et al., 2006). Consequently, increased wind speeds may lead to a lower ambient thermal environment, and the correlated reduction in relative thermal tolerances as a result.

Despite observing significant changes to knockdown times due to changes in photoperiod and food availability at the level of the clone, the relative changes in thermal tolerance along the latitudinal transect remained largely unaffected (Figure 3). In general, differences in the thermal tolerance of clones sampled along the cline far outweighed any changes in thermal tolerance that might be induced by variation in photoperiod or to a lesser extent food availability, as initially described in scenario (i) of our hypothesis. Transitioning from winter to summer photoperiods, for example, affected the thermal tolerance of clones at each location differently, but did not affect the strength of correlation between latitude and heat tolerance. Instead, a subtle increase in the strength of this correlation arose when animals were food limited, consistent with the idea that thermal limits are condition dependent (as reviewed in Chown & Nicolson, 2004). Unlike other environmental factors that reversed or weakened signals of local adaptation (Hector et al., 2019; Kuo & Sanford, 2009; Thawley et al., 2019), the general robustness of the observed clinal relationship to signals of seasonal change reinforces the utility of using clinal data for understanding and predicting the response of populations to climate change (Deutsch et al., 2008; Kingsolver & Buckley, 2017; Moran et al., 2016). This is of particular importance as predictions for the change in

species distribution in response to climate change, commonly assume that thermal tolerances remain constant across time and the changing of seasons (Kingsolver & Buckley, 2017).

Finally, while we detected a significant latitudinal cline for thermal resistance across nine populations that was largely robust to seasonal signals, and similar in strength to that of other studies in the field (Chakraborty et al., 2020; Hoffmann et al., 2002; Sgrò et al., 2010). Several opportunities exist to improve the generality of our results. First, the use of represented clones (but see also Seefeldt & Ebert, 2019; Williams et al., 2012; Yampolsky et al., 2013) is a key limitation and needs to be complemented by further study of the within-population variation in thermal tolerance at multiple locations along the cline, and ideally extending the cline beyond nine populations. This will formally quantify if the variation between populations is greater than the variation within, the main assumption of our study, as well as provide evidence for the adaptive potential of each population in the face of global change (Hoffmann & Sgrò, 2011). Second, thermal stress can result from a short exposure in extreme environments, which our static heat shock assays assess, but also slow incremental increases in temperature toward stressful levels (Chown et al., 2009; Hector et al., 2021; Terblanche et al., 2011). Prolonged exposure can elicit a different response in thermal tolerances due to either the plasticity of individuals or the accrued physiological damage (Hoffmann et al., 2003; Terblanche et al., 2011). The analyses of ramping thermal assays, which quantify critical thermal maxima and minima (CT_{max} and CT_{min} , as described in Hector et al., 2021), would offer the opportunity to assess how populations along the cline tolerate exposure to gradual changes in temperature and, if the impact of seasonality, or the lack of, was consistent across static and ramping thermal assays (e.g., Chown et al., 2009; Hector et al., 2021).

In conclusion, we observed clone-specific effects of photoperiod as well as general increases in knockdown times due to increases in food availability, signifying the important influence that seasonal change, beyond just temperature, can have on thermal resistance. We also showed local adaptation to thermal conditions in this system varied along a latitudinal cline, a relationship that persisted across all seasonal manipulations. Our results contribute to a growing awareness of the ecological conditions under which latitudinal clines in heat resistance will remain robust, or not, adding to the studies which have shown how warming generally elicits a plastic increase in the heat tolerance of populations equally (Hoffmann & Parsons, 1994; van Heerwaarden et al., 2016), whereas immune activation (Hector et al., 2019) or invasive species (Thawley et al., 2019) may erode any clinal signal.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data and code (Lush et al., 2024) are available in Monash University's Bridges repository at <https://doi.org/10.26180/25246543>.

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SUPPORTING INFORMATION

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

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