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Sex differences in the thermal acclimation and tolerance of *Daphnia* clones along a latitudinal cline

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Sex differences in thermal tolerance may significantly impact population responses to climate change, yet most studies examining geographic variation in thermal tolerance focus on a single sex. Here, we investigate how males and females might vary in their capacity to tolerate the heat along a latitudinal cline using the Australian water flea, *Daphnia carinata*. We measured heat knockdown times in males and females using clones of *Daphnia* from six populations spanning eastern Australia, raised under two acclimation temperatures (20°C and 25°C). Females consistently showed higher thermal tolerances than males, with greater capacity for thermal acclimation. Critically, we discovered that latitudinal clines in thermal tolerance were sex-specific: females showed a much steeper decline in heat tolerance with increasing latitude compared with males. As a result, sexual dimorphism in thermal tolerance was more than twice as large in northern populations compared with southern populations. These results suggest that sex-specific selection, potentially driven by differential habitat use or the female-biased demography of cyclical parthenogenesis, may be driving divergent thermal adaptation between the sexes. Our findings highlight the importance of considering both sexes when predicting population vulnerability to climate change, as sex-averaged estimates may misrepresent the heat tolerance of populations along environmental gradients.

1. Introduction

Global change is exerting unprecedented pressure on natural populations [1,2]. Understanding which populations are most at risk begins by measuring their innate capacity to cope with warmer temperatures or the upper temperature at which survival will be impaired (i.e. knockdown times or critical thermal maxima, respectively [3,4]). Indeed, research has shown that for many species populations closer to the equator have better heat tolerances (e.g. [5–7]; but see reviews [8,9]), which in concert with an understanding of their likely thermal exposure, can be used to assess a population's vulnerability to rising temperatures (i.e. thermal safety margins, [10]). However, the utility of these measures relies on the robustness of each populations' estimated tolerances to both abiotic and biotic sources of variation. Environmental factors such as food availability, thermal fluctuations or signals associated with seasonality have all been explored as potential modifiers of our ability to detect and quantify between-population variation in thermal tolerances [11–13]. Nevertheless, the heterogeneity that arises due to innate differences between individuals within a population also warrants consideration.

For many species, sex differences represent a significant source of within-population heterogeneity in thermal tolerances. Owing to either the exploitation of different ecological niches, competition over limited resources or the contrasting roles that each play in reproduction [14–16], males and females

commonly differ in behaviour, life-history, morphology and sexual displays. These differences extend to both their innate thermal tolerances and their capacity to acclimate or plastically respond to rising temperatures, but with considerable between-sex variability [17–21]. A study of 11 closely related *Drosophila* species, for example, showed that females were the more heat-resistant sex in five species, versus only two species for males, with no overt differences between males and females for the remaining five species [19]. Similarly, a recent meta-analysis has also shown that the sexes can vary in their capacity to acclimate to rising temperatures, but across all studies neither males nor females were necessarily more plastic on average [17]. Despite the evidence indicating that males and females might respond differently to rising temperatures, the impact these differences have on our understanding of between-population variation in thermal tolerances remains relatively underexplored (but notably see Lasne *et al.* [22], discussed below).

The study of thermal tolerances across large geographic areas, such as latitudinal clines, provide an ideal context for examining how sex differences might shape a population's capacity to cope with rising temperatures. Indeed, many studies have documented latitudinal and spatial variation in the difference between male and female size or morphology (reviewed in Blanckenhorn *et al.* [23]). However, few studies have investigated this context with regards to thermal tolerances. Lasne *et al.* [22] used Australian populations of the fruit fly, *Drosophila melanogaster*, to show how the relative changes in thermal tolerances across a latitudinal cline were parallel between males and females, and thus the impact of sex was consistent and predictable. By contrast, for the closely related *Drosophila simulans*, nonlinear clines for chill-coma recovery were significant for females, but not for males, indicative of emerging differences between the sexes (although formal comparisons were not made, see [24]). Similar population-specific differences between each sex were also suggested ($p = 0.051$) for the copepod *Acartia tonsa*, but limited to only a two-population comparison [25]. Overall, formal explorations of latitudinal variation in male–female thermal tolerances remain limited, with most studies focusing only on one sex, commonly females (e.g. [6,7,13,26,27]). It remains unclear whether sex differences in thermal limits are consistent across geographic regions or instead if the unique responses of males and females might disrupt our assessment of a population's ability to withstand elevated temperatures.

In this study, we used the Australian water-flea species, *Daphnia carinata*, to contrast the intrinsic thermal tolerances of both males and females, as well as their capacity to acclimate to elevated temperatures, along a latitudinal cline. *Daphnia* present an ideal test case for this question. They rapidly adapt to their local thermal environment [28], with the temperature of their main growing season in a lake or pond positively linked to a population's average thermal tolerance [13,26]. As a result, *Daphnia* from regions with higher average temperatures, such as from populations closer to the equator, are better able to tolerate the heat [13,26]. Much like with other study systems, however, the thermal tolerances of males have not been explored in this context [13,26,29], even though males are known to have lower tolerances to heat [30]. Here we used *Daphnia* genotypes sampled from along a latitudinal cline in Australia to approximate the predictable decline in thermal tolerance with increasing latitude that has been found in this species and others [6,7,13,27]. We then took advantage of the clonal nature of *Daphnia* to produce genetically identical males and females, before measuring the thermal tolerance of each sex along the cline, via static heat shock assays [13,30–32], at two different acclimation temperatures (20°C and 25°C). We examined whether the relative differences in thermal tolerances among *Daphnia* genotypes and their sampling latitude are consistent between males and females, or if each sex demonstrates a distinct response.

2. Material and methods

Our study organism is the aquatic water flea, *D. carinata*, a cyclical parthenogen found in freshwater ponds and lakes throughout Australia, New Zealand and Asia. This experiment used six clones of *D. carinata* (PLD, BWD, WWD, BLD, YYD, OZD; [33,34], but see electronic supplementary material, figure S1 and table S1 for sampling locations), previously collected in Eastern Australia along an 8° latitudinal cline from northern NSW (PLD, Tamworth, latitude –30.922, longitude 150.518) to southern Victoria (OZD, Geelong, latitude –38.166, longitude 144.338). Before beginning the experiment, stock animals from each genotype were grown individually in 70 ml glass jars filled with 50 ml of artificial *Daphnia* solution (ADaM, [35]; modified by [36]) for three generations to minimize any maternal effects. All animals were raised under standard conditions in a climate-controlled incubator (Snijders Labs Economic Premium: 20 ± 2°C with lights on, with a 16:8 h light:dark cycle), fed daily with *Scenedesmus* algae, and provided with fresh media twice weekly.

(a) Production of males and females and experimental design

Males and females from each genotype were produced by exposing mothers to the juvenile hormone methyl farnesoate (product ID: S-0153, Echelon Biosciences), following previous methods [37,38]. Briefly, once a mother produced their first clutch, they were transferred into 50 ml of hormone-treated media (300 µg l⁻¹). This media was refreshed every 2 days, and any offspring produced were collected, sexed and placed individually in jars of hormone-free media. From each genotype, individuals of each sex were randomly allocated from birth to either the 20°C or 25°C static temperatures and raised in these conditions. The artificial media was refreshed twice a week, and animals were fed daily with green algae. This process was repeated across two blocks, with each genotype, sex and temperature combination replicated up to 12 to 15 times per block, for a total sample size of 622 individuals overall (2 sexes × 2 temperatures × 6 genotypes × 2 blocks × 12–15 replicates).

(b) Thermal tolerance assays

Static heat shock assays and the time until immobilization occurs (herein, knockdown times, in minutes) were used to assess the thermal tolerance of male and female *Daphnia*, following previously established approaches [30–32]. Knockdown times measure an individual's ability to resist incapacitation during temperature extremes and have been used to compare thermal tolerance across regions in both *Daphnia* [13,26] and a variety of *Drosophila* species [6,7]. For each static shock assay, up to 48 individuals were placed in 5 ml glass vials filled with room temperature artificial media (20°C) and then placed inside a tank of artificial media held at 37°C via a programmable immersion heater circulator (model TH8500, Ratek). This temperature was chosen as it is an acute heat stress that is lethal to animals in several hours or less. Two individuals from each treatment were randomly assigned a glass vial in each assay. The media was constantly oxygenated using an aquarium pump, and vials were covered by mesh to minimise oxygen depletion over the experiment duration. Knockdown time was derived from the time when vials were fully submerged in the tank until the *Daphnia* were observed to have ceased all movement, including filter-feeding. Following the heat shock assay, the body size of each animal was measured using a stereomicroscope from above the eye to the base of the tail spine. Due to differences survival as well as handling errors, the complete set of data (knockdown times, and body size) was available for 564 individuals in total (see electronic supplementary material, figure S2), with samples sizes per treatment varying between eight and 28 individuals (median 26).

(c) Statistical analysis

All statistical analyses were performed in R (version 4.4.2; R Development Core Team available at <https://www.r-project.org/>) using the mixed-effect model package glmmTMB [39]. In all following models, the assay day per block (four levels: capturing both block and assay day variance) was modelled as a random effect, and the significance of all fixed effects evaluated using an analysis of variance (type II) via the car package [40]. The DHARMA package (<https://github.com/florianhartig/dharma>) was used to evaluate model fit, and subsequently a heterogeneous diagonal residual structure was used with separate variances for females and males. First (model 1), we investigated sex differences in the acclimation capacity of different genotypes using a model with knockdown time as the response variable, and biological sex (two levels: female and male), temperature (two levels: 20°C and 25°C) and *Daphnia* genotype (six levels: PLD to OZD, etc.) as interacting fixed-effects. Next (model 2), we investigated the influence of sex differences and acclimation on knockdown times along a latitude cline, using the same basic model as above, but with the latitude of the population that a *Daphnia* genotype was sampled from instead included as a covariate. Additional models controlling for the effects of body size are included in the electronic supplementary material, table S2. Finally, *post hoc* tests and targeted contrasts were estimated using the emmeans package ([rvlenth.github.io/emmeans/](https://rvinth.github.io/emmeans/)).

3. Results and discussion

(a) Genetic background and biological sex are dominant drivers of variation in thermal tolerances

Despite rising interest in the response of males and females to global change [17,21], most studies fail to report or confounded the sex of individuals, such as when studying thermal acclimation [17]. Not surprisingly, explorations of male and female thermal tolerances across large geographic regions, such as along latitudinal clines, remain rare, with current best evidence pointing towards males and females adapting to local thermal conditions in the same manner [22]. We first tested how the response of males and females to rising temperatures may depend on the genetic background of an animal (see also electronic supplementary material, figure S2 for knockdown time distributions per clone). Knockdown times were found to be significantly higher in females than males (figure 1B), and for animals acclimated to the warmer temperature treatment (figure 1A). However, the magnitude of change in thermal tolerances depended on significant pairwise interactions between acclimation temperature and biological sex with *Daphnia* clone (table 1, model 1).

The *Daphnia* clones, sampled from different locations along a cline, contributed the greatest source of variation in knockdown times, both in terms of the differences between clones (e.g. from 30.9 ± 3.83 to 75.13 ± 14 min for females on average, figure 1), and in the capacity of each clone to respond to warmer temperatures (table 2) or display sex differences (table 2). For example, although knockdown times were higher in females than males, the degree of sexual dimorphism ranged from 3.93 ± 3.9 min for genotype YYD, up to 27.1 ± 2.48 min for genotype WWD (table 2). The sex of an animal also influenced any acclimation to warming, with females, on average, showing a significantly greater improvement in their thermal tolerances under rising temperatures than males (e.g. increases of 11.3 ± 1.99 versus 7.4 ± 1.06 min, table 2). Our results highlight the complexities involved in establishing broad rules for how males and females should respond to rising temperatures. While females, on average, show a greater capacity than males to acclimate to rising temperatures, the relative thermal tolerances of males and females appear to be entirely dependent on the genetic background of the *Daphnia*. This result persists even after correcting for size difference (body size covariate $p < 0.05$, see electronic supplementary material, tables S2 and S3).

(b) Latitudinal clines in thermal tolerances are expressed more strongly in females than males

This variation in the strength of sex difference in thermal tolerances, with differences in knockdown times ranging from 3.9 min to 27.1 min, suggests that selection for thermal tolerance along the cline might be operating differently in males and females. For

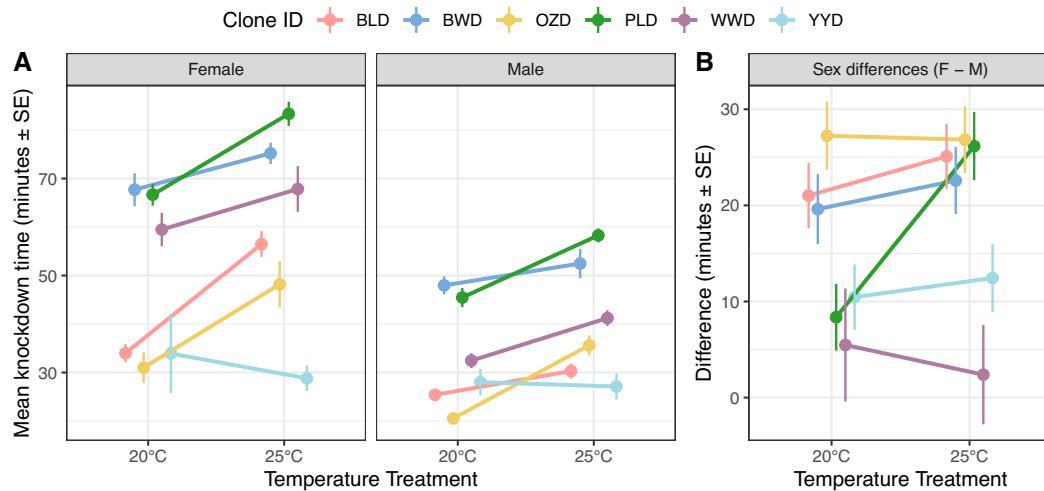


Figure 1. Reaction norms for (A) the effect of biological sex (males and females) and thermal acclimation (20°C or 25°C) on the thermal tolerances and (B) the predicted difference between males and females for each treatment combination. Shown are the mean knockdown times ($\pm$ SE) as measured via a 37°C static heat shock assay for *Daphnia carinata* sampled from six populations along a latitudinal cline in eastern Australia. Clone IDs, shown in the top legend, are arranged according to the latitude of the clones' sampling locations, with lower latitudes positioned on the left.

Table 1. Linear models for the analysis of variation in knockdown times, as partitioned by interactions between biological sex (two levels: female and male), temperature (two levels: 20°C and 25°C), and either the identity of the population a *Daphnia* clone was sampled from (six levels: PLD to OZD etc., model 1), or its latitude (a covariate, model 2). Models were run with assay day as a random effect (variance: model 1 = 9.44; model 2 = 9.49), using a heterogeneous residual structure with separate variances for females (variance: model 1 = 241.48; model 2 = 289.83) and males (variance: model 1 = 72.14; model 2 = 90.83), and evaluated (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$) as a Type II analysis of variance (model 1) or covariance (model 2).

fixed effects	χ^2	DF	p -value
model 1: analysis of variance with clone effect			
biological sex	312.369	1	< 0.001***
temperature	111.690	1	< 0.001***
clone population	667.324	5	< 0.001***
sex $\times$ temperature	4.817	1	0.028*
sex $\times$ clone	39.950	5	< 0.001***
temperature $\times$ clone	28.405	5	< 0.001***
sex $\times$ temperature $\times$ clone	9.451	5	0.092
model 2: analysis of covariance with latitudinal cline			
biological sex	249.383	1	< 0.001***
temperature	89.445	1	< 0.001***
latitude of sampling	488.729	1	< 0.001***
sex $\times$ temperature	3.746	1	0.053
sex $\times$ latitude	16.734	1	< 0.001***
temperature $\times$ latitude	0.223	1	0.637
sex $\times$ temperature $\times$ latitude	0.004	1	0.947

both sexes, the observed variation in knockdown times among the *Daphnia* clones declined with the latitude of the population that the *Daphnia* was originally sampled from, indicative of a latitudinal cline in thermal tolerances (see also [13,26]). However, the analysis of covariance model (table 1, model 2) revealed that the change in knockdown time with latitude depended on the sex of the individual (sex-by-latitude interaction, $p < 0.001$). While both males and females from populations closer to the equator were significantly more tolerant of the heat (figure 2, table 2), females had a much steeper decline in knockdown times than males ($\beta = -5.76 \pm 0.41$ for females, $\beta = -3.86 \pm 0.41$ for males). As a result of the sex-specific clines, sexual dimorphism in thermal tolerances was more than twice as large for *Daphnia* sampled from populations at lower latitudes (e.g. at 30.9° S, female–male difference = 26.20 ± 2.28 min) that those further from the equator (e.g. at 38.2° S, female–male difference = 12.40 ± 1.91 min).

Our results provide an interesting counterpoint to Lasne *et al.* [22], where male and female clines were parallel, suggesting that adaptation to local thermal environments in *Daphnia* operates differently in each sex. Parallel clines in male and female traits are expected to evolve when the trait optima for each sex shifts in a similar way, whereas divergent sex-specific clines will

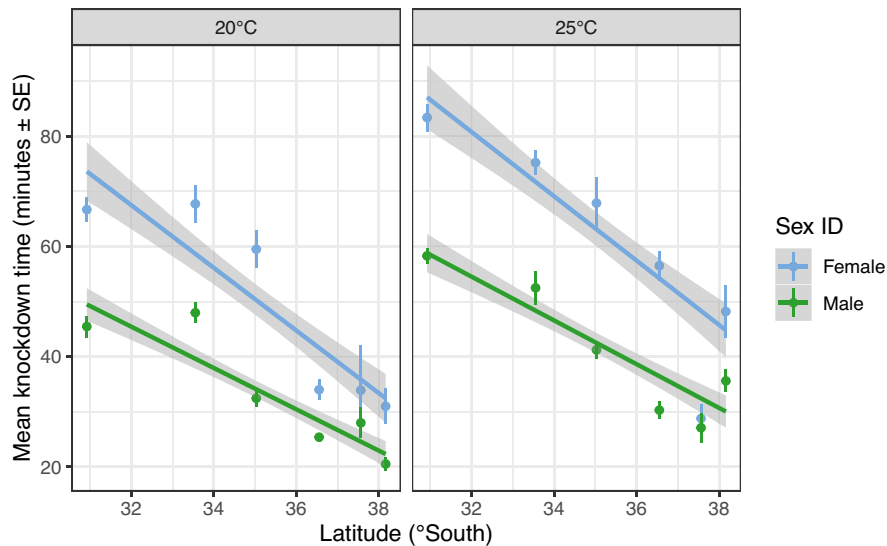


Figure 2. Thermal tolerance along a latitudinal cline ($^{\circ}$ S) for males and females of *Daphnia carinata* raised at two acclimation temperatures (20 $^{\circ}$ C and 25 $^{\circ}$ C). Shown are the mean knockdown times ($\pm$ SE), estimated via a static heat shock assay at 37 $^{\circ}$ C, for the locations at which each *Daphnia* clone was sampled (from left to right: PLD, BWD, WWD, BLD, YYD and then OZD, see electronic supplementary material, table S1), as well as the predicted trends across latitude ($\pm$ 95% confidence bands) as estimated via regression.

Table 2. Pairwise contrasts chosen to illustrate significant interaction terms derived from the analysis of variation in knockdown times due to interactions between biological sex (two levels: female and male), temperature (two levels: 20 $^{\circ}$ C and 25 $^{\circ}$ C), and the identity of the population a *Daphnia* genotype was sampled from (six levels: PLD to OZD etc., model 1 above, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

contrast	treatment	difference $\pm$ SE	p-value
temperature effect by sex, averaged over clone			
25 $^{\circ}$ C–20 $^{\circ}$ C	female	11.293 ($\pm$ 1.991)	< 0.001***
25 $^{\circ}$ C–20 $^{\circ}$ C	male	7.398 ($\pm$ 1.057)	< 0.001***
sex effect by clone, averaged over temperature			
female–male	BLD	17.265 ($\pm$ 2.481)	< 0.001***
female–male	BWD	21.100 ($\pm$ 2.522)	< 0.001***
female–male	OZD	11.449 ($\pm$ 2.453)	< 0.001***
female–male	PLD	23.060 ($\pm$ 2.399)	< 0.001***
female–male	WWD	27.046 ($\pm$ 2.481)	< 0.001***
female–male	YYD	3.930 ($\pm$ 3.908)	0.315
temperature effect by clone, averaged over sex			
25 $^{\circ}$ C–20 $^{\circ}$ C	BLD	13.456 ($\pm$ 2.481)	< 0.001***
25 $^{\circ}$ C–20 $^{\circ}$ C	BWD	6.126 ($\pm$ 2.522)	0.015*
25 $^{\circ}$ C–20 $^{\circ}$ C	OZD	15.954 ($\pm$ 2.452)	< 0.001***
25 $^{\circ}$ C–20 $^{\circ}$ C	PLD	14.530 ($\pm$ 2.399)	< 0.001***
25 $^{\circ}$ C–20 $^{\circ}$ C	WWD	8.467 ($\pm$ 2.480)	< 0.001***
25 $^{\circ}$ C–20 $^{\circ}$ C	YYD	–2.460 ($\pm$ 3.908)	0.529

arise when these optima do not shift in parallel and the trait is partially correlated between the sexes [22,41]. Here, the mixed mode of reproduction exhibited by *Daphnia* may facilitate this divergence in thermal tolerance. Males appear sporadically throughout a season, as male production is determined by several stimuli, including population density, food competition, and to a lesser extent, temperature [33,42]. This can lead to strongly female-biased sex ratios throughout most of the year, with females likely to experience a wider range and higher frequency of thermal extremes as a result, driving stronger selection for thermal tolerance and greater acclimation capacity. Even when both sexes coexist in a population, however, alternative selection mechanisms may be facilitating sexual differences in thermal tolerances. In other species, for example, behavioural differences can lead each sex preferring different thermal microhabitats, driving sex-specific selection in thermal physiology (e.g. anoles; [43]). Similar processes may occur in *Daphnia*, as females prefer the warmer, upper layers of thermally stratified water columns, while males are typically found in deeper, cooler waters [44].

4. Conclusion and future directions

In summary, our results highlight how heterogeneity due to male and female differences can potentially shape the distribution of thermal tolerances along a cline, and thus the relative risk each population may face under warming. In the case of *Daphnia*, the most appropriate thermal tolerances to use for predicting any risks under climate change would be females, due to the dominate role that the female-driven asexual life cycle has on population growth (i.e. female demographic dominance, [45]). Expanding beyond the use of representative clones (but see also [13,26]) to formally study within- and between-population genetic variability in thermal limits will help further resolve the extent to which females might be driving the evolutionary response to climate change in the long term. But for species in which population growth rates are linked more closely to both male and female fitness, it will be important to consider the thermal tolerances of the two sexes in combination, especially as the differences between them increases.

Increasing the generality of our results begins by considering the duration of thermal stress (ramping assays or cumulative heat stress, [31,46,47]) and its impact on the measurement of thermal tolerances in males and females. Heat shock assays, which form the basis of our study, represent just one metric for assessing an organism's tolerance to rising temperatures. Although previous research in *Daphnia* has shown that the results from heat shock assays closely align with those of more dynamic thermal assays with respect to biotic and abiotic determinants of thermal tolerance variation, especially at faster ramping rates [31], it remains unknown whether the degree of sex differences is influenced by the measurement approach (static versus dynamic, or via the cumulative impact of temperature). Ultimately, however, to fully unravel the conditions that underpin parallel (as in [22]) or divergent (as suggested in this study) thermal adaptation in males and females, more comparisons between populations (e.g. [22,24]) and across species (e.g. [19]) are needed, as these critical gaps currently limit our understanding of the risks males and females face under climate change.

Ethics. This work did not require ethical approval from a human subject or animal welfare committee.

Data accessibility. Data and code are available from the Monash Bridges Digital Repository [48].

Supplementary material is available online [49].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. M.D.H.: conceptualization, formal analysis, funding acquisition, methodology, project administration, supervision, visualization, writing—original draft, writing—review and editing; N.V.: conceptualization, data curation, investigation, project administration, writing—original draft; J.L.: conceptualization, investigation, methodology, writing—original draft; K.J.M.: conceptualization, data curation, methodology, project administration, writing—original draft.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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