**Responses of *Littorina* spp. intertidal snails to thermal extremes indicate countergradient variation in fitness**

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## Abstract

Global change models predict not only a steady increase in temperatures but also an increase in the occurrence of hot and cold extremes. Organisms’ responses to thermal extremes will depend on species-specific traits and the degree of within-species variation (among populations), with populations from warmer latitudes often predicted to have higher thermal tolerance than populations from colder latitudes. The evolution of population-specific responses, however, can be limited by gene flow that homogenises populations. Here, we investigate this relationship with a study of the survival of *Littorina littorea*, *L. obtusata*, and *L. saxatilis* – marine snails with varying dispersal potential – collected on either side of a known biogeographic break. Snails were lab-acclimated for several weeks before undergoing exposures to extreme heat, extreme cold, or ambient conditions, and individual mortality was recorded after each exposure. In line with common predictions, we observed that the degree of population divergence in survival under thermal extremes was negatively related to dispersal potential, and that populations from the colder latitude generally had higher survival of sub-freezing temperatures. Contrary to common predictions, however, we observed greater survival after extreme heat in populations from colder latitudes than in their warmer-latitude counterparts, a pattern known as countergradient variation. This experiment highlights counterintuitive responses to thermal extremes, emphasising that colder-latitude populations could experience population growth under more extreme climates due to higher survival at both hot and sub-freezing thermal extremes.

**Keywords:** countergradient variation, local adaptation, thermal tolerance, latitudinal gradient,

marine

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Editors-in-Chief

Ecology and Evolution

Dear Editor,

We are pleased to resubmit our manuscript **“Responses of *Littorina* spp. intertidal snails to thermal extremes indicate countergradient variation in fitness”** to be considered for publication as a research article in *Ecology and Evolution*. Our original submission was assigned the manuscript reference number **ECE-2023-12-02160**. This research provides valuable insight into the adaptive potential of marine invertebrate populations in the face of global climate change, and we believe that the content and findings of this study, as revised, make it well-suited for publication in your journal. We confirm that our manuscript presents original research that has not been submitted or published elsewhere.

Our study aimed to investigate population divergence across three intertidal snail species by comparing their survival after exposure to thermal extremes. Our findings illuminated a countergradient pattern of thermal tolerance, where the level of population divergence covaried with species’ dispersal potential. One of the key strengths of this research article is its comparative approach: by juxtaposing the responses of three congeneric species, we were able to provide an analysis of the role of organismal life history in either facilitating or limiting potential for local adaptation. With thanks to our three reviewers and associate editor, we have thoughtfully revised and strengthened our manuscript and believe that it represents a valuable contribution to the scientific discourse on climate change impacts on biodiversity. Our detailed responses to the reviewers’ and editor’s comments are included beginning on the following page.

We would be very glad if you would consider our revised article for publication in *Ecology and Evolution*. Thank you for your time and consideration, and we look forward to hearing from you.

Sincerely,

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**Response to Editor and Reviewers**

**Associate Editor**

**Comments to the Author:**

The manuscript explores heat and cold tolerance divergence in littorinid snails, offering valuable insights into thermal adaptation. The reviewers did a careful job with suggestions to improve the presentation and justification of some methods (although one reviewer recommended rejection). Having worked with snails as study model, I would add that is a pitty that authors did not perrform an individually based analysis of phenotypic selection, as for snails this is very easy to do (just put bee marks in each individual and check survival). This, together with the most recent spline/non-parametric adjustments, gives very good statistical power. I really encourage authors to try this kind of experiments in the future. In terms of the present ms, I would tone-down those claims that most studies do not compare multiple populations or species. There are a LOT of such studies, in a range of organisms and acclimation settings (ecto and endotherms). Given the comments of the reviewers (very detailed, with concerns regarding several methodological and interpretation aspects), I am recommending rejection with resubmission possibility.

***Author response:*** *We thank the editor for your valuable feedback and your insight into the design of future studies. As suggested, we have modified the statements relating our study to previously published work, in addition to incorporating various additional references to existing studies.*

**Reviewer: 1**

**Comments to the Author**

This study aims to detect survival differences on different sympatric species of littorinids (L. littorea, L. obtusata and L. saxatilis), from two localities (at both sides of a know biogeographic break, "Cape Cod"), when these are exposed to extreme hot and extreme cold treatments ("thermal extremes") in the laboratory. The results showed greater survival of L. littorea (with greater dispersal than the other spp) in extreme conditions and greater survival of the northern populations at both extreme (hot and cold) temperatures.

Although the study is interesting for the scientific community, the experimental design seems limited to make such strong claims as stated along the manuscript. The main problems are the lack of replicates in terms of populations (localities), the lack of a better representation of the environmental gradient (latitudinal) (including more localities further north and further south), the use of only one extreme temperature for cold or hot and using survival rate as a phenotypic measure (instead of other physiological measurements). On the other hand, the use of species that occupy different niches (like the ones in the manuscript) make it more complicated to compare the results, even though they belong to the same genus.

***Author response:*** *We appreciate this important perspective and have substantially revised the manuscript to provide clearer acknowledgement of the limitations of the our study’s experimental design. Our point-by-point revisions are detailed below.*

Besides these comments I still think that the study is worth of publishing in Ecology and Evolution after major revision of the manuscript. I include here all the comments on the manuscript:

Line 27. Multiple populations? As far as I can see in the manuscript, you only study two populations (localities), therefore you are not filling this gap in research. Please reword this or the next sentences accordingly.

***Author response:*** *This phrase has been removed in conjunction with other suggested revisions.*

Line 31. Two latitudes. When I read this for the first time it looks like very different latitudes, which they are not really if we take into account the distribution of these spp. Please reword.

***Author response:*** *This statement has been revised to reflect the proximity of the two sampling locations. The revised text on line(s) 28-29 now reads, “collected on either side of a known biogeographic break”.*

For example line 91-93 talks about intraspecific variation and environmental gradients, the phenotypes studied here (survival) might not be a good or reasonable representation of intraspecific variation in thermal adaptation as it is not a continuous variable and the two localities studied here are not either a good representation of an environmental gradient although they represent different thermal regimes.

***Author response:*** *These lines have been revised to more accurately convey the framework of this study, and they read as follows on lines 604-606:*

*“[...] therefore, it is essential that we first gain a stronger knowledge of the extent of vulnerability among natural populations in differing environmental regimes.”*

Methods

The introduction to the "study species" is rather short, and since great part of the importance of the study comes from the "multi-species comparisons", more background on these littorinids could certainly be added (here or in the general introduction). On the other hand, I think that the reference "The systematics and evolution of Littorina" (Reid 1996) should be included somewhere in the manuscript as it represents a key review for littorinid gastropods. Littorinids are becoming models in ecology, evolution and other fields and maybe this should be highlighted along the manuscript with other recent references. It is also important to discuss somewhere in the manuscript the fact that these species, although closely related in their phylogeny, they represent quite different niches in terms of temperature, and these niches they might even change locally within species as different localities might have very different intertidal habitats. Therefore the comparison of the life history traits in terms of dispersal might not be directly comparable as their microhabitats within shore are different. On the other hand extreme temperatures might influence differently larvae/embryos depending on the type of reproduction. These two issues should be discussed in the manuscript.

***Author response:*** *The background on the focal littorinids has been expanded (and moved to the introduction for organisation). We agree that David Reid’s 1996 book is a highly relevant reference, and we have incorporated it into the manuscript in several contexts (line 680, line 878). We have also added an array of more recent references throughout the manuscript, primarily in the discussion. Additionally, the possibility of microhabitat variation that inhibits a direct comparison of the organisms’ dispersal-related life history traits is now addressed in the discussion (lines 897-901), as is the potential for different modes of reproduction to be differentially impacted by thermal extremes (lines 846-856).*

I could not find along the manuscript a reasoning for the experimental design in terms of the localities chosen. The authors mention that they are at both sides of the biogeographic break represented by Cape Cod. For example in the case of L. saxatilis, previous studies (Doellman et al. 2011 doi: 10.1098/rspb.2011.0346; Panova et al. 2011 10.1371/journal.pone.0017511) showed a mixture of two highly divergent haplogroups (lineages) in Maine. This might be also happening for other littorinids with restricted dispersal like L. obtusata. This previously described high genetic divergence (mtDNA) might affect temperature adaptation in different ways, this should be discussed also. Fig. 2 shows certain differences between the localities although I think that other temperature variables should be also included, as max. temp. might not represent the most important selective force in intertidal non-sesile species. Littorinids are known for active ways to avoid dessication during low tide. I don´t have experience in the case of low temperatures but maybe they might have other sort of adaptations as the geographical distribution of these species reaches really high latitudes. For these reasons I think that these two topics, the genetic divergence across that geographical region and the fact that those localities might not represent an important latitudinal gradient in temperature, should be discussed in the manuscript in relation to the results obtained, but maybe already mentioned in the introduction.

***Author response:*** *We thank the reviewer for highlighting these considerations and have added a discussion of the divergent intra-population lineages both to our explanation of the study sites chosen (lines 707-713) and to our interpretation of the population-level results (lines 889-892). The populations included in this study come from localities with different patterns of haplotype diversity, as Cape Cod & Cape Cod Bay represent a division in the extent of mixing between haplogroups (as per the suggested references Doellman et al. 2011 and Panova et al. 2011). Incorporating the patterns presented in prior studies, we explain that our southern locality – geographically proximate to the Jamestown, Rhode Island population in Doellman et al. and the “Mass-3” population in Panova et al. – includes snails likely exhibiting very low haplotype diversity, while our northern locality – best represented by Doellman et al.’s Nahant, Massachusetts population and Panova et al.’s “Mass-1” population – likely represents a population mixing two distinct lineages, which we now discuss in the context of population-level observations. We have also included a more specific acknowledgement of the limitations of our study with respect to the localities chosen (lines 873-874).*

Another potential issue in the design is the use of only two temperatures (room temp. represents the control), although they were extreme temperatures this might not be the best way to test adaptation in these species. I think that this issue should be discussed in the manuscript, and also by mentioning that in the future different temperatures should be evaluated and maybe not only death and other physiological variables associated to fitness. I think that the authors should go through previous studies such as Dwane et al. (2023).

***Author response:*** *We agree that both of these aspects limit the inferences that can be drawn from this manuscript and have added to the discussion to provide specific guidance for future work to improve on our study design with regard to the range of temperatures tested and the use of mortality rather than a continuous physiological parameter, including references to highly relevant studies (lines 923-935). We have also added a more explicit link between physiological approaches and mortality by way of Cicchino et al.’s 2023 study (lines 637-638).*

MINOR COMMENTS

Line 193-195. These data were not included in the analyses, as far as I understood, so what was the goal of including this paragraph in the manuscript?

***Author response:*** *We thank the reviewer for catching this oversight and have removed the irrelevant lines from the manuscript.*

Line 203-205. "persistence of populations", which populations/species? This is a key issue because this sentence is used as a working hypothesis for the study. How likely are extreme thermal events to affect littorinids? many of these extreme thermal events might be avoided through different behaviours during exposure to high temperatures and maybe also during low temperatures. Please discuss this issues in the manuscript.

***Author response:*** *This sentence has been revised to clearly refer to marine intertidal gastropods according to Denny et al. (2009) and Wethey et al. (2011) (line 740). Considering studies including Stafford and Davies (2004) and Chapperon et al. (2017), the behavioural adaptations of high-shore littorinids such as* Melarhaphe neritoides *and* L. saxatilis *provide the benefits of reduced desiccation stress, dislodgement risk, and predation risk. Although temperature and desiccation likelihood no doubt correlate with one another (i.e. warmer conditions = more desiccating), the snails’ behavioural adaptations have been found to have limited effect on their body temperatures, such that their successful avoidance of desiccation might at some point be superseded by the thermoregulatory challenge of extremely high temperatures in and of themselves. Discussion of this context has now been added to the manuscript (lines 668-674).*

Line 305-307. As I mentioned previously, these snails have active ways to avoid high temperatures, therefore to me this hypothesis needs further support from previous studies and not just assume something that might not be true.

***Author response:*** *This is a valuable note about needed clarification; thank you. As described in our previous response, we now address this context in the manuscript in lines 668-674.*

Line 316. L. brevicula is broadcast-spawner but lives in a different habitat and it would also be interesting to know the latitudes included in Chiba et al. (2016). This info should be included in the discussion.

***Author response:*** *Chiba et al. (2016) included localities ranging from 31° N to 44° N (a notably wider range than those in our study, perhaps contributing to the disparity). This information has been added to the discussion. Additionally, a consideration of the mechanism of inter-population variation proposed by Chiba et al. and its potential relevance to* L. littorea *has also been included (lines 868-873).*

Line 317-321. Assuming greater admixture based in a study from 1973 is not reasonable to me. This discussion should be elaborated a little bit more based on other findings or other hypotheses.

***Author response:*** *Our phrasing, “greater admixture than was initially assumed”, was poorly worded and conflicted with our stated hypothesis that* L. obtusata *would exhibit a degree of variation between that of* L. littorea *and* L. saxatilis*. The sentences have been reworded (lines 875-880), and additional citations of studies that demonstrated detectable, low-level genetic divergence among populations of* L. obtusata *have been included (Wares and Cunningham 2001, Schmidt et al. 2007).*

Line 328-333. This part of the discussion goes against the experimental design, therefore the current design should be justified further.

***Author response:*** *In conjunction with the reviewer’s earlier comment regarding the divergent haplogroups known to exist in* L. saxatilis*, discussion of the differing haplotype diversity between the two sampling localities has been incorporated into the methods section for added clarification (lines 707-713).*

Line 338. See Dwane et al. (2023)

***Author response:*** *Discussion of Dwane et al.’s 2023 findings has been incorporated throughout the manuscript, and in this instance into the “Patterns of countergradient variation” section of the discussion (lines 942-944).*

**Reviewer: 2**

**Comments to the Author**

This study examines how heat and cold tolerance have diverged in three species of littorinid snail, collected from two sites. The variation in life history and intertidal range distributions across these species provides an interesting case study in thermal adaptation, although the authors focus the conceptual dimension of the paper on the comparison of Genetic-Environment covariance across species. Combined, this study is a valuable contribution, and I enjoyed reading it. I have a few suggestions, however, on places where the authors could improve the clarity, or the accessibility of the manuscript for a generalist audience.

Background information:

It would be useful to have one or two more sentences in the introduction to better describe CovGE to a generalist audience, and highlight why it matters in the context of climate change.

***Author response:*** *Additional context describing the effects of CovGE and their relevance to climate change has been added to the introduction (lines 610-612, 615-617), hopefully providing clearer explanation.*

I'd suggest the authors cite a recent study on linking acute thermal limits to chronic effects of temperature in lines 121-123.

Cicchino, A. S., Ghalambor, C. K., & Funk, W. C. (2023). Linking critical thermal maximum to mortality from thermal stress in a cold-water frog. \_Biology Letters\_, \_19\_(6), 20230106.

***Author response:*** *This study provides valuable clarification of the link between mortality and physiological measurements of tolerance and has been added to the manuscript (lines 638-639).*

In places where patterns in thermal limits are described (e.g. lines 134 & 346), I'd suggest the authors cite a recent meta-analysis on population-level divergence in thermal tolerance in addition to the Sunday paper, which is a species-level analysis.

Sasaki, M., Barley, J. M., Gignoux-Wolfsohn, S., Hays, C. G., Kelly, M. W., Putnam, A. B., ... & Cheng, B. S. (2022). Greater evolutionary divergence of thermal limits within marine than terrestrial species. \_Nature Climate Change\_, \_12\_(12), 1175-1180.

***Author response:*** *This is an excellent additional reference and has been incorporated into the manuscript in appropriate places (lines 651-652, 659).*

Methodological clarifications:

The temperature data acquisition and processing should be described in the methods. Related to this, why were water temperatures not examined? It might be expected that for species with distributions in the lower intertidal, water temperature may have a larger effect on patterns in temperature tolerance (providing an alternative explanation to increased admixture for the observed lack of divergence in L. obtusata).

***Author response:*** *Intertidal organisms in all zones experience the most extreme temperature stresses during low tides when they are exposed to air, both in hot conditions and freezing conditions; as such, their distributions are driven strongly by air temperature (Sokolova & Pörtner 2001, Somero 2002, Helmuth et al. 2006, Helmuth et al. 2011). We chose to examine air temperatures for this reason.*

Is there information about the water and air temperatures just prior to collection? A key assumption of this study is that all previous environmental effects are erased by the ~40 day acclimation period. This is probably a safe assumption, but should be stated somewhere.

***Author response:*** *Mean air temperatures over the week prior to collection were 20.6°C and 22.8°C in the southern and northern localities respectively, and mean water temperatures were 17.0°C and 17.7°C. Thus, neither locality appeared to be experiencing a particularly notable thermal event (now acknowledged in line(s) 699-701). We have also included in the methods section the explicit clarification that the role of the acclimation period was to remove the influence of possible phenotypic plasticity stemming from the snails’ previous environment (lines 721-722).*

How many sea tables were used in the study? It was a little unclear how the blocks were structured - Initially, two blocks (two sea tables) were described (lines 184-186). However, in the next section, two temporal blocks were described, pulling from three sea tables (lines 197-198).

***Author response:*** *We thank the reviewer for this comment – the sentence previously on lines 197-198, “Two temporal blocks were assembled, with samples in each block randomly chosen from the three seatables,” appears to have been erroneously left in from an earlier version of the manuscript and has been removed.*

Why were two three-hour temperature exposures used? Can the authors provide details on how quickly temperature adjusted during the exposures? How long did snails experience the target temperature for? Were tanks shielded from the extreme light levels during the heat exposures? If not directly affecting the snails, these light levels may have influenced the algae (e.g. photo-inhibition and the production of reactive oxygen species).

***Author response:*** *Two three-hour exposures were used to mimic successive daytime open-air exposures occurring around low tide (see lines 758-760). Snails experienced the target temperature for the full duration of their three-hour exposure, as the exposure chambers were allowed to reach temperature before any snails were exposed to the treatment (see lines 747-748). Additionally, snails were shielded from extreme light in the heat treatment by opaque lids. This was not noted in the manuscript, and a sentence has been added to provide this information (lines 749-750).*

I would suggest the authors specify somewhere in the methods that they are using a static temperature stress assay, rather than a dynamic assay. These assays examine different, but comparable, aspects of thermal biology.

Rezende, E. L., Bozinovic, F., Szilágyi, A., & Santos, M. (2020). Predicting temperature mortality and selection in natural Drosophila populations. \_Science\_, \_369\_(6508), 1242-1245.

***Author response:*** *We appreciate this suggestion, and this clarification has been added to the methods section (lines 734-735). We also discuss Rezende et al.’s 2020 study later in the discussion (lines 930-935).*

Could the authors also clarify the phenotypic metric used? The methods describe using mortality (in the experiments, and in the stats (e.g. - line 228)), but in the results, the phenotypic metric is described as "the proportion of days survived" (lines 248 & 293). Should this be "proportion of snails survived"?

***Author response:*** *Yes, the reviewer is absolutely right – the metric used was the proportion of snails surviving post-exposure. Both instances of incorrect wording have been revised (line 788, line 832).*

Results and Discussion:

In general, I think the authors did a really nice job organizing the complex set of results into a logical structure. I'd suggest, however, that the authors keep the species- and population-level comparisons separate. Line 270-272 appears to mix species comparisons (L. littorea and L. saxitalis) with population comparisons (northern vs. southern saxitalis). The population level comparison is then described again in the next paragraph (lines 280-282).

***Author response:*** *Thank you for your kind feedback. Per this suggestion, we have revised this portion of the results section so as not to mix species- and population-level comparisons.*

I found the wording in lines 315-316 a little awkward. Perhaps "exhibit" instead of "experience" differentiation?

***Author response:*** *The wording has been revised in accordance with this suggestion.*

The authors suggest the lack of divergence in L. obtusata indicates higher than expected admixture. Is there genetic data to back this up? Otherwise, there are other alternatives as well that are worth mentioning. Are these populations relatively recently diverged? Is there just not that strong of selection, given the lower intertidal distributions?

***Author response:*** *Our phrasing, “greater admixture than was initially assumed”, was poorly worded and conflicted with our stated hypothesis that* L. obtusata *would exhibit a degree of variation between that of* L. littorea *and* L. saxatilis*. The sentences have been reworded (lines 875-880), and additional citations of studies that demonstrated detectable, low-level genetic divergence among populations of* L. obtusata *have been included (Wares and Cunningham 2001, Schmidt et al. 2007).*

There's a sentence fragment in lines 321-322.

***Author response:*** *This has been amended.*

I'd suggest a brief section outlining potential confounding effects, or why they're not expected to be confounding in this case. For example, with the single collection from each of the two populations, the results may be affected by the environmental conditions directly proceeding collection (a particularly warm day at the northern site, for example). There is also likely variation in the quantity and quality of food at the sites that may influence the results (e.g. partially explaining the observed counter-gradient variation if snails developed in a food rich environment in the north, but a food poor environment in the south). The age of the snails is also a potential factor - you might expect the snails from the northern site to be younger than the snails from the southern site given a later start to the reproductive/hatching/dispersal season in the colder water. Could younger snails be more resistant to environmental extremes?

***Author response:*** *Thank you for this suggestion. We have incorporated discussions of potential confounding effects as relevant throughout the discussion, including the possible effect of age (lines 853-856), the likely genetic diversity within our northern population (lines 889-896), the potential for the range of microhabitat variation to differ between localities (lines 897-901), and the likelihood of varying food availability/quality (lines 901-902). We also now address the similarity of pre-collection temperatures between localities in lines 699-701 of the methods.*

Code:

The code provided by the authors is a nice narrative describing the analyses. There is some irrelevant information (e.g. the alternate method from Line 260 onwards in `LittorinaStatistics.Rmd` and suggestions for how to report results in `LittorinaCovGE.Rmd`) but overall I found the code easy to work through and leave it up to the authors to decide whether changes are made.

***Author response:*** *We appreciate these suggestions and have revised both scripts accordingly. The up-to-date version (v2) of the code is accessible at* [*https://doi.org/10.5281/zenodo.13738093*](https://doi.org/10.5281/zenodo.13738093)*; the manuscript’s data accessibility statement has likewise been revised to reflect this.*

**Reviewer: 3**

**Comments to the Author**

MS ECE-2023-12-02160 deals with a study on temperature acclimation in three intertidal marine species. The authors use two extreme temperatures above/below cero (44.75 and -12.25) to check the degree of mortality that can affect to different species and populations (southern and northern populations from Cape Cod). The study presents several interesting opportunities, like the option to study potential adaptive strategies of survivorship at extreme air temperature related to intertidal species that live in sympatry in that area, as well as incorporating some geographical replication (southern and northern populations, etc). However, as I will argue below the study shows several strong flaws that prevent its publication.

***Author response:*** *We thank the reviewer for your thoughtful and detailed feedback. We are grateful for the care and clarity of your comments, and we believe our manuscript has been substantially strengthened following the insights received from you and our other two reviewers.*

1. The approach to infer specimen acclimation and survivorship in extreme temperatures.

The study presents two extreme air temperatures to specimens of several intertidal species. However, the used temperatures have apparently never been experienced in the wild (see maximum and minimum monthly temperatures experienced in the two locations from Figure 2) at least for the high temperature and rarely for the low one. Moreover, the classical approach to infer the thermal tolerance of different organism is by using thermal performance curves (TPCs; see for example Science of the total environment, 863: 160877). Under such approach the organisms are experienced at least a range of 3-4 different temperatures in order to infer the curve of the relationship. Moreover, often some physiological measurements can be simultaneously checked (like heart rates) in order to confirm that the response is physiological. In addition, the temperature acclimation in those kind of studies should be done on at least two geographically distinct populations and two distinct temperatures, in order to distinguish genetic versus plasticity adaptation. This last point is also relevant as the present MS is being revised for evolutionary ecology, while they do not know anything about whether the observed effects are genetic or plastic (a key factor for being “evolutionary”). To characterize the species thermal tolerance, the authors use only two, and geographically very close, populations, while typically more populations are used (see references below). Actually the two populations show very similar environmental parameters (see Figure 2), which limits the interest of the comparison. In addition, to use just a simple freezer to check low temperatures seems to too simple and unrealistic, as they are not included in algae like in nature. Finally, but still important, the treatment is produced in sets of specimens together rather than by individual snails, and so they do not have proper errors of the estimates.

***Author response:*** *We agree that the incorporation of a thermal performance curve and the inclusion of additional more geographically distinct populations would provide valuable information and a broader perspective in future studies. In the context of this study, we have taken care to discuss the limitations of our experimental design in greater detail in the discussion (lines 923-935), including comparisons to other studies that robustly examined particular species. We have also incorporated Cicchino et al.’s 2023 paper, “Linking critical thermal maximum to mortality from thermal stress in a cold-water frog” into the manuscript introduction to better situate our methodology in the context of classical approaches (lines 638-639). Further, we have clarified in the text that the snails were exposed to both treatments while in their environmental microcosms, such that they had the option to be in fresh algae if they chose. This was not originally made clear in the manuscript; thank you for noting it.*

2. This study has ignored most recent specialized bibliography in relation to this topic in the same or related species, see for example:

Lee & Boulding 2010. Latitudinal clines in body size, but in thermal tolerance or heat shock cognate 70 (HSC70), in the highly dispersing intertidal gastropod Littorina keenae (Gastropoda: Littorinidae). Biol. J. Linn. Soc. 100: 494-505.

Dwane et al. 2021. Divergence in thermal physiology could contribute to vertical segregation in intertidal ecotypes of Littorina saxatilis. Physiol. Biochem. Zool. 94: 353-365)

Dong et al. 2021. An integrated, multi-level analysis of thermal effects on intertidal molluscs for understanding species distribution patterns. Biol. Rev. 3:

Dawne et al. 2023. Thermodynamic effects drive contergradient responses in the thermal performance of Littorina saxatilis across latitude. Science of the Total Environment, 863: 160877.

And this is an example and I did not pretend to be exhaustive, the authors should check these references and search for other similar studies or better to do a new bibliographic search and incorporate it to introduction and results discussion. Notice for example that Dwane et al. (2023) have observed similar results to present study in L. saxatilis but with a right design (see former point above).

***Author response:*** *In our revision provess, we have reviewed and incorporated a number of recent relevant studies into the introduction and discussion sections of the manuscript. This has allowed us to better relate our study to the existing literature and, we believe, has distinctly strengthened the manuscript.*

*In addition to the four references provided above by the reviewer, our additions include:*

*Sasaki et al. 2022. Greater evolutionary divergence of thermal limits within marine than terrestrial species. Nature Climate Change 12:1175–1180.*

*Blakeslee et al. 2021. Population structure and phylogeography of two North Atlantic* Littorina *species with contrasting larval development. Marine Biology 168:117.*

*Reid & Harley 2021. Low temperature exposure determines performance and thermal microhabitat use in an intertidal gastropod (*Littorina scutulata*) during the winter. Marine Ecology Progress Series 660:105–118.*

*Urban et al. 2020. Evolutionary origins for ecological patterns in space. Proceedings of the National Academy of Sciences 117:17482–17490.*

*Chapperon et al. 2017. Mitigating thermal effect of behaviour and microhabitat on the intertidal snail* Littorina saxatilis *(Olivi) over summer. Journal of Thermal Biology 67:40–48.*

3. The discussion should change considerably, by adding all the references mentioned in point two, by considering ontogenetic and latitudinal effects already studied in L. saxatilis (see Dwane et al. 2021 and 2023), etc. In addition, most results observed cannot be excluded to be caused by differences in phenotypic plasticity between species and population and so this should be detailed considered at all levels.

***Author response:*** *In conjunction with this comment and suggestions from the other reviewers, the discussion has been extensively revised and expanded. The ontogenic effects on thermal tolerance presented in Dwane et al. (2021) have been incorporated into our discussion of the potential inter-species variation existing among the focal littorinids (lines 851-853) and our discussion of the possible confounding effects influencing the inter-population variation observed in* L. saxatilis *(lines 889-902). Likewise, the results of Dwane et al.’s 2023 study are now discussed in the context of this study’s observation of countergradient variation between populations (lines 942-944). Additionally, we agree that phenotypic plasticity is likely at play in the premise of this study, as we suggest that our observations may stem from evolved differences in phenotypic plasticity (i.e., different populations have evolved to have different extents of plasticity). The framework and metric presented in Albecker et al. (2022) provide a way to measure how phenotypic plasticity covaries with genetic differentiation, and as the populations respond significantly differently to one another when exposed to the same thermal conditions (i.e. a common garden), we find this indicative of genetic differentiation between populations.*

4. In addition, I found some other minor points that could be perhaps considered.

. Cape Cod is rather atypical geographical area, with two environmental apparent different sites (although not so following present figure 2), perhaps some figure showing the locations of the study and several further information about the two sites could be needed in M&M.

***Author response:*** *Thank you for this suggestion. Figure 2 has been updated to include an inset map displaying the locations of the two specimen collection sites relative to Cape Cod, which we hope provides helpful context regarding the study localities.*

. local adaptation does not seem and adequate keyword, as actually plasticity cannot be considered adaptation itself.

***Author response:*** *As discussed above, we suggest that while the focal species may indeed (and almost certainly do) have the capacity for phenotypic plasticity, this plasticity does not negate the local adaptation demonstrated by the two populations of* L. saxatilis *(in both thermal conditions) and the two populations of* L. obtusata *(in the cold condition) in this study. An individual from a given population would likely moderate its response depending on the environment in which it was placed (i.e., phenotypic plasticity), and individuals from different populations also responded significantly differently to one another under the same environmental conditions (i.e., suggestive of local adaptation).*

. Snail and seaweeds Images from figure 1 seems of too low quality for a formal figure, although the general idea seems fine.

## *Author response: Thank you for this feedback: we will upload a higher-resolution version of Figure 1 with our resubmission.*

## Introduction

Predicting species vulnerability to climate change, as well as the vulnerability of populations within species, is a fundamental goal in biodiversity conservation. Local adaptation plays a valuable role in mediating species’ responses to climate change and refers to the process by which a given population evolves to be better adapted to its environment than populations of the same species from other environments (Razgour et al. 2019, Thompson and Fronhofer 2019). More often than not, however, climate change projections exclude factors like local adaptation, largely due to a lack of necessary data on underlying mechanisms such as population differentiation and the impacts of environmental variation (Urban et al. 2016). To offer representative predictions of species’ future success, therefore, it is essential that we first gain a stronger knowledge of the extent of vulnerability among natural populations in differing environmental regimes.

One factor contributing to local adaptation is the occurrence of covariance between genetic and environmental effects on phenotypes (CovGE), which can cause genotypes to be distributed nonrandomly across an environmental gradient (Conover and Schultz 1995, Trussell and Etter 2001). Spatial CovGE may emerge in a pattern that either amplifies environmental variation or dampens it, respectively maximising or minimising phenotypic variation across environments (Urban et al. 2020). Past studies (Conover et al. 2009, Hoffmann and Sgrò 2011, Savolainen et al. 2013, Hu et al. 2020) have demonstrated that organisms’ specific genotypic and environmental relationships may form the basis for their differing responses to environmental gradients; thus, as the magnitude and direction of CovGE can play a fundamental role in determining organisms’ responses to changing or novel environments, an understanding of these interactions is essential to robustly predicting species outcomes under climate change.

When CovGE is negative, a system demonstrates countergradient variation, in which the environmental influence on the phenotype is opposed by the genetic influence, thereby minimising phenotypic variation from environment to environment. As such, countergradient variation evolves counterintuitive patterns in nature, such as the consistently faster growth rates of northern (cold-water) Atlantic silversides than those of their southern (warm-water) counterparts (Conover and Schultz 1995). Countergradient variation is predicted to be common in nature, having already been observed in many species, with many more likely candidates that have yet to be examined (Conover et al. 2009). In particular, multi-species comparisons of countergradient variation are lacking, although they are highly relevant to understanding local adaptation in the context of environmental change.

Temperature is frequently an important factor in the occurrence of local adaptation and plays a driving role in the biogeography of ectotherms (Pörtner 2002). As such, changing temperatures concomitant with global climate change have the potential to substantially alter ectotherms’ distribution patterns, especially those in marine ecosystems (Fields et al. 1993). Notably, ectothermic inhabitants of the rocky shore intertidal zone must also cope with their habitat’s extreme temperature fluctuations, which vary geographically (Helmuth et al. 2002, Sunday et al. 2011). Further, the body temperatures of marine ectotherms are closer to their upper thermal limits than those of terrestrial ectotherms (Pinsky et al. 2019). As the threat presented by climate change often correlates positively with an organism’s proximity to its upper thermal limit (Diamond et al. 2012, Hamblin et al. 2017), and estimates of organismal thermal tolerance have been shown to correlate closely with mortality from chronic heat stress (Cicchino et al. 2023), heat tolerance is therefore an important metric for understanding populations’ ability to persist under consistently warming temperatures. Moreover, the general pattern of increasing temperatures under climate change is accompanied by the occurrence of opposite extremes, including extremely cold winter temperature events (Firth et al. 2011, Wethey et al. 2011), which therefore necessitates an understanding of species’ cold tolerance as well.

For marine intertidal species, the environmental conditions influencing species’ thermal tolerance are a product of the organism’s geospatial location, which can include vertical and latitudinal positions. Species’ vertical distribution in the intertidal zone contributes to their physiological tolerance of thermal extremes, as higher zones are exposed to terrestrial conditions for a greater portion of each tidal cycle and thereby experience greater fluctuations in temperature (Murphy 1979, Stillman 2002, Stickle et al. 2015). Further, research has found a negative relationship between increasing latitude and downward shifts in both upper and lower thermal limits at the species level (Sunday et al. 2011) in addition to further divergence at the population level (Sasaki et al. 2022). This contrasts with the trend of increasingly variable temperatures as latitude increases (Stevens 1989, Gaston and Chown 1999), as unlike in terrestrial ectotherms, the ranges of thermal tolerance for higher-latitude marine ectotherms appear not to widen along with their habitats’ greater extents of temperature variability (Sunday et al. 2019). Previous studies comparing related species of marine invertebrates have demonstrated latitudinal variation in thermal tolerance (Sinclair et al. 2004, Dennis et al. 2014), including several studies that have explored divergence among populations within a species (Kuo and Sanford 2009, Brahim and Marshall 2020, Dwane et al. 2021, Sasaki et al. 2022, Dwane et al. 2023). However, these studies have typically focused specifically on either heat tolerance or cold tolerance, highlighting the need for more studies that compare the responses of a consistent set of species and populations to both hot and cold extremes.

*Littorina* species are among the most abundant marine gastropods worldwide and inhabit the intertidal zone of rocky shores in northern temperate waters (Reid 1989). Of the three species in this study, *L. obtusata* is most abundant in lower tidal zones, *L. littorea* is typically present in low and mid-tidal zones, including tide pools, and *L. saxatilis* primarily occurs in the upper intertidal, thus experiencing the greatest amount of air exposure during a single tidal cycle (Yamada and Mansour 1987, Kozminsky 2013) (Figure 1). *L. saxatilis* notably exhibits behavioural adaptations to avoid desiccation during low tide, principally the tight closure of their operculum in a “standing” posture and the formation of aggregations, that together reduce evaporative water loss (Newell 1979, Atkinson and Newbury 1984). However, these behaviours have limited utility in relieving heat stress: the standing posture has been shown to lower body temperatures by an average of only 1–2°C, and aggregation behaviour appears not to provide a thermoregulatory benefit (Chapperon et al. 2017).

Additionally, the population connectivity of *Littorina* species is impacted by divergent larval development strategies. *L. littorea* reproduces through broadcast spawning with pelagic larvae that undergo long-range dispersal, while in contrast, *L. obtusata* forgoes a dispersive larval period and instead directly develops from eggs into crawling juveniles. *L. saxatilis* also undergoes direct development, but rather than laying eggs, it is the sole littorinid to brood and birth live young (Reid 1996, Johannesson 2003) (Figure 1). According to population genetic theory, dispersal ability generally relates inversely to population structure (Bohonak 1999), suggesting that these littorinids likely exhibit differing levels of inter-population variation between species.

Here, we take advantage of a system of sympatric intertidal snails with diverse ecological and life-history traits to test hypotheses about the effects of thermal extremes on mortality among species and among populations within species. Among species, we predicted that the higher a species lives in the intertidal zone, and therefore the greater its exposure to thermal extremes during the tidal cycle, the lower its demonstrated mortality would be at both thermal extremes. Within species, we predicted that the level of divergence in thermal tolerance among populations would be negatively associated with dispersal potential. As such, this study expands our understanding of the species- and population-level variation in thermal tolerance among marine invertebrate species and provides key information about the geographic dynamics that may play an important role in the persistence of marine ectotherms under climate change scenarios.

## Methods

### Sample Collection

Individuals of *L. littorea*, *L. obtusata*, and *L. saxatilis* were collected in late June to early July of 2019 at two rocky shore sites north and south of the biogeographic break at Cape Cod (Allee 1923, Pappalardo et al. 2015). In the week prior to collection, mean air temperatures between sites were within 2.5°C of one another (National Data Buoy Center 2019a, National Data Buoy Center 2019b). The first site, in Nahant, MA on Massachusetts Bay (42.4194°N 70.9069°W), USA, was defined as the “northern” population (Figure 2 inset, triangle), and the second, in Acoaxet, MA on Rhode Island Sound (41.5069°N 71.0889°W), USA, was defined as the “southern” population (Figure 2 inset, circle). Over the ten years prior to sampling, the northern locality’s maximum and minimum monthly mean temperatures were each 5.4°C lower than in the southern locality (Figure 2), representing the likely variation in thermal extremes between sites. Notably, existing genetic work on *L. saxatilis* indicates that populations of *L. saxatilis* south of Cape Cod – the origin of our southern population – represent a single lineage and have very low haplotype diversity, while a second haplogroup is also present among those in the southern Gulf of Maine (i.e., north of Cape Cod) – the origin of our northern population (Doellman et al. 2011, Panova et al. 2011). While similar work has not been conducted on *L. obtusata*, it is possible that similar patterns may exist between the northern and southern sides of Cape Cod due to *L. obtusata*’s somewhat restricted dispersal.

The snails were placed in prelabeled, perforated specimen containers (120 mL) with a layer of aquarium gravel and fresh thalli of brown algae (*Fucus spiralis* and *Ascophyllum nodosum*), and only individuals without barnacles or other epizoic organisms were collected to avoid external stressors on the organisms.

### Acclimation

Our experiment was set up in a block design with the 120-mL specimen containers placed into seatables (blocks) for acclimation in June 2019. Snails were acclimated for 40 to 47 days (Figure 3; Block 1: 40 days, Block 2: 47 days) with a twelve-hour light cycle and at ambient air temperature (20 to 21°C) to reduce the possible influence of phenotypic plasticity on results. The seatables were gradually drained each morning and refilled with filtered seawater from Massachusetts Bay in the afternoon to simulate the tidal cycle. When full, the seatables were under constant flow-through, and the double layer of specimen containers was completely submerged in seawater. The flow-through seawater temperature averaged 17.8 ± 0.51°C (mean ± SE). The snails were provided with fresh thalli of *F. spiralis* and *A. nodosum* at roughly three-week intervals, and thalli were cleaned with warm freshwater to remove dirt and clinging animals before being placed in the sample containers.

During acclimation, the wet weight (g) of each snail was measured using a digital balance, and snails exceeding 1g were excluded from the analyses to minimise the confounding effect of body size.

### Thermal exposure

After a block’s acclimation period, a static temperature stress assay (Lutterschmidt and Hutchison 1997) was conducted in which snails were exposed to one of three thermal treatments on a 48-hour exposure-recovery timeline: extreme heat (mean ± SE: 44.75 ± 1.11°C, n = 4 observations over 2 blocks), extreme cold (mean ± SE: -12.25 ± 0.25°C), or control (ambient) temperature (mean ± SE: 20 ± 0.41°C) (Figure 3), with 10 snails per treatment-by-population-by-species replicate for a total of 180 snails per block.

Since the long-term persistence of marine intertidal gastropods is more dependent on extreme thermal events than on mean temperatures (Denny et al. 2009, Wethey et al. 2011), the thermal treatment temperatures were determined based on known thermal limits of *Littorina* species snails. The heat shock temperature was set at 45°C based on a study exploring the vulnerability of intertidal snails including *L. brevicula* to heat stress (Dong et al. 2017) and a study examining the lethal high temperatures for several marine invertebrates including *L. littorea* (Fraenkel 1960). The heat exposure was implemented in a wooden box with four 70-watt PAR38 flood light bulbs, which were turned on 30 minutes before exposure to allow the box to reach temperature. Snails, in specimen containers holding rocks and fresh algae, were placed in the heat treatment with their opaque container lids in place to shield organisms from direct light exposure. The cold shock temperature was set at -12°C in accordance with studies investigating freezing-induced muscle injury in *L. littorea*, *L. obtusata*, and *L. saxatilis* and the roles of freezing temperature and duration on *L. littorea* mortality (Murphy 1979, Murphy and Johnson 1980). The cold exposure was implemented by placing snails, likewise in lidded specimen cups containing rocks and fresh algae, in a -12°C freezer. In the control treatment, snails were placed in a dry sea table at ambient air temperature. In all treatments, a temperature probe was inserted through the lid of an additional specimen container included in each treatment to measure the temperature inside.

At the beginning of the 48-hour experimental timeline, snails were exposed to their respective treatments for three hours and then returned to the seatables, mimicking an open-air exposure around the low tide. Mortality was checked for all snails 21 hours post-exposure, with survival recorded if a snail (1) was attached by its foot to the container wall, base, or lid; (2) responded by moving or opening its operculum when submerged in water; or (3) responded to a physical stimulus. All surviving snails were exposed once again to their respective treatments for another three hours, after which they were returned to the seatables; after another 21 hours, mortality was again checked and recorded.

### Statistical analysis

To test for the effects of species, population, and thermal treatment on snail mortality, we created a generalised linear mixed-effects model using the package *lme4* (Bates et al. 2015) with species (*L. littorea*, *L. obtusata*, or *L. saxatilis*), population (northern or southern), treatment (heat exposure, cold exposure, or control), and block (A or B) all treated as fixed effects. We included block as a fixed effect instead of a random effect because two levels are too few to accurately estimate the variance of random effects (Crawley 2002). The data demonstrated overdispersion with unequal variance across data points. We therefore used a bias-reduced generalised linear model with the R package *brglm2* (Kosmidis and Firth 2021, Kosmidis et al. 2021) and modelled the data as a quasibinomial distribution with the logit link function. The bias-reduced model was able to improve estimates of standard error compared to a traditional generalised linear model’s high standard error values for all coefficients, due to the control treatment’s near-100% survival.

We then proceeded with model selection by removing first the four-way interaction, and then subsequent three-way and two-way interactions in a model selection approach based on Akaike information criterion (AIC) values. Following the model selection process, we calculated specific contrasts to examine our *a priori* hypotheses about species and population effects on survival within the best model. We then corrected for multiple comparisons (18 tests conducted) using the Benjamini-Hochberg correction(1995).

### CovGE calculations

CovGE estimates were calculated using the framework laid out in Albecker et al. (2022). The phenotypic data (proportion of snails surviving) were standardised by subtracting the overall mean phenotype from each phenotypic data point and then dividing by the standard deviation of group means, where the “group” referred to the genotype (e.g., each species and location group) and experimental environment (thermal exposure treatment) pair. The genotypic mean phenotype was determined by calculating the mean phenotype for each genotype across environments, and the experimental environment mean phenotype was calculated as the mean phenotype for each environment across genotypes. An ANOVA was used to extract estimated marginal means for each genotype and environmental mean. Finally, 95% confidence intervals were generated using bootstrapping with 999 runs, and hypothesis testing was conducted using permutation to determine statistical significance testing the null hypothesis that CovGE = 0, with results interpreted as significant if the 95% confidence interval did not include zero.

## Results

After conducting our model selection process using AIC comparisons, we determined the best model to be a bias-reduced generalised linear model that included the main effects of species, population, treatment, and block, along with the interactions of species:population, species:treatment, population:treatment, and treatment:block. We conducted a series of *a priori* contrasts to help interpret the multiple two-way interactions (Table 1).

In the control conditions, the survival of all species was almost 100%, and there were no significant differences across species, across populations within a species, or across populations overall (Figure 4). Meanwhile, the snails’ heat and cold tolerances were similar across species with several notable contrasts. Following heat exposure, the higher survival of *L. littorea* compared to *L. saxatilis* represented a significant difference (*P* < 0.001) (Figure 4, red lines). Following cold exposure, the higher survival of *L. littorea* compared to *L. obtusata* and of *L. obtusata* compared to *L. saxatilis* likewise represented significant differences (*P* < 0.01) (Figure 4, blue lines).

Examining the populations within each species, the northern and southern populations of *L. littorea* were not significantly different in either the hot or cold treatments (Figure 4, left panel). The northern and southern populations of *L. obtusata* similarly did not demonstrate significantly different mortality following heat exposure, but following cold exposure, the northern population fared significantly better than the southern population (*P* < 0.01) (Figure 4, centre panel). Meanwhile, the northern population of *L. saxatilis* exhibited significantly higher survival after both heat and cold exposure than their southern counterparts (*P* < 0.01, *P* = 1 × 10-5, respectively) (Figure 4, right panel).

We also observed a main effect of population in the cold exposure, which must be interpreted in light of the interaction. Across populations, snails did not show significant differences in heat tolerance between the northern and southern groups; however, the southern populations’ overall survival following cold exposure was significantly lower than the northern populations’ survival (*P <* 1 × 10-4), a result that was driven in large part by the significant differentiation between the two populations of *L. obtusata* and *L. saxatilis*.

### Patterns of countergradient variation

Across all species studied, the northern populations of snails exhibited higher survival than their respective southern populations in both the cold and heat exposures, indicating a pattern of countergradient variation among these species (Figure 5). Of the three species, *L. saxatilis* exhibited the strongest countergradient variation in the proportion of snails surviving (CovGE = -0.995 (95% CI [-1.000, -0.299]) (Figure 5, right panel). *L. obtusata* showed slightly weaker but still distinct evidence of countergradient variation (CovGE = -0.750 (95% CI [-0.982, -0.001]) (Figure 5, centre panel), while *L. littorea* exhibited no gradient variation (CovGE = -0.010 (95% CI [-0.265, 0.242]) (Figure 5, left panel).

## Discussion

Here we took a comparative approach to investigating species- and population-level variation in organisms’ tolerance of high and low thermal extremes. Counter to our initial hypothesis, the vertical distribution of the three species in the intertidal zone showed only a limited correlation with their tolerance of upper and lower thermal extremes, with three specific pairings presenting as significant. In two of these cases, the species exhibiting significantly lower survival was *L. saxatilis*, which was contrary to the expectation that it should have the greatest tolerance of extremes due to the species’ greater air exposure during a tidal cycle (Table 2). At the same time, this study’s focus on mature individuals precluded observation of the potential variation among species due to influences on their earlier life stages. As the three species reproduce through three distinct modes, exposure to thermal extremes might induce differential impacts during their earlier stages corresponding to the vulnerability of their respective eggs, embryos, or veligers (larvae). *L. obtusata* larvae, for example, have been found to become more physiologically sensitive as they develop (Bitterli et al. 2012), and populations of *L. saxatilis* in the eastern north Atlantic displayed increased inter-ecotype divergence in heat tolerance as they progressed through their life history stages (Dwane et al. 2021). Further, if these patterns of physiology and development persist once snails are adults but continue to age, then potential variability between the age structures of the focal *Littorina* populations could complicate an interpretation of the inter-species variability.

Population-level divergence within species was largely consistent with our expectations based on each species’ potential for larval dispersal. As a broadcast spawner, *L. littorea*’s lack of population variance in survival rates aligns with their pelagic larval stage in accordance with prior research demonstrating that *Littorina* species with planktonic larvae often show less genetic structure than direct-developing species (Kyle and Boulding 2000, Blakeslee et al. 2021), in addition to a minimal latitudinal cline in thermal tolerance (Lee and Boulding 2010). Thus, this dispersal ability allows for panmictic populations that demonstrate minimal local adaptation, at least on the scale of the latitudinal difference in this study. Interestingly, Chiba et al. (2016) found that *L. brevicula*, another broadcast-spawning *Littorina* species, exhibited variation in cold tolerance among populations that experienced latitudinally different cold extremes, suggesting that even species with pelagic larvae may have the capacity to exhibit differentiation among populations distributed across a broader latitudinal gradient. Notably, though, these population localities covered a wider range of latitudes (31° N to 44° N) than those included in this study; thus, Chiba et al.’s proposed mechanism of variation, wherein individuals randomly recruit to different locations and those with locally adaptive traits survive (intragenerational selection; Kurihara et al. 2006), might still be at play among populations of *L. littorea* on a broader geographic scale. As such, future work would do well to examine populations from multiple localities across a broader latitudinal gradient, including population replicates.

The relative lack of differentiation between populations of *L. obtusata* suggests that their populations may experience an intermediate degree of admixture, likely because both egg masses and adult snails on macroalgae are liable to raft longer distances should the algae become detached (Reid 1996). This outcome agrees with previous findings that populations of *L. obtusata* exhibit low but nonetheless discernable genetic variation compared to either *L. littorea* or *L. saxatilis* (Berger 1973, Wares and Cunningham 2001, Schmidt et al. 2007). The much stronger divergence observed in this study between northern and southern populations of *L. saxatilis* fits with their contrastingly low dispersal, with live birth of offspring facilitating local adaptation of individual populations to their local conditions. Further, the direction of population-level variation in northern and southern *L. saxatilis* snails’ cold tolerance was consistent with data indicating that organisms from higher latitudes are often better adapted to extreme cold. The lack of a significant difference in northern and southern *L. saxatilis* survival following heat exposure, however, suggests that the differential in maximum temperatures between the two sampling locations may not be substantial enough to have resulted in differing thermal tolerance. Alternatively, the patterns observed may be influenced by the variable genetic lineages underlying each population: this level of genetic divergence may translate to contrasting degrees of thermal adaptation among individuals within the single focal population, thereby confounding a population-level assessment of tolerance. Relatedly, Dwane et al. (2021) measured different degrees of thermal tolerance between two *L. saxatilis* ecotypes, and while care was taken in this study to collect specimens from a consistent region of the intertidal zone, the potential inclusion of differently adapted ecotypes could likewise confound patterns of inter-population variation.

It is worth noting as well that the ability to directly compare these species’ divergence due to dispersal potential could be inhibited by microhabitat structuring that differs among localities (Lathlean et al. 2014), for example offering more or less availability of thermal refugia that can allow intertidal gastropods to somewhat reduce the costs of exposure to extreme temperatures (Reid and Harley 2021, Dong et al. 2022). A differential in food availability and/or quality between localities could similarly influence the observed inter-population results. Moreover, as several studies have demonstrated that air temperature alone may not fully predict intertidal organisms’ body temperatures (Gilman et al. 2006, Helmuth et al. 2006, 2011), it is also possible that additional factors such as regional tidal patterns or organisms’ body temperature sensitivity may vary between the two population sites, offsetting the difference in temperatures.

Overall, the higher survival of both *L. littorea* populations following heat exposure suggests that this species has the potential to respond better to increasing temperatures across populations, representing a likely advantage of high dispersal and probable higher gene flow. Meanwhile, *L. saxatilis* appears to have the potential for higher survival only in its northern population, which presents a possible downside or constraint to the local adaptation demonstrated by species with lower dispersal. Valuably, however, the countergradient variation observed among these species suggests that northern populations’ adaptation to an overall cooler climate does not seem to leave them at a disadvantage in tolerating increasingly extreme high temperatures. In fact, they may even have an advantage: counterintuitively, other studies have shown that some of the most heat-adapted marine organisms may be most at risk under rising temperatures due to constraints on their ability to tolerate increased heat (Pinsky et al. 2019, Dong et al. 2022), while organisms at higher latitudes have a greater capacity to acclimatise.

Through these results, this study highlights an excellent system to be used in further exploration of pressing questions around species’ potential for adaptation under climate change, and in pursuing parallel lines of inquiry such as analyses of population differentiation at the genetic level (e.g. Blakeslee et al. 2021). Subsequent work would do well to evaluate the impacts of heat or cold exposure at more than two temperatures: although the temperatures used in this study were opposite extremes, the incorporation of a thermal performance curve would provide a more nuanced picture of the species’ thermal tolerance. Additionally, as survival alone provides limited insight into organismal fitness, future studies would benefit from the use of continuous physiological metrics such as cardiac activity or activity coefficients. Dwane et al. (2023) provide an excellent model of this style of experimental design applied to *L. saxatilis*, as do Reid and Harley (2021) in their study on *L. scutulata*. Future work could also consider the incorporation of additional successive exposures to extreme temperatures beyond the two 24-hour cycles implemented here: in the natural world, organisms are threatened not only by individually stressful thermal events but also by the cumulative impact of recurring thermal stress (Rezende et al. 2020), so such a design could offer added perspective on the extent of focal organisms’ thermal tolerance.

### Patterns of countergradient variation

Multiple-species comparisons of countergradient variation provide valuable insight into questions about local adaptation and environmental change. As such, this study broadens our basis by which to understand intra-species responses to the changing climate. While northern populations might be expected to have greater cold tolerance due to their native environment, the greater tolerance of northern populations to heat conditions presented a distinctive pattern of countergradient variation. This outcome concurred with the findings of Dwane et al. (2023) that the thermal optima and upper thermal limits of focal *L. saxatilis* populations increased with latitude to their highest values in the northernmost population. Countergradient variation has also previously been observed in the growth of *L.* *obtusata* snails in response to water velocity, although not to thermal conditions, setting a precedent that gradient variation is possible in the species (Trussell 2002). Further, as temperatures tend to vary more dramatically with increasing latitude, it is reasonable that populations at higher latitudes may undergo adaptation to exhibit greater tolerance for both extreme hot and extreme cold temperatures (although prior studies have shown otherwise, e.g. Sunday et al. 2019). There is no theory yet that describes why countergradient variation evolves in some species but not in others; nonetheless, understanding these interactions between genotype and environment is critical to improving our accuracy in predicting species responses to climate change.

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## Conflict of Interest Statement

The authors declare that they have no conflicts of interest.

## Data Accessibility

The data that support the findings of this study are available in Dryad at <https://doi.org/10.5061/dryad.ht76hdrnx> (while in review, <https://datadryad.org/stash/share/hMgu45MpvJZN9dXzYBiP4sWd5CnZYwViUIxF5Ctf704>). All code used for analyses is available at <https://doi.org/10.5281/zenodo.13738093>.

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**Table 1. Effect sizes and significance values for all *a priori* contrasts.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Question** | **Contrast**  **(group 1 – group 2)** | **Direction of effect (group 1 – group 2)** | ***P*** | ***Pcorrected*** |
| In control conditions, is there a pairwise species difference in logit-transformed mortality? | *littorea* – *obtusata* | NS | 0.33 | 0.41 |
| *littorea* – *saxatilis* | NS | 0.28 | 0.39 |
| *obtusata* – *saxatilis* | NS | 0.88 | 0.88 |
| In control conditions, is there a pairwise population difference in logit-transformed mortality? | *littorea* N – *littorea* S | NS | 0.23 | 0.35 |
| *obtusata* N – *obtusata* S | NS | 0.09 | 0.17 |
| *saxatilis* N – *saxatilis* S | NS | 0.02 | 0.05 |
| In control conditions, are there overall population differences in logit-transformed mortality? | N – S | NS | 0.07 | 0.15 |
| In heat conditions, is there a pairwise species difference in logit-transformed mortality? | *littorea* – *obtusata* | NS | 0.05 | 0.12 |
| *littorea* – *saxatilis* | + | 5 × 10-4 | 0.003 |
| *obtusata* – *saxatilis* | NS | 0.08 | 0.15 |
| In heat conditions, is there a pairwise population difference in logit-transformed mortality? | *littorea* N – *littorea* S | NS | 0.47 | 0.54 |
| *obtusata* N – *obtusata* S | NS | 0.64 | 0.71 |
| *saxatilis* N – *saxatilis* S | + | 0.009 | 0.03 |
| In heat conditions, are there overall population differences in logit-transformed mortality? | N – S | NS | 0.32 | 0.41 |
| In cold conditions, is there a pairwise species difference in logit-transformed mortality? | *littorea* – *obtusata* | – | 0.004 | 0.02 |
| *littorea* – *saxatilis* | NS | 0.87 | 0.88 |
| *obtusata* – *saxatilis* | + | 0.002 | 0.01 |
| In cold conditions, is there a pairwise population difference in logit-transformed mortality? | *littorea* N – *littorea* S | NS | 0.21 | 0.35 |
| *obtusata* N – *obtusata* S | + | 0.005 | 0.02 |
| *saxatilis* N – *saxatilis* S | + | 1 × 10-5 | 2 × 10-4 |
| In cold conditions, are there overall population differences in logit-transformed mortality? | N – S | + | 8 × 10-5 | 7 × 10-4 |

**Table 2. Comparison of hypotheses and results for the respective species’ levels of thermal tolerance and population divergence.**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Species** | **Mortality in cold shock** | | **Mortality in heat shock** | | **Population divergence** | |
| **Hypothesis** | **Result** | **Hypothesis** | **Result** | **Hypothesis** | **Result** |
| *L. littorea*  *(middle intertidal)* | medium | high | medium | low | lowest | lowest |
| *L. obtusata (low intertidal)* | high | medium | high | medium | middle | middle |
| *L. saxatilis*  *(high intertidal)* | low | high | low | high | highest | highest |