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Role of climatic variability in shaping intraspecific variation of thermal tolerance in Mediterranean water beetles

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Abstract The climatic variability hypothesis (CVH) predicts that organisms in more thermally variable environments have wider thermal breadths and higher thermal plasticity than those from more stable environments. However, due to evolutionary trade-offs, taxa with greater absolute thermal limits may have little plasticity of such limits (trade-off hypothesis). The CVH assumes that climatic variability is the ultimate driver of thermal tolerance variation across latitudinal and altitudinal gradients, but average temperature also varies along such gradients. We explored intraspecific variation of thermal tolerance in three typical Mediterranean saline water beetles (families Hydrophilidae and Dytiscidae). For each species, we compared two populations where the species coexist, with similar annual mean temperature but contrasting thermal variability (continental vs. coastal population). We estimated thermal limits of adults from each population, previously acclimated at 17, 20, or 25 °C. We found species-specific patterns but overall, our results agree with the CVH regarding thermal ranges, which were wider in the continental (more variable) population. In the two hydrophilid species, this came at the cost of losing plasticity of the upper thermal limit in this population, supporting the trade-off hypothesis, but not in the dytiscid one. Our results support the role of local adaptation to thermal variability and trade-offs between basal tolerance and physiological plasticity in shaping thermal tolerance in aquatic ectotherms, but also suggest that intraspecific variation of thermal tolerance does not fit a general pattern among aquatic insects. Overlooking such intraspecific variation could lead to inaccurate predictions of the vulnerability of aquatic insects to global warming.

Key words acclimation capacity; aquatic insects; climate change; heat coma temperature; supercooling point; thermal plasticity

Introduction

Climate change and biodiversity loss are escalating global threats (Turney *et al.*, 2020). To face these challenges, we need a mechanistic understanding of species' response to climate change (Kearney & Porter, 2009). Therefore, understanding what factors affect organisms' thermal limits and acclimation capacity and hence, the

conditions that define species' fundamental niches, geographical distribution and evolutionary dynamics, is essential to determine their vulnerability to climate change (Hutchinson, 1981; Soberón & Nakamura, 2009; Duarte *et al.*, 2012; Seebacher & Franklin, 2012; Bennet *et al.*, 2018). Among the multiple hypotheses proposed to explain species variation in thermal tolerance, the mountain passes hypothesis (Janzen, 1967) and the climatic variability hypothesis (CVH; Stevens, 1989) represent a cornerstone of thermal ecology. Both hypotheses predict that organisms that have evolved in environments subject to higher thermal variability have greater thermal tolerance

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breadths and acclimation capacities than those from more stable environments. However, more recently, it was suggested that due to evolutionary trade-offs, taxa that have evolved greater absolute thermal limits (especially upper ones) may have done so at the expense of the plasticity of such limits, showing limited acclimation capacity (trade-off hypothesis hereafter; Stillman, 2003; Magozzi & Calosi, 2015; Comte & Olden, 2017; Armstrong *et al.*, 2019).

Janzen's hypothesis and the CVH assume that climatic variability is the ultimate driver of intra and interspecific variation in thermal tolerance across latitudinal and elevation gradients. However, such gradients usually vary in parallel with changes in mean annual temperature, which is another key driver of thermal tolerances and biodiversity patterns (Jacobsen *et al.*, 1997; Angilletta, 2009; Payne *et al.*, 2016; Comte & Olden, 2017). Indeed, Payne and Smith (2017) put the focus on mean temperature; they suggested that given the temperature dependence of biological rate processes, which in turn determines organismal performance, the performance decrease from optimum (T_{opt}) to critical (T_{crit}) temperatures will be quicker for higher values of T_{opt}, so that warm-adapted species will have narrower thermal tolerance breadths than those from colder environments. Therefore, it is challenging to discriminate the relative role of each type of selective pressure in driving thermal adaptations.

Despite the overwhelming number of studies testing the above-mentioned hypotheses (e.g., Addo-Bediako *et al.*, 2000; Gutiérrez-Pesquera *et al.*, 2016; Colado *et al.*, 2022), there are still important knowledge gaps and inconsistencies in our understanding of thermal tolerance regarding several aspects, such as the taxa and habitats analyzed and the kind of traits evaluated. For example, the relationship between thermal tolerance and climatic stability has been studied to a lesser extent in aquatic (especially freshwater) than terrestrial organisms, likely due to the general assumption that aquatic habitats are more buffered from thermal fluctuations than terrestrial ones. Among freshwater organisms, most studies concern vertebrates (e.g., Comte & Olden, 2017; Nati *et al.*, 2021; Pintanel *et al.*, 2022), while those on invertebrates are scarcer and generally show a high context-dependence. For example, support for the CVH has been found when comparing thermal tolerance breadths between freshwater insects from temperate and tropical streams (Shah *et al.*, 2017b), and variation in thermal plasticity across latitudinal and elevation gradients was also consistent with CVH predictions in mayflies, but not in stoneflies (Shah *et al.*, 2017a). In several lineages of diving beetles, thermal tolerance is positively correlated to species geographical range size, which implies a greater thermal

range, also providing support to the CVH (Calosi et al., 2008a, 2010), and thermal limits do not seem to trade-off with acclimation capacities (Calosi *et al.*, 2008a; Verberk *et al.*, 2018). Improving our understanding of thermal tolerance patterns in freshwater taxa is a key and urgent issue in a climate change context, given the worrying rates of biodiversity decline in these habitats, which are higher than those from other ecosystems (Dudgeon, 2019; Albert *et al.*, 2021). Also importantly, current predictions of the effects of climate change on species distribution frequently assume that all populations have identical thermal tolerance ranges, but due to intraspecific variation in thermal tolerance, such assumption might inaccurately estimate species' ability to cope with climate change (Naya & Bozinovic, 2012; Violle *et al.*, 2012; Buckley *et al.*, 2015). This question deserves more attention as, despite the evidence that thermal tolerance can vary at the intraspecific level as a consequence of local adaptation (Hoffmann *et al.*, 2003), comparisons within species have yielded conflicting results (Angilletta, 2009).

Studies testing the CVH in aquatic taxa have generally covered large latitudinal or altitudinal gradients, usually overlooking the potential confounding effect of average temperature. Recently, Birrell *et al.* (2023) compared thermal breadths of one mayfly and one stonefly from adjacent streams (minimizing differences in mean temperature) with greatly different levels of thermal variation (annual thermal ranges differed around 20 °C between sites). However, climatic variability seems to be relevant for species thermal tolerance traits also within much narrower climatic gradients, as found for subterranean invertebrates (e.g., Raschmanová *et al.*, 2018; Mammola *et al.*, 2019; Pallarés *et al.*, 2021; Jones *et al.*, 2021; Beasley-Hall *et al.*, 2022; Colado *et al.*, 2022), or even at the microhabitat level, as demonstrated for ant rainforest species (Kaspari *et al.*, 2015). Gradients of climatic variability at a given latitude, where daily and seasonal thermal variability increases with continentality (i.e., from coastal to inland areas), provide an ideal setting for testing the CVH across environments with similar average thermal conditions and thus controlling for the effect of mean temperature on organisms' performance (Payne & Smith, 2017).

Inland water bodies in regions with warm, arid climates such as the Mediterranean Basin are subjected to nonnegligible daily and seasonal thermal variations (Paskoff, 1973; Hertig & Jacobeit, 2011; Bonada & Resh, 2013), offering an interesting study system to test the CVH in aquatic taxa. Aquatic biodiversity loss is particularly worrying in the Mediterranean Basin (García *et al.*, 2010; Nogueira *et al.*, 2021), one of the global biodiversity hotspots (Myers *et al.*, 2000; Barrios *et al.*, 2014).

Multiple anthropogenic pressures, increasing demands for food production and urban areas, together with the increasing aridification of the area, exert an extreme pressure on aquatic ecosystems (Drobinski *et al.*, 2020). Biodiversity loss in some particular Mediterranean aquatic habitats, such as inland saline waters, would involve a loss of the biological singularity of this region. These ecosystems, which are rare in a global context but common in the southern Mediterranean Basin, hold unique species assemblages with a high number of rare and endemic species (Millán *et al.*, 2011). Insect communities in saline waters are comprised of halotolerant and halophilic taxa, adapted to the naturally stressful conditions imposed by the particular geological and climatic conditions (Pallarés *et al.*, 2015; Arribas *et al.*, 2019). However, such conditions are becoming more extreme, as regional climatic models for southern Europe stress that the Mediterranean is an especially vulnerable region to global change (Sánchez *et al.*, 2004; Giorgi & Lionello, 2008). Higher, more variable and unpredictable temperatures are anticipated, resulting in novel environmental conditions in the aquatic ecosystems of this area. In this context, it is essential to improve our knowledge about thermal tolerance and plasticity in Mediterranean populations of aquatic insects, as these traits will be key drivers of their vulnerability to climate change (Arribas *et al.*, 2017).

Here, we compared thermal limits and acclimation capacity between Mediterranean populations of three of the most typical saline beetle species in this area: *Enochrus jesusarribasi* Arribas & Millán, 2013 and *Berosus hispanicus* Küster, 1847 (fam. Hydrophilidae) and *Nebrioporus baeticus* Schaum, 1864 (fam. Dytiscidae). These species are excellent study models for the purpose of this study, as they coexist in saline streams exposed to similar annual mean temperature but different thermal variability (continental vs. coastal populations, with greater and lower thermal variability, respectively). Furthermore, two of the species (*E. jesusarribasi* and *N. baeticus*) are narrow-range endemisms of Southeast Spain. Endemic saline species, which often occur as highly isolated populations in these fragmented habitats (e.g., Abellán *et al.*, 2007), may be particularly vulnerable to climate change. This may be especially true for lotic species, which have low dispersal capacity (Ribera, 2008) and therefore limited potential for range shifts under climate change (Arribas *et al.*, 2017). According to the CVH, both thermal tolerance range and acclimation capacity are expected to be higher in the continental than the coastal populations of the study species. Alternatively, if plasticity in thermal tolerance trades off with inherent (basal) tolerance in these taxa (trade-off hypothesis), we would expect that the continental populations show higher absolute thermal limits but a lower plasticity of such limits than the coastal ones.

Materials and methods

Study species and localities, collection, and housing

This study was performed with adults of three water beetle species: the Southeastern Iberian Peninsula endemics *E. jesusarribasi* and *N. baeticus* and the transiberian *B. hispanicus*, which occurs also in South France and North Africa (Millán *et al.*, 2014). The three species are aquatic in all life stages (true water beetles *sensu* Jäch & Balke, 2008) and are present in shallow saline streams in South and East Spain, usually in high densities.

We used the Iberian water beetle distributional database ESACIB (Millán *et al.*, 2014; Sánchez-Fernández *et al.*, 2015) to select localities in Spain where (i) the three species coexist and (ii) have a similar average annual temperature but differ in their range of annual thermal variation. For this, we explored the values of two temperature variables from the WorldClim v. 2.0 database (Fick & Hijmans, 2017): BIO1 (Mean Annual Temperature) and BIO7 (Temperature Annual Range, that is, the difference between the maximum temperature of the warmest month and the minimum of the coldest month). The selected localities, both located in Spain and separated by ∼200 km, were *Arroyo de Brujuelo* (continental site hereafter), a continental saline stream in Jaen province (latitude: 37°53′1.464″ N, longitude: 3°40′10.56″ W) and *Rambla del Reventón* (coastal site hereafter), a saline stream located in the coastal part of the Murcia province (latitude: 37°38′134.851″ N, longitude: 1°22′1.21″ W). Both localities have a mean annual temperature around 17 °C and similar summer and winter average temperatures, but differ in maximum and minimum temperatures: the annual thermal range of the continental site (31.5 °C) is higher than that of the coastal one (26 °C) (Fig. 1).

A minimum of 40 specimens of each species and population were collected using an aquatic hand net, and transported to the laboratory within the same day in 500 mL containers with moistened filter paper, in a portable cooler. Water temperature and conductivity, measured *in situ* with a conductivity-meter (HACH/Hq40d, Hach®, USA) were 20 °C, 56 mS/cm in the continental site, and 21 °C, 30 mS/cm in the coastal one. In the laboratory, beetles were maintained for 3 d in aerated aquaria at 12 g/L (∼20 mS/cm), 17 °C (the annual mean temperature of both localities) and 12 : 12 L : D photoperiod in a climatic
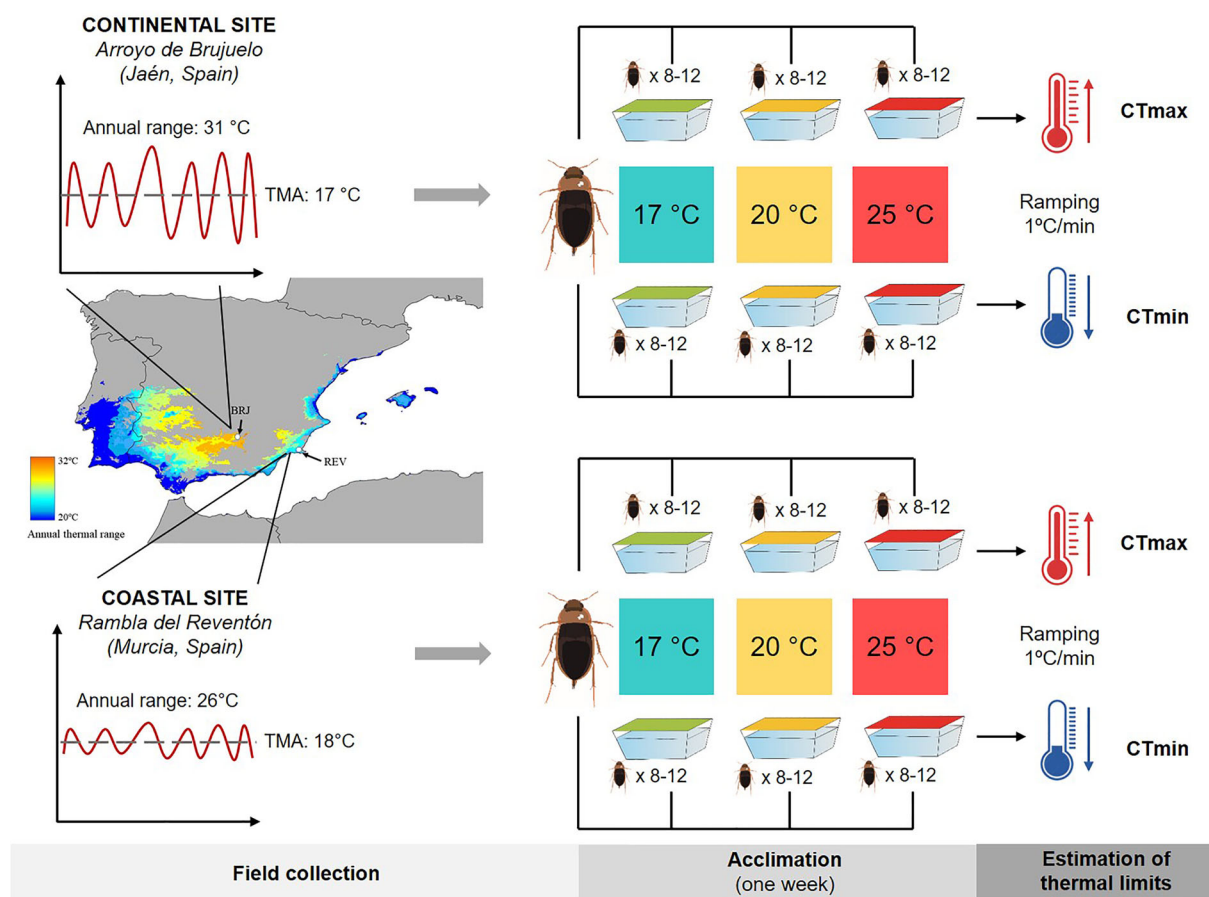
Fig. 1 Schematic representation of site selection and experimental design. The map shows the climatic variability of the areas of the Iberian Peninsula with a mean annual temperature of 17 ± 1 °C (colored area). The color gradient represents the annual thermal range varying from blue (lower) to orange (higher). The experimental design is shown for one hypothetical species, for simplicity. TMA: Annual Mean Temperature; CTmax, CTmin: critical thermal maximum and minimum, respectively.

chamber (SANYO MLR-351). Saline solutions were prepared by dissolving an appropriate quantity of marine salt (Ocean Fish, Prodac) in distilled water. Beetles were fed with frozen chironomid larvae (*N. baeticus*) or macrophytes from the collection sites (*Cladophora* sp.) (*B. hispanicus* and *E. jesusarribasi*). The aim of this preacclimation period was the habituation of the beetles to the laboratory, to reduce the effects of transport, recent thermal history and other previous field conditions. We chose an acclimation salinity within the tolerance range of the study species, which are euryhaline osmoregulators with wide salinity tolerance ranges, and close to the isosmotic point with their hemolymph (Céspedes *et al.*, 2013; Pallarés *et al.*, 2015), in order to minimize osmotic stress.

Thermal tolerance and acclimation capacity experiments

Groups of 8−12 individuals of each species and population were randomly assigned to six experimental groups, defined by three different acclimation temperatures (17, 20, and 25 °C) and two thermal limits (upper and lower), in different aquaria (Fig. 1). Acclimation temperatures ranged from the approximate mean annual temperature of both localities (17 °C, control treatment) to the mean summer temperature in both localities (25 °C, WorldClim 2.0, Fick & Hijmans, 2017). Salinity and photoperiod were maintained as previous to the acclimation, and food was provided ad libitum except from the day before measuring thermal limits, because gut content may affect thermal tolerance (Chown & Nicolson, 2004).

Upper thermal limit and acclimation response ratios

The heat coma temperature (HCT), which is the temperature at which the individuals experience paralysis prior to death, preceded by spasmodic movements of legs and antennae (Chown & Terblanche, 2006), was used as the upper thermal limit. Specimens were washed with distilled water, gently dried with tissue paper and placed upside down in a rectangular piece of pottery using a small amount of nontoxic glue (ErichKrause) (Fig. S1). Trials were carried out in air in a controlled-temperature chamber (BINDER MK53. BINDER GmbH, Tuttlingen, Germany) coupled with an infrared thermographic camera (FLIR SC305) to record body temperature. The infrared camera converts body surface radiation into a thermal pattern and was capable of detecting temperature differentials of 0.1 °C. A dynamic method was employed, using a heating rate of 1 °C/min, starting at the corresponding acclimation temperatures. This is a standard ramping rate, widely used in thermal tolerance assays with arthropods (e.g., Diaz *et al.*, 2002; Calosi *et al.*, 2008b; Wehner & Wehner, 2011). A video camera (Sony DCR-DVD110E, Sony Co., Tokyo, Japan) was also used to record high quality images to determine the moment at which the movement of legs, antennae, and palps of each individual ceased. Plasticity of the upper thermal limit was calculated as the acclimation response ratio (ARR), which describes the change in thermal limits with a given change in acclimation temperature (Gunderson & Stillman, 2015). It provides a comparative measurement of the potential of plasticity of heat tolerance to buffer the species from global warming. For each species and population, we estimated ARR values for the 20 and 25 °C acclimation treatments, compared with the control (17 °C), as:

$$ARR = \Delta HCT / \Delta Acclimation\ temperature.$$

Mean values of heat coma temperature for the corresponding acclimation treatments were used.

Lower thermal limit

As the lower thermal limit, we determined the supercooling point (SCP), that is, the temperature at which the body fluids of the organism begin to freeze when specimens are exposed to a cooling rate (Sinclair *et al.*, 2015). The same methodology as for heat coma temperature was used, but using a cooling rate of -1 °C/min starting from the acclimation temperature. Supercooling point was determined as the body surface temperature just before the

freezing moment, when a sudden temperature increase occurs due to the latent heat released by body fluids melting.

Thermal images (Fig. S1) were analyzed with the software ThermaCAM Researcher Professional 2.10.

After the experiments, dry mass of all the tested specimens (previously dried at 50 °C for 48 h) was measured with an electronic high-precision balance ($\pm$ 0.00001 g) and beetles were sexed by examination of the genitalia.

Data analyses

We used two-way ANOVAs to determine differences in heat coma temperatures or supercooling points between populations and acclimation treatments in each species, including their interaction. Sex and body mass were included as covariates. Pairwise comparisons were made with Bonferroni-adjusted post hoc tests. Data conformed to a normal distribution, so no transformation was required. Normality and homoscedasticity assumptions of model residuals were also validated (Zuur *et al.*, 2009). All statistical analyses were conducted in R v.4.1.2 (R Core Team, 2021) using default packages and the *phia* package for pairwise comparisons (De Rosario-Martinez, 2015).

Results

The thermal range (difference between the mean heat coma temperature and supercooling point) ranged between 53.7 and 60.3 °C across the studied species, populations and acclimation treatments and was in general higher in the continental, variable population than in the coastal, more stable one (Table 1). On average, *E. jesusarribasi* had the widest thermal range of all the species.

Upper thermal limit (heat coma temperature) and acclimation response ratios

In *B. hispanicus*, heat coma temperature (average of all acclimation treatments) was significantly higher in the continental, variable population than in the coastal one (Table 2). In this species and in *E. jesusarribasi*, the effects of acclimation temperature differed between the two populations (significant interaction Population × Temperature, Table 2). Heat coma temperatures were higher in the continental than the coastal (more stable) population after acclimation at the mean annual temperature of both localities (17 °C) in both species, as well as in the 20 °C treatment in *B. hispanicus*. No differences were

Table 1 Heat coma temperature (HCT) and supercooling point (SCP) data.

Species	Population	Temperature (°C)	HCT (mean ± SE) (°C)	SCP (mean ± SE) (°C)	Thermal range (°C)
B. hispanicus	Continental	17	49.10 ± 0.17 (8)	−8.90 ± 1.14 (8)	58.00
		20	49.36 ± 0.12 (9)	−9.87 ± 0.98 (9)	59.23
		25	48.97 ± 0.09 (9)	−8.36 ± 0.94 (9)	57.33
	Coastal	17	48.38 ± 0.16 (11)	−7.73 ± 0.95 (8)	56.11
		20	48.53 ± 0.16 (10)	−7.88 ± 0.72 (11)	56.41
		25	49.1 ± 0.15 (11)	−4.62 ± 0.52 (11)	53.72
E. jesusarribasi	Continental	17	50.3 ± 0.13 (12)	−9.21 ± 1.04 (12)	59.51
		20	50.29 ± 0.11 (12)	−7.78 ± 0.48 (12)	58.07
		25	50.21 ± 0.08 (12)	−9.94 ± 0.78 (12)	60.15
	Coastal	17	49.56 ± 0.20 (8)	−10.76 ± 0.58 (8)	60.32
		20	50.23 ± 0.15 (8)	−7.84 ± 0.53 (9)	58.07
		25	50.67 ± 0.14 (10)	−9.23 ± 0.75 (12)	59.9
N. baeticus	Continental	17	46.29 ± 0.15 (11)	−11.14 ± 0.61 (11)	57.43
		20	46.25 ± 0.08 (11)	−9.76 ± 0.52 (9)	56.01
		25	46.23 ± 0.12 (12)	−10.8 ± 0.34 (12)	57.03
	Coastal	17	46.03 ± 0.16 (12)	−10.7 ± 0.94 (8)	56.73
		20	46.3 ± 0.20 (12)	−10.67 ± 0.7 (11)	56.97
		25	46.58 ± 0.08 (12)	−9.98 ± 0.72 (12)	56.56

The number of replicates (specimens) for each group is indicated in parentheses. The thermal range is estimated as the difference between the mean HCT and SCP for each experimental group.
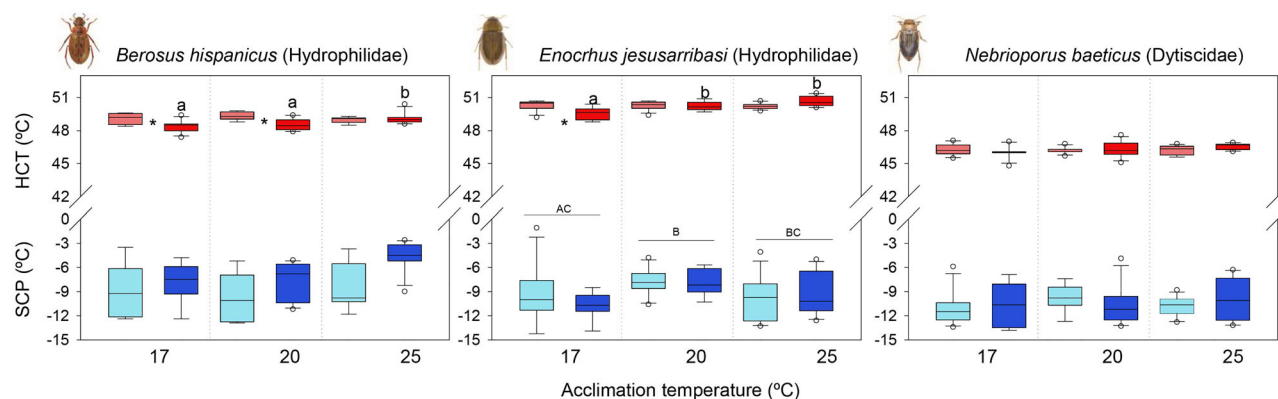

Fig. 2 Heat coma temperature (HCT) and supercooling point (SCP) of the more variable (Brujuelo, continental site, darker colors) and more stable population (Reventón, coastal site, lighter colors) of the three studied species. Boxplots represent Q25, median and Q75, whiskers are Q10 and Q90 and dots are outliers. Significant pairwise comparisons for each thermal limit and species, determined by Bonferroni-corrected post hoc tests, are shown as follows: asterisks for differences between populations within each acclimation treatment, lowercase letters for differences between acclimation treatments within a given population and capital letters for differences between acclimation treatments across both populations. Photo credits: Paula Arribas, Lech Borowiec and José A. Carbonell.

found at 25 °C in any of the species (Fig. 2). There was a significant increase of heat coma temperature with increasing acclimation temperature in the coastal population in both species, but not in the continental one (Fig. 2). In contrast, no significant effects of neither population or acclimation temperature were found in *N.*

baeticus (Fig. 2), whose heat coma temperatures were higher in males than females and increased with body mass (Table 2).

ARR of the upper thermal limit were lower than 0.5 in all cases, and generally higher in the coastal than the continental population (Table 3).

Table 2 ANOVA results for heat coma temperature.

Species	Factor	df	SumSq	MeanSq	*F*	*P*
Berosus hispanicus	Population	1	3.133	3.133	15.549	**<0.001**
	Temperature	2	1.160	0.580	2.878	0.066
	Sex	1	0.079	0.079	0.391	0.535
	Dry mass	1	0.551	0.551	2.736	0.104
	Population × Temperature	2	2.481	1.241	6.157	**0.004**
	Residuals	50	10.073	0.202		
Enochrus jesusarribasi	Population	1	0.083	0.084	0.466	0.498
	Temperature	2	1.850	0.925	5.164	**0.009**
	Sex	1	0.186	0.186	1.039	0.313
	Dry mass	1	0.348	0.348	1.944	0.169
	Population × Temperature	2	3.338	1.669	9.316	**<0.001**
	Residuals	54	9.675	0.179		
Nebrioporus baeticus	Population	1	0.069	0.069	0.374	0.543
	Temperature	2	0.900	0.450	2.435	0.096
	Sex	1	0.936	0.936	5.064	**0.028**
	Dry mass	1	2.271	2.271	12.295	**0.001**
	Population × Temperature	2	0.713	0.356	1.929	0.154
	Residuals	61	11.269	0.185		

Bold *P* values indicate significant differences ($P \leq 0.05$).

Table 3 Acclimation response ratios (ARR) of the heat coma temperature (HCT) for 20 and 25 °C acclimation treatments.

Species	Population	Acclimation treatment (°C)	ARR (HCT)
B. hispanicus	Continental	20	0.09
		25	0.02
	Coastal	20	0.05
		25	0.09
E. jesusarribasi	Continental	20	0.00
		25	0.01
	Coastal	20	0.22
		25	0.14
N. baeticus	Continental	20	0.01
		25	0.01
	Coastal	20	0.09
		25	0.07

Lower thermal limit (supercooling point)

Supercooling points showed a higher interindividual variation within groups than heat coma temperatures

(Table 1, Fig. 2). In *B. hispanicus*, supercooling points were significantly lower in the continental, variable population than in the coastal one, and there was a significant effect of acclimation temperature (Table 4). Supercooling points increased with increasing temperature (Fig. 2); however, no significant differences among acclimation treatments were found in pairwise comparisons, which could be due to the small sample size or the use of a conservative method to adjust *P* values. In *E. jesusarribasi*, supercooling points did not differ among populations, but did vary with acclimation temperature (Table 4). In this species, supercooling points were significantly lower in individuals acclimated at 17 °C than in those acclimated at 20 °C (Fig. 2). No significant effects of any of the tested predictors were found in *N. baeticus* (Table 4, Fig. 2).

Discussion

By comparing lethal thermal limits and acclimation capacity between populations of water beetles with contrasting annual thermal regimes, we found that climatic

Table 4 ANOVA results for supercooling point.

Species	Factor	df	SumSq	MeanSq	*F*	*P*
Berosus hispanicus	Population	1	81.570	81.570	12.619	**0.001**
	Temperature	2	65.370	32.690	5.057	**0.010**
	Sex	1	16.000	16.000	2.476	0.122
	Dry mass	1	2.810	2.810	0.434	0.513
	Population × Temperature	2	21.070	10.530	1.630	0.207
	Residuals	47	303.790	6.460		
Enochrus jesusarribasi	Population	1	2.100	2.129	0.341	0.561
	Temperature	2	47.300	23.672	3.796	**0.029**
	Sex	1	2.400	2.440	0.391	0.534
	Dry mass	1	2.900	2.862	0.459	0.501
	Population × Temperature	2	10.600	5.283	0.847	0.434
	Residuals	56	349.200	6.236		
Nebrioporus baeticus	Population	1	0.010	0.010	0.003	0.959
	Temperature	2	3.450	1.727	0.465	0.631
	Sex	1	2.560	2.561	0.690	0.410
	Dry mass	1	5.100	5.102	1.374	0.246
	Population × Temperature	2	18.590	9.293	2.503	0.091
	Residuals	54	200.460	3.712		

Bold *P* values indicate significant differences ($P \leq 0.05$).

variability is an important factor in shaping their thermal limits, in particular upper ones, while trade-offs between absolute thermal breadths and thermal plasticity seem to constrain their acclimation capacity. However, support for the CVH or the trade-off hypotheses was not consistent across the studied species. Interspecific comparisons of thermal tolerance across climatic variability gradients have yielded contrasting results among aquatic taxa (e.g., Shah *et al.*, 2017a). Likewise, our results suggest that intraspecific variation of thermal tolerance is also species-specific and so, does not seem to fit a general pattern among aquatic insects.

In general, individuals from the continental population, characterized by a larger annual thermal range, had a wider thermal window (i.e., difference between the upper and lower thermal limit) than those from the coastal one, in agreement with the CVH. However, in *E. jesusarribasi* and *N. baeticus*, the thermal windows differed by less than 1 °C between the studied populations. Thermal tolerance results in *B. hispanicus* were clearly consistent with the CVH, as the absolute upper and lower thermal limits were significantly higher in the continental population than in the coastal, more stable one. To a lesser

extent, thermal variability also appears to shape the thermal tolerance breadth in *E. jesusarribasi*, but not in *N. baeticus*. Such differences in intraspecific variation of the thermal breadth among the studied species could be, in part, attributed to their different geographical range sizes: while the geographical distribution of *B. hispanicus* spans from North Africa to South France, *E. bicolor* and *N. baeticus* are narrowly distributed Iberian endemics. Therefore, given the broader thermal range experienced by *B. hispanicus* over evolutionary time, this species may retain greater intraspecific variation in its thermal tolerance (Nati *et al.*, 2021). On the other hand, the fact that we found more similar patterns (and similar thermal limit values) between *B. hispanicus* and *E. jesusarribasi* (fam. Hydrophilidae) than those with *N. baeticus* (fam. Dytiscidae) could reflect different evolutionary histories of these beetle families. Hydrophilids are pressumed to have more recent terrestrial ancestors than diving beetles (Dytiscidae). Secondary colonizations of the terrestrial medium (and back to water again) seem to have been frequent within the family Hydrophilidae (Bernhard *et al.*, 2006; Short & Fikácek, 2013; Bloom *et al.*, 2014), while the aquatic Adephaga (which includes dytiscids) apparently

entered the aquatic environment only once (Shull *et al.*, 2001; Hunt *et al.*, 2007; Jäch & Balke, 2008). Therefore, hydrophilids might conserve typical traits associated to more variable, terrestrial environments (e.g., Pallarés *et al.*, 2022), like higher thermal tolerance. In this sense, studies across different lineages controlling for phylogenetic effects would be illuminating.

Regarding acclimation capacity, again, we found consistent patterns in *B. hispanicus* and *E. jesusarribasi*, in which the more thermally variable population, with higher absolute thermal limits, showed no acclimation capacity of the upper thermal limit. In contrast, individuals from the less variable population were capable of increasing their heat coma temperatures upon acclimation at high temperatures. These results are consistent with the trade-off hypothesis (Stillman, 2003). In contrast, but in line with previous findings in other dytiscids (Calosi *et al.*, 2008a; Verberk *et al.*, 2018), no trade-off between thermal limits and acclimation capacity was apparent in *N. baeticus*. Nevertheless, more populations across the studied species' geographical ranges should be examined for a more meaningful exploration of the relationship between upper thermal limits and thermal plasticity.

As for the differences in acclimation capacity between the populations of the hydrophilid species studied, they might be related to the higher intensity and frequency of extreme events with ongoing climate change, such as heat waves, which could have an impact on the plasticity of the upper thermal limit at the micro-evolutionary level (Baudier *et al.*, 2018; Dai *et al.*, 2022). Empirical research has demonstrated that extreme events can drive directional selection toward more resistant phenotypes (Pauls *et al.*, 2013; Coleman & Wernberg, 2020) and cause a loss of genetic diversity (e.g., Coleman *et al.*, 2020; Gurgel *et al.*, 2020). This might occur in the more thermally variable population studied here, for which more extreme heat waves could exert a strong selective pressure exposing the species to temperatures closer to their physiological limit more frequently. Consequently, individuals with higher basal heat tolerance could be positively selected, at the expense of losing more plastic phenotypes. Nevertheless, it has also been argued that increased climate variation can provide strong selection pressure on traits that are related to increased phenotypic plasticity as organisms need to adapt to a less predictable environment (Pauls *et al.*, 2013).

Notwithstanding the differences in thermal plasticity between the studied populations, it is important to highlight that such plasticity appears to be very limited in all species, at least regarding heat tolerance, as the low ARR values of upper thermal limits reflect. Such values were overall much lower than those estimated for other freshwater, terrestrial, and marine taxa (Gunderson & Stillman, 2015). Nevertheless, in a context of climate change, a limited physiological thermal plasticity of upper thermal limits might be compensated by other forms of plasticity (e.g., behavioral, developmental) or plasticity in other temperature-dependent traits. Indeed, one of the species here studied (*E. jesusarribasi*) displays some degree of plasticity in metabolic and locomotor thermal performance curves upon acclimation at different temperatures, although the extent to which such acclimation capacity might aid in coping with climate change also appears to be reduced (Pallarés *et al.*, 2021).

Some limitations of our approach should be acknowledged. First, salinity levels differed between the two localities, being higher in the continental population. Salinity has been shown to influence thermal tolerance in water beetles (e.g., Sánchez-Fernández *et al.*, 2010; Arribas *et al.*, 2012). However, we expect this potential effect to be negligible compared with that of acclimation temperature, as beetles from both populations were acclimated at a common salinity for one week (plus three preacclimation days) before performing thermal tolerance measurements. In addition, the studied species are effective osmoregulators across a wide range of salinities (Pallarés *et al.*, 2015) and the acclimation salinity was close to the isosmotic point with respect of their hemolymph, so that the required osmoregulatory effort is minimum in such conditions. Second, by exploring acclimation responses using a limited range of acclimation treatments, we only obtain a simplified vision of a complex process such as plasticity of thermal tolerance. The positive ARR of the upper thermal limits indicate the studied species have some ability to increase their heat tolerance limits as acclimation temperature increases; however, we cannot assume such increase to be lineal. Indeed, acclimation capacity is expected to decrease as acclimation temperature approaches physiological limits and so, the studied populations might differ in the slopes and thresholds of their acclimation response. Also, we cannot rule out the possibility that the species show different patterns of plasticity of the lower thermal limit under colder acclimation temperatures. Describing such acclimation response would require a greater range of acclimation temperatures to be tested. Third, microclimatic variability within sites was not considered in our study. Yet though the difference in annual thermal variability between the studied sites is modest (ca. 5 °C), microclimatic variation within sites is expected to be lower across aquatic habitats, which tend to have less spatial variability in operative thermal conditions than terrestrial ones (Gunderson & Stillman, 2015). Finally, exposure to temperature

variation or acute heat shock can also adjust thermal tolerance limits (Giomi *et al.*, 2016; Kingsolver *et al.*, 2016). Further experiments including acclimation at constant vs. variable thermal regimes with different daily variability (e.g., Bozinovic *et al.*, 2016) would allow us to obtain a more accurate estimate of the impact of increasing environmental variability and climatic extremes (IPCC, 2022) on species persistence.

Regardless the factors driving the contrasting thermal responses among the studied species, our results have important implications in a context of climate change (Sunday *et al.*, 2012; Khaliq *et al.*, 2014) and the alarming biodiversity loss in freshwater habitats (Dudgeon, 2019; Albert *et al.*, 2021), particularly in the Mediterranean region (García *et al.*, 2010; Nogueira *et al.*, 2021). Assuming that all populations have identical thermal tolerance ranges and acclimation capacities might lead to inaccurate estimates of species vulnerability to climate change and so, the effect of thermal conditions at local levels on species performance is key to understanding biodiversity responses to global warming. Studies replicating our approach (i.e., comparing populations from sites with similar average temperatures but contrasting thermal variability) would provide significant insights into the field of thermal biology in freshwater invertebrates. It would be of particular interest to identify trade-offs between basal thermal tolerance and thermal plasticity among aquatic insects. Such a pattern, which seems to be taxon-specific, could leave populations from variable environments more vulnerable in the face of climatic change.

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Disclosure

The authors have no conflicts of interest to declare.

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Supporting Information

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

Fig. S1 Example of the plate with the specimens glued dorsally to determine upper and lower thermal limits (upper) and screenshot of a thermal image being analyzed with the software ThermaCAM Researcher Professional 2.10 (lower).