

Thermal tolerance does not explain the altitudinal segregation of lowland and alpine aquatic insects

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ABSTRACT

Elevation gradients provide powerful study systems for examining the influence of environmental filters in shaping species assemblages. High-mountain habitats host specific high-elevation assemblages, often comprising specialist species adapted to endure pronounced abiotic stress, while such harsh conditions prevent lowland species from colonizing or establishing. While thermal tolerance may drive the altitudinal segregation of ectotherms, its role in structuring aquatic insect communities remains poorly explored. This study investigates the role of thermal physiology in shaping the current distribution of high-mountain diving beetles from the Sierra Nevada Iberian mountain range and closely related lowland species. Cold tolerance of five species from each altitudinal zone was measured estimating the supercooling point (SCP), lower lethal temperature (LLT) and tolerance to ice enclosure, while heat tolerance was assessed from the heat coma temperature (HCT). Alpine species exhibited wider fundamental thermal niches than lowland species, likely associated with the broader range of climatic conditions in high-mountain areas. Cold tolerance did not seem to prevent lowland species from colonizing higher elevations, as most studied species were moderately freeze-tolerant. Therefore, fundamental thermal niches seem not to fully explain species segregation along elevation gradients, suggesting that other thermal tolerance traits, environmental factors, and biotic interactions may also play important roles.

1. Introduction

Understanding species responses to environmental gradients is a key topic in ecology (Vellend 2010). Species assemblages result from various processes, including environmental filters, biotic interactions and dispersion, often displaying complex interactions (Chase and Myers 2011). Elevation gradients provide powerful study systems to investigate how communities are structured by the environment, as environmental conditions change rapidly within short distances, offering insights into the potential effects of global climate change on biodiversity (Sundqvist et al., 2013; Álvarez et al., 2024). Elevation is considered a strong environmental filter, associated with several climatic trends and gradients, such as reduced temperature and oxygen availability and increased UV radiation (Körner 2007). Moreover, other abiotic factors such as geographical barriers and patchy habitat availability impose filters on upwards range expansions (Fourcade et al., 2021). High-mountain habitats, at the extreme of such gradients (e.g. alpine and subalpine belts), are characterized by harsh living conditions,

including very low temperatures, long periods of snow or ice cover and short growing seasons (Larson et al., 2019). These environmental conditions represent strong abiotic filtering that shapes specific high-elevation assemblages, often composed of specialist species adapted to endure pronounced abiotic stress, and prevent lowland species from colonizing or establishing in these harsh environments (Montaño-Centellas et al., 2021).

One crucial niche dimension determining species survival in harsh conditions is thermal tolerance, especially significant for ectothermic animals like insects, as temperature directly influences their development, reproduction and survival (Chown et al., 2004; Angilletta Jr., 2009). Thermal niches may explain species assemblages along elevation gradients (e.g. Khaliq et al., 2023). However, our understanding of how temperature affects the altitudinal limits of species, particularly aquatic macroinvertebrates, remains limited (Freeman 2016). According to Hutchinson's (1957) concept of the realized and fundamental niche, a species' fundamental (i.e. physiological) thermal niche represents the range of environmental temperature under which the species could

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survive, while the species' realized thermal niche is defined by the environmental temperatures at which it actually lives, reflecting the constraining effects of biotic interactions and dispersal limitations (Gvoždík 2018). The extent to which the fundamental thermal niches of ectotherms reflect their realized thermal niches indicates the relative role of temperature on distribution against other factors. Thus, the upper and lower altitudinal range boundaries of any insect species will be determined by its capacity to match its thermal tolerance range to the altitudinal temperature profile of its habitat, unless other factors such as biotic interactions intervene (Hodkinson 2005; Jankowski et al., 2013).

Alpine ponds and glacial lakes are some of the most remote and undisturbed aquatic habitats in Europe, hosting highly specific and usually species-poor aquatic communities, less complex compared to similar lowland ecosystems (Hinden et al., 2005). These extreme environments provide ideal systems to study how elevation shapes the assembly of unique communities. Several environmental variables contribute to the harsh living conditions in these water bodies, including low oxygen partial pressure, highly diluted waters, limited nutrients and autotrophic production, large variations in irradiation and high spatial isolation. However, low temperatures are the primary factor driving abiotic stress (Catalán et al., 2006). With a mean annual air temperature close to 0 °C, these habitats experience a winter snow and ice cover that may last for more than half a year at mid-latitudes (Catalán et al., 2006). Consequently, organisms in these habitats are expected to be adapted to a short ice-free season and to endure persistent low temperatures as well as rapid and wide daily temperature changes (Hodkinson and Coulson 2004).

In this study, we compare for the first time the thermal biology of high-mountain diving beetles (family Dytiscidae) from Sierra Nevada mountain range (southern Iberia) and closely related species from lowlands, with the aim to determine the role of thermal physiology in shaping the current distribution of species between both altitudinal zones (i.e. whether thermal tolerances prevent alpine specialists to occupy lowland habitats and lowland species to colonize alpine environments). The Sierra Nevada massif hosts a system of ponds and lakes of glacial origin between approximately 2800 and 3100 m.a.s.l., which harbours a specific community of water beetles, very distinct from those

inhabiting Iberian lowland ponds (Millán et al., 2013, 2014, Abellán et al., 2022). Thus, alpine and lowland ponds present non-overlapping beetle assemblages, suitable to investigate whether differences in the thermal biology of species (e.g. thermal tolerances and cold hardiness strategies) can explain this altitudinal segregation. We propose three alternative hypotheses to explain the current species sorting along the low-high temperature gradient (alpine to lowlands) (Fig. 1): 1) Species fundamental and realized thermal niches are quite similar and both alpine and lowland species occupy their maximum thermal range, but species from different altitudinal areas are segregated because they differ in their fundamental thermal limits (Fig. 1a). This scenario is quite unlikely, as realized and fundamental physiological niches of aquatic insects differ significantly in general (Araújo et al., 2013); 2) The fundamental thermal niches of alpine and lowland species are similar, but species from both altitudinal ranges are segregated by biological interactions so that their realized thermal niches do not overlap (Fig. 1b). This pattern means species from lowland could physiologically inhabit cold alpine regions and alpine species could inhabit warmer lowlands, but interactions like resource competition or predation shape the species distribution along the altitudinal gradient; 3) The fundamental thermal niche of alpine species is wider than that of lowland species, which is narrower and displaced towards warmer temperatures, but the realized thermal niche of alpine species only covers lower temperature habitats in which they can avoid biological interactions (Fig. 1c). In some aquatic insect groups, high-elevation species have been reported to present wider thermal breadths compared to counterparts from lowlands, which may have evolved as a result of the greater climatic variation typical of high mountain regions (Shah et al., 2017). This is consistent with one cornerstone hypothesis in thermal ecology: the climatic variability hypothesis (Stevens 1989), which predicts a positive relationship between species' thermal tolerance breadth and the degree of climatic variability they experience. The observed wider thermal niche of alpine species might be the result of the evolution of increased cold tolerance and the conservation of upper thermal tolerance, which seems to be a common pattern among ectotherms (Araújo et al., 2013; Hoffmann et al., 2013; Lancaster et al., 2015). This scenario would also agree with the asymmetric abiotic stress limitation

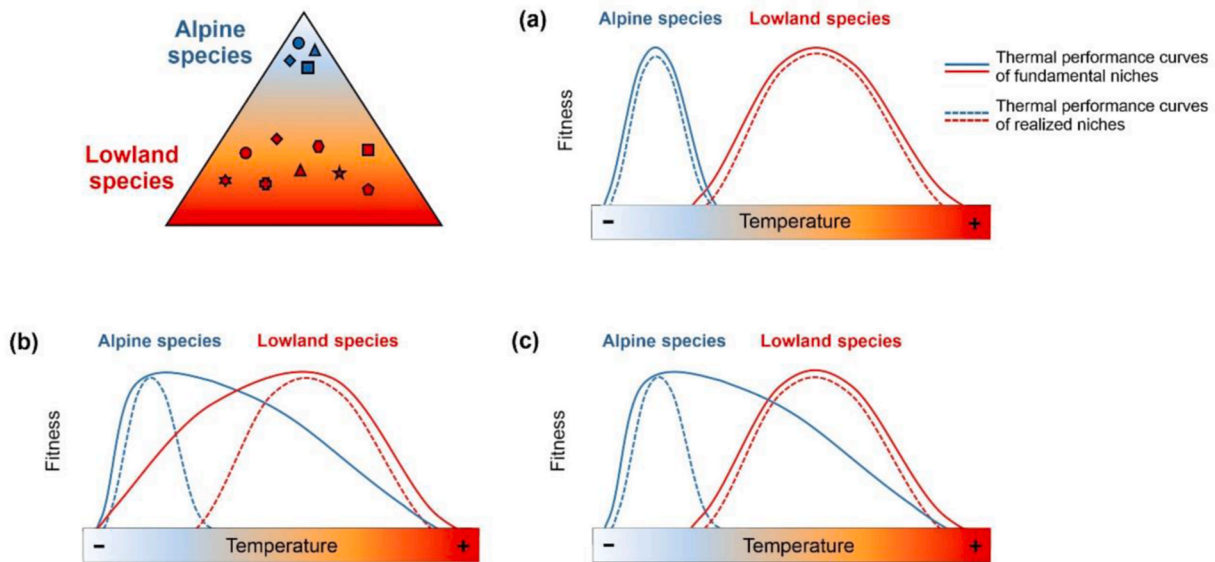


Fig. 1. Aquatic macroinvertebrate assemblages are segregated along the altitudinal gradient according to three main scenarios: (a) species fundamental and realized thermal niches almost match and alpine species and species from lowlands occupy their maximum thermal range, but species from each altitudinal area are segregated along the temperature gradient as they differ in their fundamental thermal limits; (b) upper and lower fundamental thermal limits of alpine and lowland species are similar, but species from both ranges are segregated along the temperature gradient by biological interactions; (c) fundamental thermal niche of alpine species is wider than that of lowland species, which is narrower and displaced to warmer temperatures, but realized thermal niche of alpine species only covers lower temperatures to avoid biological interactions.

hypothesis (Normand et al., 2009), according to which physiological tolerance is the primary constraint towards the more stressful ends of environmental gradients (e.g. upper-altitudinal range boundaries), while biotic interactions assume a greater role towards less stressful

conditions (see e.g. Machac et al., 2011).

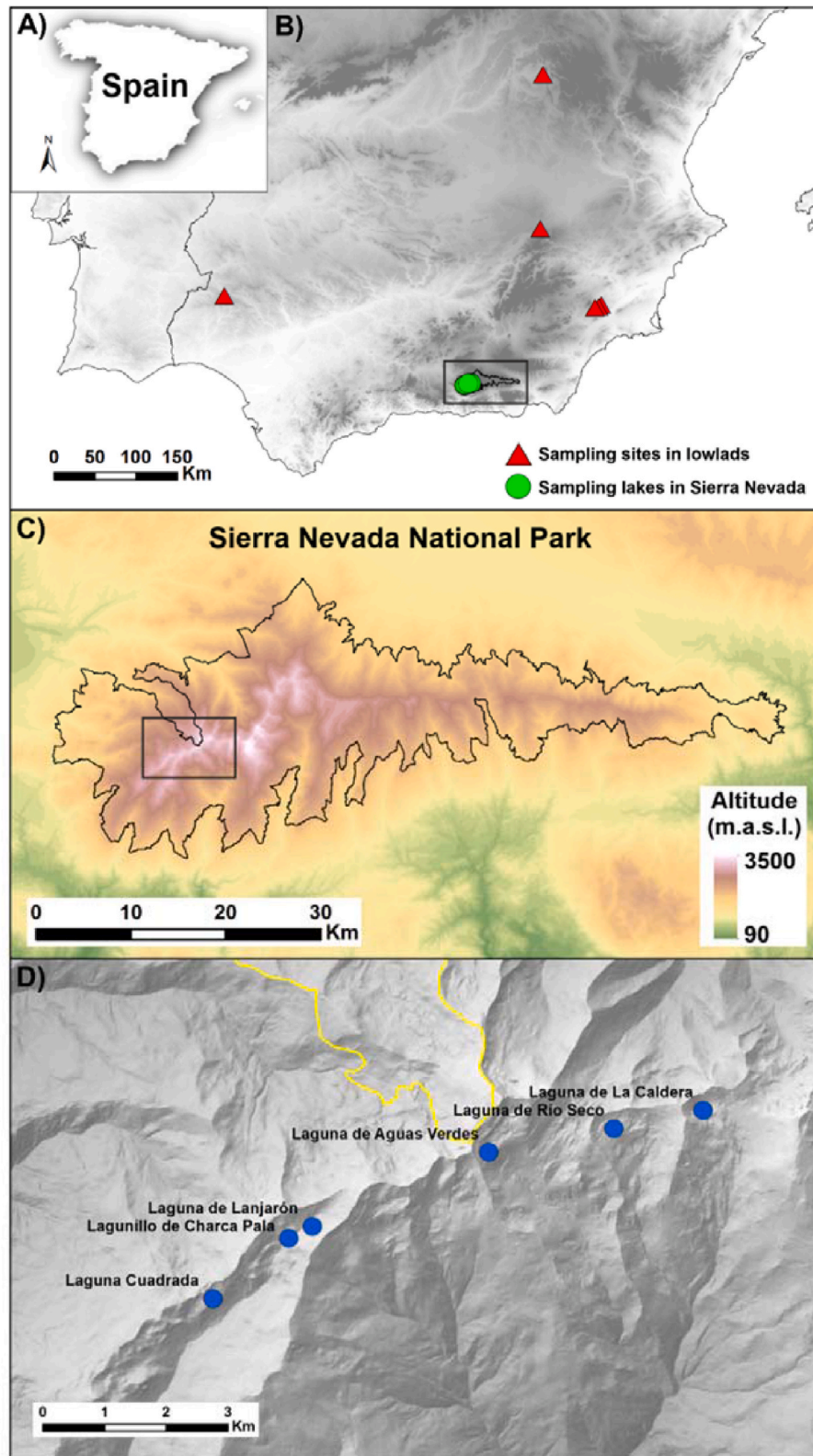


Fig. 2. Study area showing the location of the sampled sites in lowlands (triangles) and Sierra Nevada massif (circles). The boundaries of the Sierra Nevada National Park are shown in c) (solid line) and d) (yellow line). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

2. Material & methods

2.1. Study system

The Sierra Nevada massif is an alpine mountain range in southern Iberia that covers a surface of approximately 2000 km² (Fig. 2). Sierra Nevada is the southernmost alpine system in Europe, and has the highest peak (Mulhacén, 3482 m.a.s.l.) in western Europe outside the Alps. Its glacial lakes host cold-adapted macroinvertebrate communities dominated by water beetles, some of which are endemic species to this mountain massif (Millán et al., 2013, 2014, Abellán et al., 2022).

To study and compare the thermal biology of alpine and lowland water beetles, five species of diving beetles (Dytiscidae) inhabiting the alpine lakes of Sierra Nevada (alpine species hereafter) and five common species of the same family inhabiting lowland or mid-altitude lentic water bodies in southern Iberia (lowland species hereafter) were studied (see below). The five alpine species selected are the most representative and common diving beetles inhabiting the system of alpine lakes in Sierra Nevada (Ruano et al., 2013; Millán et al., 2014, Abellán et al., 2022). While some of them are not exclusively alpine, they all inhabit high mountain water bodies and are rarely found in lowland areas. On the other hand, the lowland species selected are widely distributed in different types of freshwater habitats in the Iberian Peninsula, but most commonly found in lowlands, and absent in the Sierra Nevada alpine lakes (Millán et al., 2014).

The studied species from Sierra Nevada alpine ponds were.

- *Agabus nevadensis* Lindberg, 1939 is a medium-sized species (body length between 8.0 and 8.5 mm). This is an Iberian micro-endemic species only found in Sierra Nevada (Millán et al., 2013, 2014).
- *Hydroporus sabaudus sierranevadensis* Shaverdo, 2004 is a small-sized subspecies (3.5–4.0 mm) belonging to the *nigrita* group. It is also an Iberian micro-endemic subspecies only found in Sierra Nevada (Millán et al., 2013, 2014).
- *Hydroporus nevadensis* Sharp, 1882 is a small-sized (3.2–3.9 mm) Iberian endemic species belonging to the *longulus* group, distributed across the main mountain ranges in the Iberian Peninsula (Millán et al., 2013, 2014).
- *Hydroporus marginatus* (Duftschmid, 1805) is a small-sized (4.2–4.5 mm) species belonging to the *marginatus* group. It has a Western Palearctic distribution, occurring in the main mountain ranges of the Iberian Peninsula (Millán et al., 2013, 2014).
- *Boreonectes ibericus* (Dutton and Angus, 2007) is a small-sized (4.3–4.9 mm) species distributed from North Africa to the Alps, including the main Iberian mountain ranges (Millán et al., 2013, 2014).

The studied species from lowlands were.

- *Agabus bipustulatus* (Linnaeus, 1767) is a species varying in size from medium to large (9.0–11.0 mm), whose distribution covers the Palearctic region. This species is widely distributed in the Iberian Peninsula, mainly in lentic waters (Millán et al., 2014).
- *Agabus didymus* (Olivier, 1795) is a medium-sized species (7.0–8.0 mm). It has a Palearctic distribution, occurring mainly in lotic waters, but also can be frequently found in lentic water bodies in the Iberian Peninsula (Millán et al., 2014).
- *Agabus conspersus* (Marshall, 1802) is a medium-sized species (6.0–8.0 mm) with Palearctic distribution. This species inhabits lentic water bodies like marshes and ponds, especially in the eastern Iberian Peninsula (Millán et al., 2014).
- *Agabus biguttatus* (Olivier, 1795) is a medium-sized species (8.5–9.0 mm) with Palearctic distribution. This species is widely distributed in the Iberian Peninsula, mainly in lotic systems, but also can be found frequently in standing waters in mountain ranges (Millán et al., 2014).

- *Hydroporus pubescens* (Gyllenhal, 1808) is a small-sized (3.1–3.6 mm) species with Palearctic distribution. This species inhabits different kinds of lotic and lentic water bodies in the Iberian Peninsula, at different altitudinal ranges (Millán et al., 2014).

2.2. Collecting and housing

Adults of the study species were collected in summer 2021 and 2022 from several lakes and ponds of Sierra Nevada massif (alpine species) and different water bodies (altitude <1150 m) in the southern half of the Iberian Peninsula (lowland species) (Fig. 2, Table A1). Localities from both altitudinal zones were selected according to records of the study species from previous studies (Millán et al., 2013; Abellán et al., 2022; Carbonell et al., 2024). Species were collected and transferred to the laboratory as in Carbonell et al. (2024), i.e. using a hand net and transported within 24 h to the laboratory placed in a portable refrigerator at approximately 10 °C. Species were held during 4–5 days at 10 °C (alpine species) and 20 °C (species from lowlands) at 12:12 L:D photoperiod in a climatic chamber. Individuals were fed daily *ad libitum* with frozen chironomid larvae. These temperatures were selected to mimic spring average water temperature in lowlands (<https://es.climate-data.org>) and the average temperature of water during ice-free season (June to October) in Sierra Nevada (<https://climanevada.obsnev.es/>).

2.3. Experimental design

Before carrying out the thermal tolerance trials, all the study species were subjected at two acclimation treatments (common garden conditions) for 7 days: 10 °C (potential stressful temperature for lowland species and non-stressful for alpine ones) and 20 °C (potential stressful temperature for alpine species and non-stressful for lowland species) (see Pallarés et al., 2020). The acclimation phase started with about 100 adult individuals of each study species per temperature. After this acclimation phase, individuals were starved for 24 h, as gut content may modify thermal tolerance (Chown et al., 2004), and groups of between 10 and 25 individuals per temperature were randomly selected to estimate different cold and heat tolerance endpoints. All the trials to estimate cold and heat tolerance endpoints were carried out individually. Cold and heat tolerance experimental data for the five alpine species at 10 °C acclimation treatment was obtained from Carbonell et al. (2024).

2.4. Assessing cold tolerance

To study the species tolerance to cold and freezing conditions, the supercooling point (SCP), lower lethal temperature (LLT) and tolerance to ice enclosure were estimated. Freeze-tolerance of species can be inferred by comparing the lower lethal temperature (LLT) and the supercooling point (SCP) (Block 2003). Species described as freeze avoidant generally present very low freezing/supercooling points, which are also close to their LLT (Block 2003), while freeze-tolerant species survive body fluid freezing and present SCP values more than 10° above their LLT (Sinclair 1999; Lee 2010).

2.4.1. Supercooling point (SCP)

The supercooling point (SCP) is the temperature at which the body fluids of the organisms begin to freeze when specimens are cooled. Trials were carried out in air on ten individuals of each species from each acclimation treatment in a controlled-temperature chamber, employing a dynamic ramping method with a cooling rate of -1 °C min^{-1} , starting from the corresponding acclimation temperature (10 °C or 20 °C) until reaching a temperature well below the expected lower thermal limits (-45 °C). The body surface temperature of the individuals was logged using an infrared video camera. SCP was determined as the body surface temperature before the freezing of body fluids (see Appendix A2 for details).

2.4.2. Lower lethal temperature (LLT)

The lower temperature tolerance limit of the species was determined by cooling them at various below-zero temperatures (temperatures of shock) and testing for freezing injuries and survival (following Van der Laak, 1982; Lencioni and Bernabò, 2017). Following methodology in Carbonell et al. (2024), groups of five individuals (replicates) from each acclimation treatment were cooled at a rate of 0.3 °C min⁻¹ starting from the acclimation temperature (10 °C or 20 °C) until five different low temperatures: 5, -10, -15 and -20 °C. After 30 min, the degree of cooling injuries and survival was recorded (see Appendix A2 for details).

2.4.3. Tolerance to ice enclosure

Being enclosed in ice is a potential environmental risk factor in alpine streams and ponds (Hotaling et al., 2021), as it can cause mortality through inoculative freezing, hypoxia or mechanical damage (Conradi-Larsen and Sømme 1973). Following the methodology in Carbonell et al. (2024), trials to study whether the studied species could survive being temporarily enclosed in ice exposed to -1 °C for 3 h were carried out (see Appendix A2 for details).

2.5. Determination of heat tolerance

2.5.1. Heat coma temperature (HCT)

The heat coma temperature (HCT) is defined as the temperature at which the insects experience paralysis prior to death, preceded by spasmodic movements of legs and antennae (Chown and Terblanche 2006). The heat coma temperature was determined as the measured body surface temperature when the individuals reached the complete paralysis, following the same methodology as for SCP, but setting a heating ramping rate of +1 °C min⁻¹ (see Appendix A3 for details).

2.6. Data analysis

The effects of species, genus, region (alpine vs lowlands), acclimation temperature and the interactions of acclimation temperature with the other factors on SCP and HCT were tested using generalized linear models (GLMs) with a normal error structure and the identity link function. To reduce the possible overfitting of the models as a result of an excessive number of predictors and interactions in relation to the relatively low number of observations, previous models were carried out and non-significant factors removed from the analyses. For the lower lethal limit (LLT), factors “species” and “acclimation temperature” were removed from the model ($P = 0.7609$ and $P = 0.1400$ respectively), while for the HCT, factor “acclimation temperature” ($P = 0.3531$) was removed. For the LLT, the response variable was binary (dead or alive), and therefore GLMs with a binomial error structure and the logit link function were used. For this response, the effects of genus and temperature of shock (i.e., the low temperature treatment tested) were tested. The interactions between acclimation temperature and temperature of shock with the other factors were also included in case they were significant in previous models. When interaction terms were significant, Fisher’s post hoc LSD t tests with a Bonferroni correction were used to identify significant differences between group pairs. Data from LLT experiments were used to estimate the temperature at which 50 % (LLT₅₀) and 100 % (LLT₁₀₀) mortality occurred (Lencioni and Bernabò 2017) by using a Probit analysis, a type of regression typically used in toxicology assays (Hahn and Soyer 2005). Statistical analyses were conducted in R version 3.5.3 for Windows (R Core Team 2015) using the packages “lme4”, “lmerTest”, “drc” and “MASS”, and SPSS for Windows, v26.0. 2019 (IBM Corp., Armonk, NY, U.S.A.).

3. Results

3.1. Cold tolerance

Alpine species presented significantly lower SCPs than lowland

species, mainly at the higher acclimation temperature (20 °C). Genera and species also showed significant differences in their SCP; the genus *Hydroporus* (especially *H. nevadensis*) showed the lowest SCP and *Agabus* species the highest values (Fig. 3, Tables 1 and 2). Species showed significantly lower SCP at higher acclimation temperature (20 °C). Species belonging to the genera *Hydroporus* and *Boreonectes* presented lower SCP than the other species at 20 °C.

In the LLT experiments, Alpine and lowland species did not show significant differences. Genera showed significant differences in survival throughout the progressively lower temperatures, with *Agabus* species showing the lowest survival. Species’ survival was lowest at -20 °C and highest at -5 °C (Fig. 4, Tables 1 and 2). All the studied species but two can be considered as moderate freeze-tolerant, as their LLT₅₀s were few degrees lower than their SCPs (Figs. 3 and 4, Table 1). For the alpine species *H. nevadensis* and *H. marginatus*, SCPs were similar to their LLTs, a typical pattern shown by freeze avoidant species. In ice enclosure tolerance trials, species’ survival was maximum (100%) for all the species but for the alpine *H. s. sierranevadensis* (80 %) (Table 1).

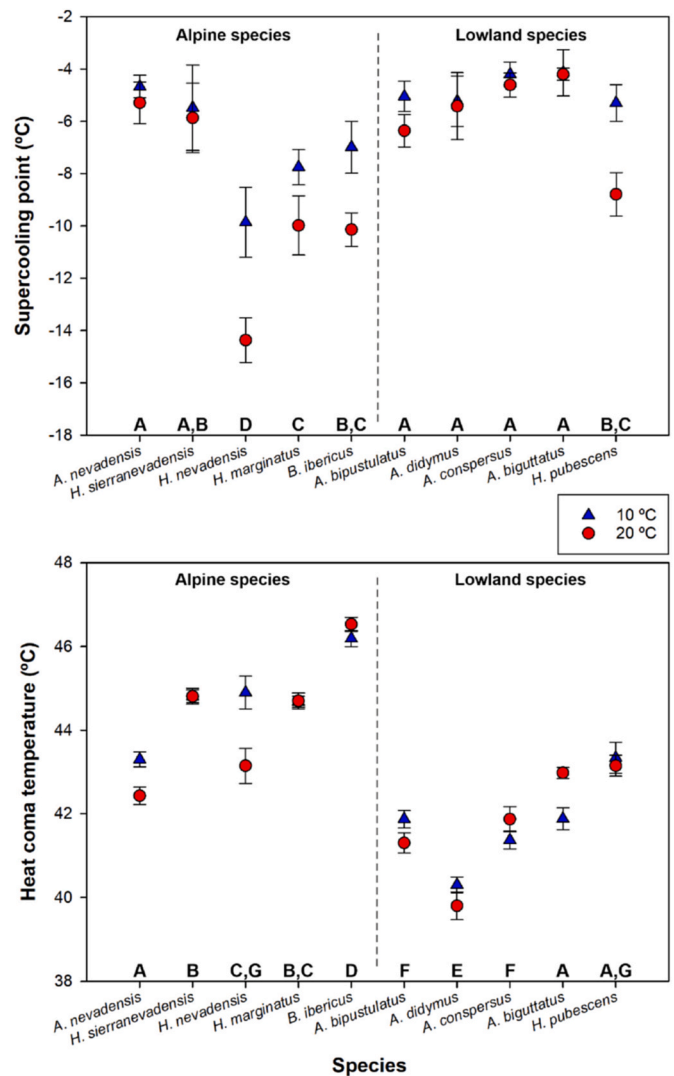


Fig. 3. Mean ± SE heat coma temperatures and supercooling points of the 10 studied species measured at a heating or cooling rate of 1 °C/min. Capital letters represent differences among species’ means, determined by Fisher’s post hoc LSD t -test with Bonferroni correction. Significant differences between genera are as follows: HCT: *Agabus* (A), *Hydroporus* (B), *Boreonectes* (C); SCP: *Agabus* (A), *Hydroporus* (B), *Boreonectes* (B). Significant differences between regions determined are as follows: HCT: Alpine region > lowlands; SCP: Alpine region < lowlands.

Table 1

Values of the different endpoints measured for the 10 studied species at the two acclimation temperatures (10 °C and 20 °C): supercooling points (SCP), lower lethal temperatures 50% (LLT₅₀) and 100% (LLT₁₀₀), ice-enclosure tolerance (% survival) and heat coma temperature (HCT). Data are presented as the mean ± SE for the SCP and HCT and the value and 95% confidence level for the LLT₅₀ and LLT₁₀₀.

	SPECIES FROM SIERRA NEVADA									
	A. nevadensis		H. s. sierranevadensis		H. nevadensis		H. marginatus		B. ibericus	
	10 °C	20 °C	10 °C	20 °C	10 °C	20 °C	10 °C	20 °C	10 °C	20 °C
SCP (°C)	-4.67 ± 0.48	-5.29 ± 0.91	-5.48 ± 1.10	-5.87 ± 1.66	-9.86 ± 1.47	-14.37 ± 1.35	-7.75 ± 1.11	-9.98 ± 1.04	-6.99 ± 0.99	-10.14 ± 0.64
LLT ₅₀ (°C)	-9.79 ± 0.05	-7.05 ± 0.66	-8.20 ± 4.29	-14.08 ± 0.21	-9.79 ± 0.05	-14.77 ± 0.06	-5.79 ± 1.51	-12.22 ± 0.76	-9.70 ± 6.39	-11.81 ± 5.37
LLT ₁₀₀ (°C)	-15	-10	-20	-15	-15	-20	-15	-20	-20	-20
Ice enclosure tolerance (% survival)	100	100	100	80	100	100	100	100	100	100
HCT (°C)	43.30 ± 0.18	42.43 ± 0.21	44.81 ± 0.19	44.81 ± 0.15	44.90 ± 0.39	43.15 ± 0.42	44.68 ± 0.13	44.70 ± 0.19	46.19 ± 0.19	46.53 ± 0.17
Thermal niche range (HCT-SCP)	47.97	47.72	50.29	50.68	54.76	57.52	52.43	54.68	53.18	56.67
	SPECIES FROM LOWLANDS									
	A. bipustulatus		A. didymus		A. conspersus		A. biguttatus		H. pubescens	
	10 °C	20 °C	10 °C	20 °C	10 °C	20 °C	10 °C	20 °C	10 °C	20 °C
SCP (°C)	-5.05 ± 0.58	-6.36 ± 0.62	-5.23 ± 0.97	-5.42 ± 1.28	-4.20 ± 0.47	-4.61 ± 0.46	-4.14 ± 0.89	-4.20 ± 0.23	-5.30 ± 0.70	-8.79 ± 0.83
LLT ₅₀ (°C)	-7.05 ± 0.66	-9.12 ± 0.24	-9.12 ± 0.24	-10.2 ± 0.07	-7.05 ± 0.66	-9.12 ± 0.24	-7.05 ± 0.66	-9.12 ± 0.24	-14.77 ± 0.06	-12.25 ± 0.58
LLT ₁₀₀ (°C)	-10	-15	-15	-15	-10	-15	-10	-15	-20	-15
Ice enclosure tolerance (% survival)	100	100	100	100	100	100	100	100	100	100
HCT (°C)	41.87 ± 0.21	41.30 ± 0.24	40.30 ± 0.19	39.80 ± 0.33	41.37 ± 0.21	41.87 ± 0.23	41.88 ± 0.26	42.98 ± 0.13	43.34 ± 0.37	43.15 ± 0.25
Thermal niche range (HCT-SCP)	46.92	47.66	45.53	45.22	45.57	46.48	46.02	47.18	48.64	51.94

Table 2

Results of the generalized linear models for the supercooling point (SCP), lower lethal temperature (LLT) and heat coma temperature (HCT). Bold *P*-values indicate significant differences (*P*-value ≤ 0.05).

Response variable	Effect	Estimate	<i>F</i> -value	df	<i>P</i> -value
<i>SCP</i>	Species	0.380	9.615	6	<0.0010
	Genus	2.898	20.814	2	<0.0010
	Acclimation Temp	0.304	18.452	1	<0.0010
	Region	3.269	49.060	1	<0.0010
	Region x Acclimation Temp	-0.310	1.862	1	0.1743
	Genero x Acclimation Temp	-0.481	3.328	2	0.0383
	Species x Acclimation Temp	-0.189	1.006	6	0.4233
<i>LLT</i>	Genus	-34.191	5.009	2	0.0067
	Tshock	4.054	319.205	1	<0.0010
	Genero x Tshock	-3.643	4.931	2	<0.0010
<i>HCT</i>	Species	-0.881	16.89	6	<0.001
	Genus	1.602	168.19	2	<0.001
	Region	-1.542	595.360	1	<0.001

3.2. Heat tolerance

Species from different altitudinal ranges showed significant differences in heat coma temperature (HCT); alpine species presented higher HCTs than lowland ones. Genera and species also differed significantly in their heat coma temperature (HCT), with *B. ibericus* presenting the highest values and *Agabus* species (especially *A. didymus*) the lowest (Fig. 3, Tables 1 and 2).

4. Discussion

This study provides, for the first time, experimentally-based data defining the fundamental thermal niche of adult alpine aquatic beetles

and related lowland species inhabiting lentic waters. The alpine species studied exhibited wider fundamental thermal niches than related lowland species, possibly due to the greater climatic variation typical of high mountain regions, supporting the third hypothesis proposed in this study. Most species from both altitudinal zones seemed to be tolerant to freezing of internal fluids and survived ice enclosure, crucial traits for facing harsh winter conditions in cold environments.

Three out of the five alpine species studied (*A. nevadensis*, *H. s. sierranevadensis*, and *B. ibericus*) seemed to exhibit moderate freeze tolerance, as their LLT₅₀s were slightly lower than their SCPs, a pattern commonly observed in alpine insects (Wharton 2011). Surprisingly, all the lowland species studied also showed moderate freeze tolerance, likely due to their higher SCPs, resulting in a higher distance from the lower lethal temperature (LLT), although their LLT did not significantly differ from that of alpine species. However, while the lowland species appeared to be freeze-tolerant in the short-term under the approach used in this study, they might not endure long-term freezing in nature compared to alpine species. Two alpine species, *H. nevadensis* and *H. marginatus*, stood out for showing very low SCPs similar to their LLT₅₀, a characteristic typical of freeze-avoidant species (Sinclair 1999). These species may endure cold temperatures by avoiding freezing through supercooling, allowing them to remain active during winter when alpine species experience freezing conditions. This strategy might enable them to overwinter outside lakes, partially protected from freezing air conditions by overlying snow. This, combined with the production of cryoprotectant molecules such as antifreeze proteins (Olsen et al., 1998; Danks 2007), could be a more effective adaptation to alpine conditions than freeze tolerance.

All the studied species survived ice enclosure during 3 h, suggesting they were able to resist physical damages derived from direct contact with ice, at least during relatively short exposure times. This resistance is likely accounted by protective mechanisms such as a thick and physically resistant cuticle or cryoprotectant molecules (like epicuticular waxes) (Olsen et al., 1998; Danks, 2007). Also, the survival under ice enclosure of the study species may be explained because their LLTs or

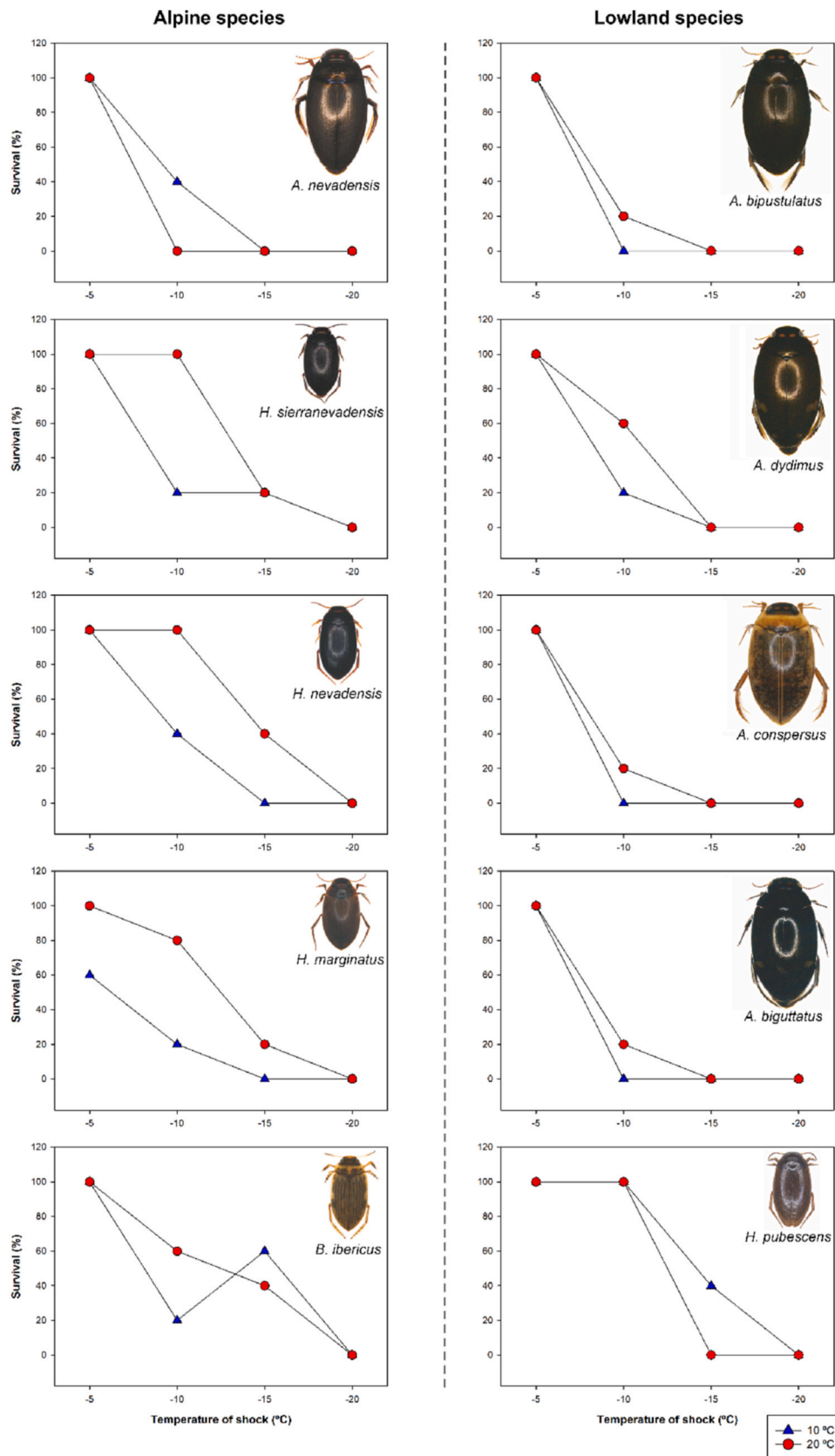


Fig. 4. Survival curves at low temperatures of the 10 studied species. Significant differences of survival between temperatures of shock are as follows: 5 °C (A) > -10 °C (B) > -15 °C (C) > -20 °C (D). Significant differences of survival between genera are as follows: *Agabus* (A) < *Hydroporus* (B); *Boreonectes* (A,B).

SCPs were not reached during the short-term trials. The capacity to survive ice enclosure during winter months seems to be common for aquatic alpine insects (Hotaling et al., 2021). For instance, it has been described for larvae of the freeze-tolerant stonefly *Nemoura arctica* (Walters Jr et al., 2009) and for adults of several species of Hemiptera and Coleoptera collected from the ice layer in alpine areas, which were able to recover in the laboratory after thawing (Mihalicz 2015).

In summary, according to our results, cold tolerance does not seem to fully prevent lowland species from colonizing alpine habitats. However, we cannot discard that other cold tolerance traits, not assessed with the approach used in this study, may play an important role in the species segregation, as species' long-term survival does not only depend on critical thermal tolerance limits, but also on the rate of cooling, the temperature fluctuations and the duration and frequency of subzero chilling episodes (Danks 2007). For instance, and independently of their cold hardiness strategy (i.e. freeze avoidance or freeze tolerance), insects in cold environments are susceptible to chilling injury (Hayward et al., 2014), which can result from rapid cooling or from long-term exposure to low temperatures (Bale 2002). Furthermore, temperature also impacts fitness traits other than survival, such as development (Zografou et al., 2022) or reproduction (Ma et al., 2017), that were not assessed in the present study. Moreover, it is important to highlight that other abiotic factors such as geographical barriers and patchy habitat availability may impose a filter for lowland species range expansion to alpine regions (Fourcade et al., 2021). In this sense, further studies on dispersal capacity of species in relation to geographical barriers and the microclimatic habitats along the altitudinal gradient should be developed to provide more insight on environmental factors which may prevent lowland species from colonizing alpine habitats.

Alpine species showed a wider fundamental thermal niche than lowland ones, attributed to a higher heat coma temperature and lower supercooling points (Fig. 3). These results align with the typical pattern for ectotherm species adapted to cooler environments, showing increased low temperature tolerance while selection on upper thermal tolerance is relaxed (Thomas et al., 2001), leading to broader thermal tolerance breadths (Araújo et al., 2013; Hoffmann et al., 2013; Lancaster et al., 2015). This finding supports the third hypothesis proposed in this study to explain species altitudinal segregation, as the realized thermal niche of alpine species only covers the lower part of their wide fundamental thermal niche, likely to avoid biological interactions. This pattern is also consistent with the climate variability hypothesis (Janzen 1967; Stevens 1989), suggesting that species from regions with higher thermal variability, such as alpine areas (Ohmura 2012; Esteban-Parra et al., 2022), would present wider thermal niches (as recently described for terrestrial arthropods in Khaliq et al., 2023).

Furthermore, other mechanisms could explain the higher heat tolerance of alpine species. For example, it could be a capacity derived from adaptation to high UV radiation, through changes in the cuticle structure or thickness related to radiation management, which could indirectly increase the heat tolerance of these species (passive thermoregulation; see Alves et al., 2018). Also, according to the Bogert effect (Huey et al., 2003), a limited capacity for thermal adjustment through behavior (for example, difficulty seeking shelter from high radiation) as a consequence of reduced microhabitat availability in very homogeneous habitats (such as alpine lakes) could enhance physiological thermal tolerance (Huey et al., 2003; Gunderson and Stillman 2015).

It is important to acknowledge that differences found in thermal tolerances between species from alpine areas and lowlands might be conditioned by phylogeny (i.e. differences among genera), as methodological limitations made it impossible to select a balanced number of species belonging to same genus from both alpine regions and lowlands. However, differences found in HCTs between regions seem not to be affected by phylogeny. The genus *Agabus*, to which most of the lowlands species belong, presented the lowest HCT, but the alpine species *A. nevadensis* showed higher HCT than most of the *Agabus* species from lowlands. Moreover, the lowlands species *H. pubescens* presented lower

HCT than most of the alpine *Hydroporus* species. It is also stressed out that the species comparison made in this study concerns only the adult stage. Although the study of the thermal niche of other life cycle stages such as larvae may be interesting, as the larvae of some alpine aquatic beetles have been described to be more sensitive to thermal stress than adults (Pallarés et al., 2020), the impossibility of obtaining larvae from all species made their study impossible. On the other hand, aquatic stages such as larvae or eggs are less exposed to harsh cold conditions, remaining at temperatures above zero under the ice layer of pond surfaces during winter (Carbonell et al., 2024), so these stages seem not to be critical for studying comparative adaptation among species from alpine regions and lowlands.

Acclimation temperatures had significant effects on the thermal limits of the studied species, particularly for heat coma temperature (HCT) and supercooling point (SCP). While one lowland species (*A. biguttatus*) exhibited some acclimation capacity, with increased heat tolerance in response to higher acclimation temperatures, 4 out of 5 alpine species showed a counter-intuitive response, displaying higher heat tolerance and lower cold tolerance when acclimated at a lower temperature (10 °C). In part, this unexpected pattern may be due to prolonged exposure to suboptimal temperatures during acclimation at 20 °C, potentially causing sublethal effects on physiological processes, such as oxidative stress, which could have impacted their fitness and thermal performance (Monaghan et al., 2009). However, the higher cold tolerance of individuals acclimated at 20 °C remains counter-intuitive even in such scenario. Additionally, inherent limitations of the methodological approach used in the study could have contributed to this unexpected pattern. Although differences in exposure time during ramping assays between acclimation treatments were minimal, other studies have shown that insects can mount an effective and rapid physiological response to thermal stress through mechanisms underlying rapid cold-hardening (Kelty and Lee Jr, 2001; Overgaard et al., 2006). Similarly, studies have described higher upper thermal limits when insects were exposed to longer trials (e.g. Kay and Whitford 1978; Pallarés et al., 2020). It is important to note that while different starting temperatures in the cold and heat ramping assays may affect thermal limit estimations, this effect should not impact the conclusions drawn from species comparison in the study, as all studied species were exposed to the same acclimation treatments and trial starting temperatures.

5. Conclusions and future directions

The alpine species studied presented wider fundamental thermal niches than lowland ones. However, while fundamental thermal niches partially explain the species distribution along the elevation gradient, our findings suggest that other factors, such as freeze tolerance in lowland species, may also play a role in their potential to inhabit alpine areas. Our results support the hypothesis that alpine species have wider fundamental thermal niches due to the broader range of climatic conditions in high-mountain areas (Gaston and Chown 1999). However, the realized thermal niche of alpine species may only cover lower temperatures, possibly to avoid biological interactions such as resource competition and intraguild predation (Fig. 1c). Additionally, environmental factors such as higher ultraviolet radiation, shortened growing and breeding seasons and hypoxia due to decreased air oxygen concentration with altitude, could create unsuitable conditions for non-adapted lowland species (Dahlhoff et al., 2019; Larson et al., 2019). Future studies should consider the contribution of species tolerance to other environmental factors and biotic interactions in explaining species distribution along the altitudinal gradient, to enhance our understanding of the factors influencing current distribution patterns and potential future alterations.

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CRediT authorship contribution statement

J.A. Carbonell: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **S. Pallarés:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **J. Velasco:** Writing – review & editing, Investigation, Conceptualization. **A. Millán:** Writing – review & editing, Investigation, Conceptualization. **P. Abellán:** Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Other (please explain: e.g. 'I have shared the link to my data as an attachment').

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jtherbio.2024.103862>.

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