



Thermal biology of aquatic insects in alpine lakes: Insights from diving beetles

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Abstract

1. High mountain areas are especially vulnerable to global warming, as they experience faster temperature changes than lowlands in a climate change context. Notably, increased temperatures and frequency of extreme flooding and droughts, and the consequent decrease in ice cover and water availability fluctuations, will induce important physical changes in alpine freshwater systems. Thus, assessing thermal limits and exploring overwintering strategies of aquatic alpine insects is pivotal to understanding how aquatic communities of high-mountain fresh waters will respond to climate change. However, knowledge on these topics is still scarce for aquatic alpine insects.
2. Here, the thermal biology of adults of five diving beetle species from alpine lakes located in the Sierra Nevada mountain range (southern Iberia) was studied. Cold tolerance was measured estimating the supercooling point (SCP), lower lethal temperature (LLT), tolerance to ice enclosure and to submersion, whereas heat tolerance was assessed from the heat coma temperature and upper lethal temperature.
3. All of the species survived ice enclosure for 3 h. Furthermore, three of the studied species had SCPs higher than their LLTs, suggesting that they could be freeze-tolerant. All species except *Agabus nevadensis* also were tolerant to submersion, which could be a key adaptation for overwintering underwater below the ice cover as adults, reducing risk from freezing conditions in the air. The species did not differ significantly in their upper thermal limits, which were similar to those of other dytiscids from lower altitudes.
4. Overall, our results suggest that increasing temperatures is not expected to be the most important threat for the water beetle populations in Sierra Nevada, but rather the colonisation of alpine lakes by lowland dytiscids in a warmer climate scenario.

KEYWORDS

climate change, Dytiscidae, glacial ponds, lower thermal limit, upper thermal limit

J. A. Carbonell and S. Pallarés—share first authorship.

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1 | INTRODUCTION

High mountain areas are especially vulnerable to global warming, as they have been shown to experience faster temperature changes than lowlands in a climate change context (Pepin et al., 2015). Increased temperatures and frequency of extreme flooding and droughts, and the consequent decrease in ice cover and increase of water availability fluctuations, will induce important physical changes in alpine freshwater systems (Collins & Tait, 2016). Therefore, aquatic alpine invertebrates will be exposed not only to higher temperatures, but also to habitat alteration and loss. Species living in these fragmented habitats could be highly sensitive to heat stress (and hence especially vulnerable to climate variations) as they seem to have a limited adaptive potential and physiological plasticity to temperature increase (Kinzner et al., 2019; Pallarés et al., 2020).

Alpine lakes are commonly regarded as sentinels of climate change, providing unique insights into physical and ecological responses to global warming (Moser et al., 2019). Such high-elevation lentic environments are subjected to extreme environmental conditions, such as low temperature, short ice-free seasons, high radiation and low nutrients (Morales et al., 1992), containing highly specific, cold-adapted species assemblages. Understanding the mechanisms of cold and heat tolerance in species inhabiting these fragile habitats is crucial in order to accurately predict ecological consequences of climate change. However, our knowledge of thermal adaptations in extreme environments such as alpine ecosystems is still scarce and partial. Most studies on this topic have focused on terrestrial vertebrates (e.g., Hof & Allen, 2019; Rehnus et al., 2018), and those conducted on aquatic species have been conducted on running waters (e.g., Giersch et al., 2014; Hotaling et al., 2021; Lencioni & Bernabò, 2015; Treanor et al., 2013; Tsyrlin et al., 2021), whilst studies on the thermal biology of standing water insects are still rare (but see Pallarés et al., 2020; Rosset & Oertli, 2011). Furthermore, few studies have considered species' physiological thermal limits, which are key determinants of insects' vulnerability to temperature increase (Arribas et al., 2012, 2017; Calosi et al., 2008; Pallarés et al., 2020; Sgro et al., 2016).

Climate change is expected not only to increase water temperatures, but also to alter snowpack and ice-free season length in these remote habitats (Hidalgo-Hidalgo et al., 2022; López-Moreno et al., 2011). The reduction or loss of ice cover over both land and water during winter, which isolates organisms from extreme air temperatures, may expose them to more extreme lower temperatures, increasing the frequency of freeze–thaw cycles and risks of ice encasement (Bale & Hayward, 2010). The effects of such changes on alpine species will depend to a great extent on their overwintering strategies. Winter conditions in cold climates in alpine regions include low subzero temperatures, ice formation, snow accumulation, and chemical and oxygen concentration changes in lake waters sealed beneath ice and overlying snow (Danks, 2007). Most of the research to date on cold hardiness involves terrestrial insects (Overgaard & MacMillan, 2017; Toxopeus

& Sinclair, 2018), yet aquatic insects also have developed different overwintering strategies and cold hardiness adaptations that allow survival at temperatures close to their lethal limit, comprising morphological, behavioural, ecological and physiological adaptations (Lencioni, 2004). Low-temperature biology has been studied in some alpine aquatic species that inhabit running waters (mostly Diptera Chironomidae; see Lencioni, 2004), but there is no information for species inhabiting alpine lentic water bodies, which are subjected to different environmental rigours. Furthermore, lower lethal limits and overwintering strategies of alpine aquatic insects with aquatic larval and adult stages have been never been studied, to the best of our knowledge. Low-temperature biology is a key determinant of insect distribution (Andersen et al., 2015) and it is known that lower lethal limits are evolutionarily less conserved in ectotherms than upper ones (Araújo et al., 2013; Hoffmann et al., 2013; Kellermann et al., 2012). Therefore, cold- and heat-tolerance limits, as well as overwintering strategies, need to be considered for drawing physiological comparisons of the thermal niche (i.e., range of body temperatures that an organism can tolerate, allowing positive population growth; Gvoždík, 2018) and climate-change sensitivity among alpine species.

In this study, laboratory experiments were performed to explore the thermal biology of five diving beetle species (family Dytiscidae) from alpine lakes in the Sierra Nevada mountain range (southern Iberia). We focused on diving beetles as models because dytiscids are one of the main biodiversity components in alpine lakes in Sierra Nevada, with three species being exclusively endemic to this massif (Millán et al., 2013). All of the life cycle stages of diving beetles are aquatic; larvae take oxygen from surrounding water through gill respiration, and adults breathe using subelytral air storage that acts as a compressible gas gill and needs to be replenished periodically with atmospheric oxygen at the water's surface (Buchwalter et al., 2019). Adults of some small diving beetles are able to tolerate submersion for weeks by extracting the oxygen dissolved in the water through respiratory pores in their integument (Madsen, 2012), which may represent an advantage to reduce the predation risk in lentic waters and to reduce the risk of being swept away during surface visits in running waters (Kehl & Dettner, 2009).

Several cold- and heat-tolerance endpoints were determined in order to characterise the fundamental thermal niche of the species, which constitutes a more realistic approach than considering only one cold- or heat-tolerance endpoint (Rezende et al., 2014, 2020), and allowed us to infer the overwintering strategies of the studied species. We studied cold tolerance measuring the supercooling point (SCP), lower lethal temperature (LLT), tolerance to ice enclosure, and tolerance to submersion (lower lethal oxygen concentration [LLO] at cold temperatures). Regarding heat tolerance, we measured the heat coma temperature (HCT) and upper lethal temperature (ULT). Our study improves our knowledge on thermal adaptations of insects in extreme alpine environments, setting the stage for future comparative studies and generating data for modelling the vulnerability of alpine aquatic insects to climate warming.

2 | METHODS

2.1 | Study system

Sierra Nevada is an alpine mountain range in southern Iberia declared as National Park in 1999. It covers a surface of approximately 2000 km² and stretches from East to West for almost 90 km (Figure 1). Sierra Nevada is the southernmost alpine system in Europe, with its highest peak, Mulhacén, at 3,482 m above sea level (a.s.l.), which is not only the highest mountain in the Iberian Peninsula, but also the highest peak in western Europe excluding the Alps. Because of these unique characteristics, it represents one of the areas with the highest values of biodiversity and endemism of the Iberian Peninsula (Millán et al., 2013). Sierra Nevada hosts a system with tens of glacial ponds and lakes (between 2,800 and 3,050 m a.s.l.), which present cold-adapted macroinvertebrate communities dominated by water beetles, some of which are Iberian endemics or micro-endemics only present in this mountain range (Millán et al., 2013, 2014).

In the present study, five of the most representative and widely distributed species of diving beetles (Dytiscidae) in the alpine

lakes of Sierra Nevada were studied (Abellán et al., 2022; Millán et al., 2013, 2014):

- *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 found only in Sierra Nevada (Millán et al., 2013, 2014). Although its taxonomic status has been questioned in relation to the widespread species *Agabus bipustulatus* (Ribera et al., 1998), according to recent studies by Drotz et al. (2010) and molecular analyses based on the COI mitochondrial gene (Bergsten et al., 2012), *A. nevadensis* is currently considered as a valid species nested within the *A. bipustulatus* complex.
- *Hydroporus sabaudus sierranevadensis* (Shaverdo, 2004) is a small-sized subspecies (3.5–4.0 mm) belonging to the *nigrita* group. This is an Iberian micro-endemic subspecies found only 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).

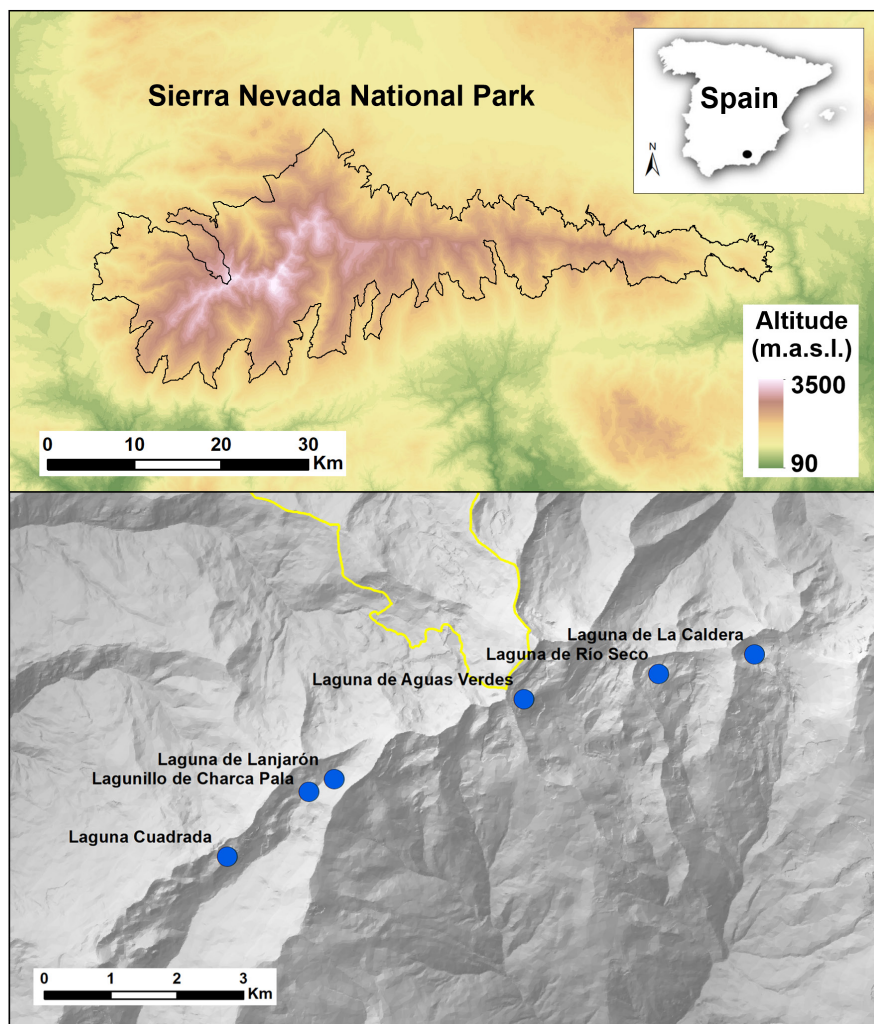


FIGURE 1 Study area showing the location of the sampled lakes.

- *Hydroporus marginatus* (Duftschmid, 1805) is a small-sized (4.2–4.5 mm) species belonging to the *marginatus* group. It has a Western Palaearctic distribution, occurring in the main mountain ranges of the Iberian Peninsula (Millán et al., 2013, 2014).
- *Boreonectes ibericus* (Dutton & 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).

2.2 | Specimen collection and housing

Adults of the study species (c. 100 individuals per species) were collected from six alpine lakes in Sierra Nevada National Park in summer 2021 (Figure 1; Table 1). All of the selected lakes belong to the cryoro-Mediterranean bioclimatic belt (>2,900 m a.s.l.) (Rivas Martínez, 1990) (see Table 1). Localities were selected based on records of the study species from previous studies (Millán et al., 2014; Pallarés et al., 2020). Specimens were collected using an aquatic hand net and transported within 24 h to the laboratory in the University of Murcia in 500-ml containers with moistened filter paper, placed in a portable refrigerator at approximately 10°C. Upon arrival, specimens were allowed to acclimatise to laboratory conditions for 10 days at 10°C under a 12h:12h, light:dark photoperiod in a climatic chamber (SANYO MLR-351). They were placed in 7-L tanks with 2 L of bottled water from Sierra Nevada (Lanjarón®), separated by species and with densities lower than 50 individuals/L. Stones from the collection sites were used as substrate. Individuals were fed daily *ad libitum* with frozen chironomid larvae. The acclimation temperature (10°C) was selected to mimic the average temperature of water during the ice-free season (June to October) in Sierra Nevada (unpublished data; see also Pallarés et al., 2020).

Individuals were starved for 24 h before trials, as gut content may modify thermal tolerance (Chown et al., 2004), and sets of individuals were randomly divided in sub-groups of between 10 and 25 individuals to estimate different cold- and heat-tolerance endpoints (see below).

2.3 | Assessing cold tolerance

In order to study the cold tolerance and overwintering strategies of the species, the SCP, lethal temperature (LLT), and tolerances to ice enclosure and to submersion were estimated.

The SCP is the temperature at which the body fluids of the organism begin to freeze when specimens are cooled. SCP has been used as an objective cold tolerance variable, comparable among species (Carbonell et al., 2016) and its measurement is often the first step in the investigation of insect cold tolerance (Sinclair et al., 2015). Moreover, as an indicator of internal ice formation, the SCP needs to be determined to distinguish among the three typical cold-tolerance strategies in insects: *chill-susceptible* species die at temperatures above their SCP; *freeze-avoidant* species typically survive temperatures slightly above the SCP; and *freeze-tolerant* species survive body fluid freezing (Lee, 2010). SCP was estimated as the lower temperature reached before the release of the latent heat of crystallisation by employing a dynamic method with a cooling rate of –1°C/min. Body surface temperature was measured using infrared thermography.

The LLT of the species was determined by cooling them in air at various shock below-zero temperatures (–5, –10, –15 and –20°C) and testing for survival and cooling injuries.

We tested whether the species could survive being temporarily enclosed in ice, exposing individuals at freezing conditions in contact to an ice cube at –1°C for 3 h. 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 internal freezing, hypoxia or mechanical damage (Conradi-Larsen & Sømme, 1973).

In order to test if the studied species are able to overwinter in their adult stage submerged without access to air under the ice layer of the lakes, tolerance to submersion was assessed as LLO. Individuals of the studied species (except for *H. nevadensis*, which was not included owing to methodological limitations) were individually maintained at 4°C in sealed bottles filled with aerated water, and oxygen concentrations and survival were recorded every 2–3 days for up to 11 days.

Full details of each experiment are shown in Appendix S1.

2.4 | Assessing heat tolerance

The HCT was estimated as the temperature at which the individuals experience paralysis before death, preceded by spasmodic movements of legs and antennae (Chown & Terblanche, 2006), under a heating rate of +1°C/min (see Appendix S2 for details).

The ULTs were determined underwater after exposing individuals to a slower heating rate of 3°C/day (see Appendix S2 for details).

TABLE 1 Sampled alpine lakes in Sierra Nevada National Park along with environmental data and species collected from each lake.

Lake	X	Y	Elevation (m a.s.l.)	Area (ha)	Species collected
Laguna de Aguas Verdes	37.048674	–3.368381	3,055	0.19	<i>Hydroporus sierranavadensis</i>
Laguna de Río Seco	37.052113	–3.345583	3,025	0.42	<i>Agabus nevadensis</i> + <i>H. sierranavadensis</i>
Laguna de la Caldera	37.054566	–3.329206	3,030	2.1	<i>Agabus nevadensis</i>
Laguna Cuadrada	37.027073	–3.418602	2,910	0.24	<i>Hydroporus nevadensis</i>
Laguna de Lanjarón	37.037718	–3.400453	2,980	0.29	<i>Hydroporus marginatus</i>
Lagunillo de Charca Pala	37.035964	–3.404770	2,945	0.04	<i>Boreonectes ibericus</i>

2.5 | Data analysis

The differences among species in their SCP, HCT and ULT were tested using generalised linear models (GLMs) with a normal error structure and identity link function. For the LLT and LLO experiments, the response variable (survival) was binary (dead or alive) and therefore GLMs with a binomial error structure and the logit link function were used. For the LLT, the effects of species, temperature of shock (i.e., the low temperature treatment tested) and their interactions were tested, whilst for the LLO, the effects of species, oxygen concentration range and their interaction were considered. We further explored significant effects for these two responses using Fisher's *post hoc* least significant difference (LSD)-tests with a Bonferroni correction. A probit analysis, a type of regression commonly used in toxicology to determine the relative toxicity of chemicals for living animals (Hahn & Soyer, 2005), was used to estimate the temperature and oxygen concentration at which 50% (LLT₅₀ and LLO₅₀, respectively) and 100% (LLT₁₀₀ and LLO₁₀₀) mortality occurred (Lencioni & Bernabò, 2015). 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, version 26.0.2019 (IBM Corp.).

3 | RESULTS

3.1 | Cold tolerance

Species showed significant differences in their SCP; *H. nevadensis* showed the lowest SCP (-9.86 ± 1.47) and *A. nevadensis* the highest value (-3.92 ± 0.62) (Figure 2; Tables 2 and 3).

In the LLT experiments, species did not show significant differences in survival throughout the progressively lower temperatures

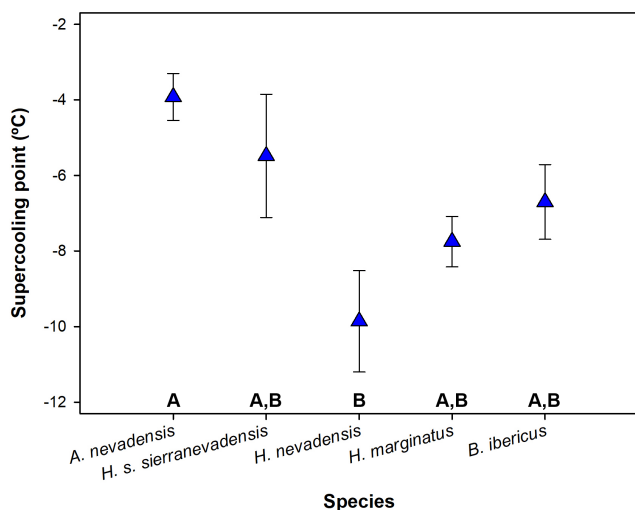


FIGURE 2 Supercooling points (mean \pm SE) of the five studied species measured at a cooling rate of 1°C/min. Capital letters represent differences among species' means, determined by Fisher's *post hoc* LSD *t*-test with Bonferroni correction.

(Figure 3; Table 3). Temperature had a significant effect on species' survival, which was lowest at -20°C and highest at -5°C , whereas no significant differences were found between -10 and -15°C (Figure 3; Table 3). Three of the studied species (*A. nevadensis*, *H. s. sierranevadensis* and *B. ibericus*) can be considered as moderately freeze-tolerant, as their LLT₅₀s were a few degrees lower than their SCPs (Figures 2 and 3; Table 2), whilst SCPs of *H. nevadensis* and *H. marginatus* were similar or lower to their LLTs. Moreover, species' survival was maximum (100%) during ice enclosure tolerance trials for all the species (Table 2).

Species' survival under submersion decreased along decreased oxygen concentration and differed significantly among the species (Figure 4; Tables 2 and 3). Adults of *A. nevadensis* showed no tolerance to submersion, and among the remaining species, adults of *B. ibericus* presented the lowest LLO₅₀ and LLO₁₀₀ (4.12 ± 4.90 mg/L and <3.21 mg/L, respectively) (Figure 4; Table 2).

3.2 | Heat tolerance

Species differed significantly in their HCT, with *B. ibericus* presenting the highest values and *A. nevadensis* the lowest (Figure 5a; Tables 2 and 3). However, ULT did not significantly differ among the species (Figure 5b; Tables 2 and 3).

4 | DISCUSSION

The present study provides experimental data to define the thermal niche and determine the cold-tolerance strategy of alpine aquatic beetles inhabiting lentic waters, which are key traits with which to assess their vulnerability under a climate-warming scenario. Adults of the species studied showed wide thermal niches. Most of the species seemed to be tolerant to both freezing of the internal fluids and to underwater submersion, which are crucial traits for facing the harsh winter conditions in alpine systems.

4.1 | Cold and freeze tolerance

Freeze-avoiding species generally present a very low freezing/SCP, which also matches closely their LLT (Block, 2003), whereas freeze-tolerant species survive body fluid freezing, presenting SCP values more than 10°C above their LLT (Lee, 2010; Sinclair, 1999). Moderate freeze-tolerance is the dominant cold-tolerance strategy among alpine insects, which typically survive a few degrees (less than 10°C) below their SCPs (Sinclair, 1999; Wharton, 2011). Therefore, on the one hand, three of the alpine diving beetles in this study (*A. nevadensis*, *H. s. sierranevadensis* and *B. ibericus*) can be considered as moderately freeze-tolerant according to their LLT₅₀s (from -8.20 to -9.79°C , with a mean of -9.23°C), which were a few degrees lower than their SCPs (i.e., a difference from -3.92 to -6.70°C , with a mean of c. -5.37°C). The freezing point and the LLT

TABLE 2 Values of the different studied endpoints for the five studied species: supercooling points (SCP), lower lethal temperatures 50% (LLT₅₀) and 100% (LLT₁₀₀), ice-enclosure tolerance, lower lethal oxygen concentrations 50% (LLO₅₀) and 100% (LLO₁₀₀), heat coma temperature (HCT) and upper lethal temperature (ULT). Results are presented as the mean ± SE for the SCP, HCT and ULT for each species. For the LLT₅₀ and LLO₅₀ the value and 95% confidence level are provided. Note that the LLO was not tested for *Hydroporus nevadensis*.

	<i>Agabus nevadensis</i>	<i>Hydroporus sabaudus sierranevadensis</i>	<i>Hydroporus nevadensis</i>	<i>Hydroporus marginatus</i>	<i>Boreonectes ibericus</i>
SCP (°C)	-3.92 ± 0.62	-5.48 ± 1.63	-9.86 ± 1.47	-7.75 ± 0.67	-6.70 ± 0.99
LLT ₅₀ (°C)	-9.79 ± 0.05	-8.20 ± 4.29	-9.79 ± 0.05	-5.79 ± 1.51	-9.70 ± 6.39
LLT ₁₀₀ (°C)	-15	-20	-15	-15	-20
Ice-enclosure tolerance (% survival)	100	100	100	100	100
LLO ₅₀ (mg/L)	11	10.67 ± 2.96	-	5.70 ± 0.04	4.12 ± 4.90
LLO ₁₀₀ (mg/L)	11	10.20	-	5.55	<3.21
HCT (°C)	43.30 ± 0.18	44.81 ± 0.19	44.90 ± 0.39	44.68 ± 0.13	46.19 ± 0.19
ULT (°C)	33.11 ± 0.59	32.57 ± 2.19	30.30 ± 1.56	34.00 ± 1.77	35.62 ± 1.33

Response variable	Effect	Wald χ^2	Df	p-Value
SCP	Species	20.104	4	<0.001
LLT	Species	6.044	4	0.196
	Tshock	60.443	1	<0.001
	Species × Tshock	6.037	4	0.196
LLO	Species	32.265	3	<0.001
	Oxygen concentration range	15.285	1	<0.001
	Species × oxygen concentration range	5.148	3	0.161
HCT	Species	82.555	4	<0.001
ULT	Species	6.068	4	0.194

TABLE 3 Results of the generalised linear models for the supercooling point (SCP), lower lethal temperature (LLT), lower lethal oxygen concentration (LLO), heat coma temperature (HCT) and upper lethal temperature (ULT).

Note: Bold p-values indicate significant differences ($p \leq 0.05$).

pattern shown by the studied species matches with that of most alpine insects, showing a moderate freeze-tolerance response. For example, this pattern has been observed in adults of freeze-tolerant alpine insects (see Wharton, 2011), like the orthopteran species *Hemideina maori*, which can be observed covered with ice crystals in its natural habitat (Leader & Bedford, 1978), and was experimentally confirmed to be freezing-tolerant (Ramløv et al., 1992). The alpine cockroach *Celatoblatta quinquemaculata* also shows a moderate freezing-tolerance and presents activity throughout the year, having been observed frozen in the field and recovering upon thawing (Sinclair, 1997). Other alpine insect species with aquatic larval stages have been described to be unequivocally freeze-tolerant, including more than 25 chironomid species (Bouchard et al., 2006; Danks, 1971), empidids, culicids, nemourids (Walters Jr et al., 2009) and even dragonflies (see Danks, 2007); however, there are species which do not present this trait (e.g. the alpine stonefly *Lednia tumana*, see Hotaling et al., 2021). On the other hand, two of the studied species (*H. nevadensis* and *H. marginatus*) showed SCPs (-9.86 and -7.75°C, respectively) similar to their LLT₅₀s (-9.79 and -5.79°C, respectively). These results seem to point out that these species are not freeze-tolerant but could be partially freeze-tolerant—thus, they survive a small proportion of their body water being converted into ice (Sinclair, 1999). *Hydroporus nevadensis* and *H. marginatus* are

widely distributed in the Iberian Peninsula, and although they occur mainly in mountain ranges, these species have been reported inhabiting lowland water bodies, even at sea level in the case of *H. marginatus* (Millán et al., 2014). Therefore, these species could be less adapted to cold temperatures than the other study species, which are restricted to alpine conditions in high mountain ranges.

The moderate freeze-tolerance capacity of most of the studied diving beetles could be a key adaptation to survive the extreme below-zero air temperatures in alpine systems. However, these species also might face the risk of ice-enclosure, which may not provoke freezing of their internal fluids, but causes mechanical damage as a result of the expansion of water as it freezes (Danks, 1971; Olsson, 1981). All species studied here survived 3 h of ice enclosure after 24-h acclimation at 4°C. This suggests they are able to avoid or resist such damage that direct contact with ice poses, at least during relatively short exposure times, something which could be accounted for protective mechanisms such as a thick and physically resistant cuticle or cryoprotectant molecules (such as antifreeze proteins or epicuticular waxes) (Danks, 2007; Olsen et al., 1998). Such capacity to survive ice enclosure seems to be common (although not universal; see Hotaling et al., 2021) among other aquatic alpine insects. For example, larvae of the freeze-tolerant stonefly *Nemoura arctica* survived contact with ice in the laboratory (Walters Jr et al., 2009).

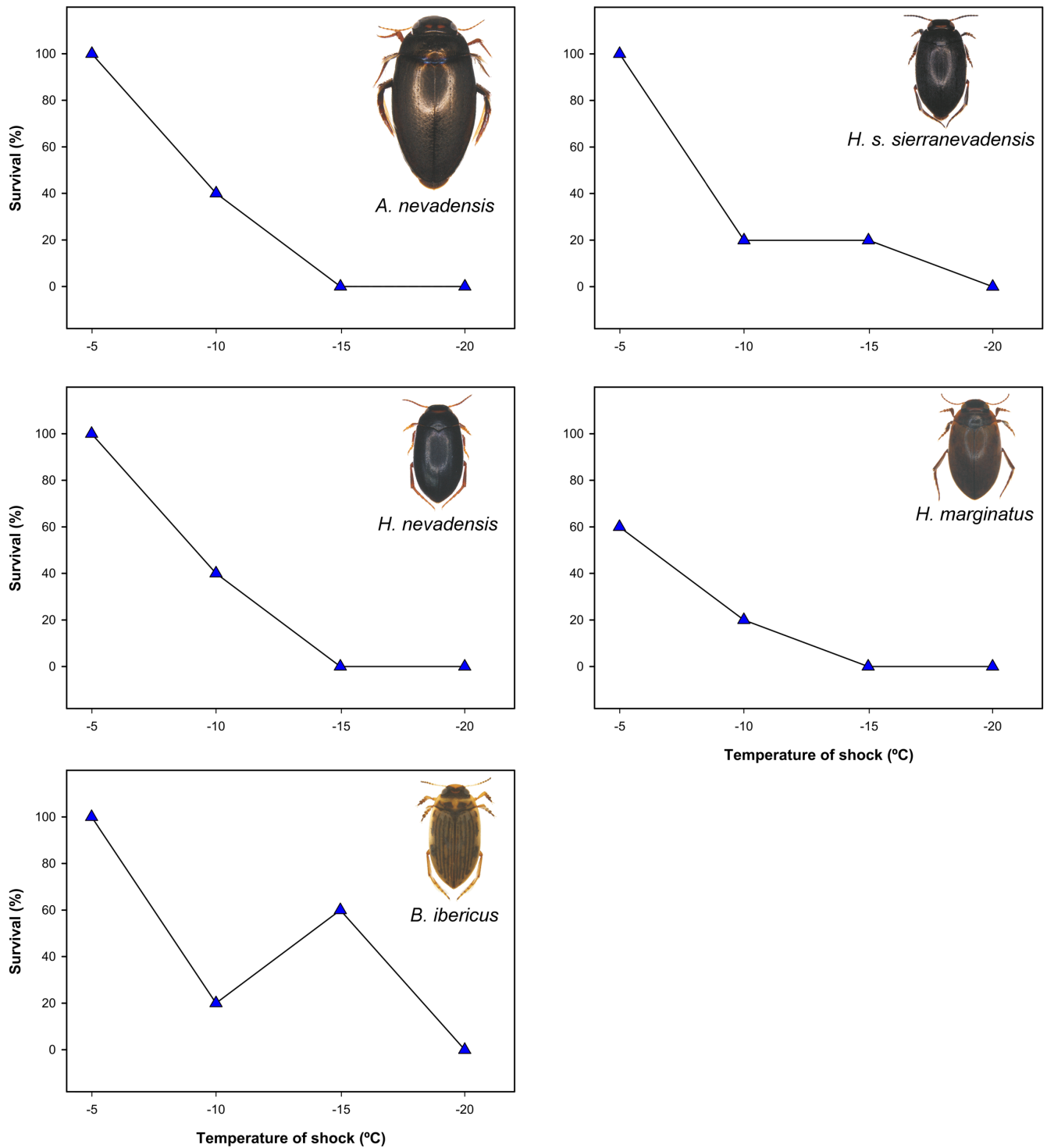


FIGURE 3 Survival curves at low temperatures of the five studied species. Significant differences between temperatures determined by Fisher's *post hoc* LSD *t*-test with Bonferroni correction are as follows: -5°C (A), -10°C (A,B), -15°C (B,C), -20°C (C).

Mihalic (2015) found that several species of Hemiptera, Coleoptera (larval stages), Diptera and Odonata retrieved from the ice and sediment, survived after thawing.

The LLT₁₀₀ of the studied species (from -10 to -20°C) were much lower than those described for other lentic diving beetles from lower altitudes. For instance, the LLT reported for several Iberian populations of non-alpine dytiscid species of genera *Agabus*

and *Nebrioporus* ranged between -3 and -8°C, with a maximum of -12°C for *Agabus brunneus* from northern Spain (Calosi et al., 2008; Hidalgo-Galiana et al., 2014; Sánchez-Fernández et al., 2010). The LLTs of lowland diving beetles are not only higher than those of their alpine relatives, but also close to their SCPs, so these species could be considered to be freeze-avoiding and/or not freeze-tolerant species. This factor could be an important physiological limitation

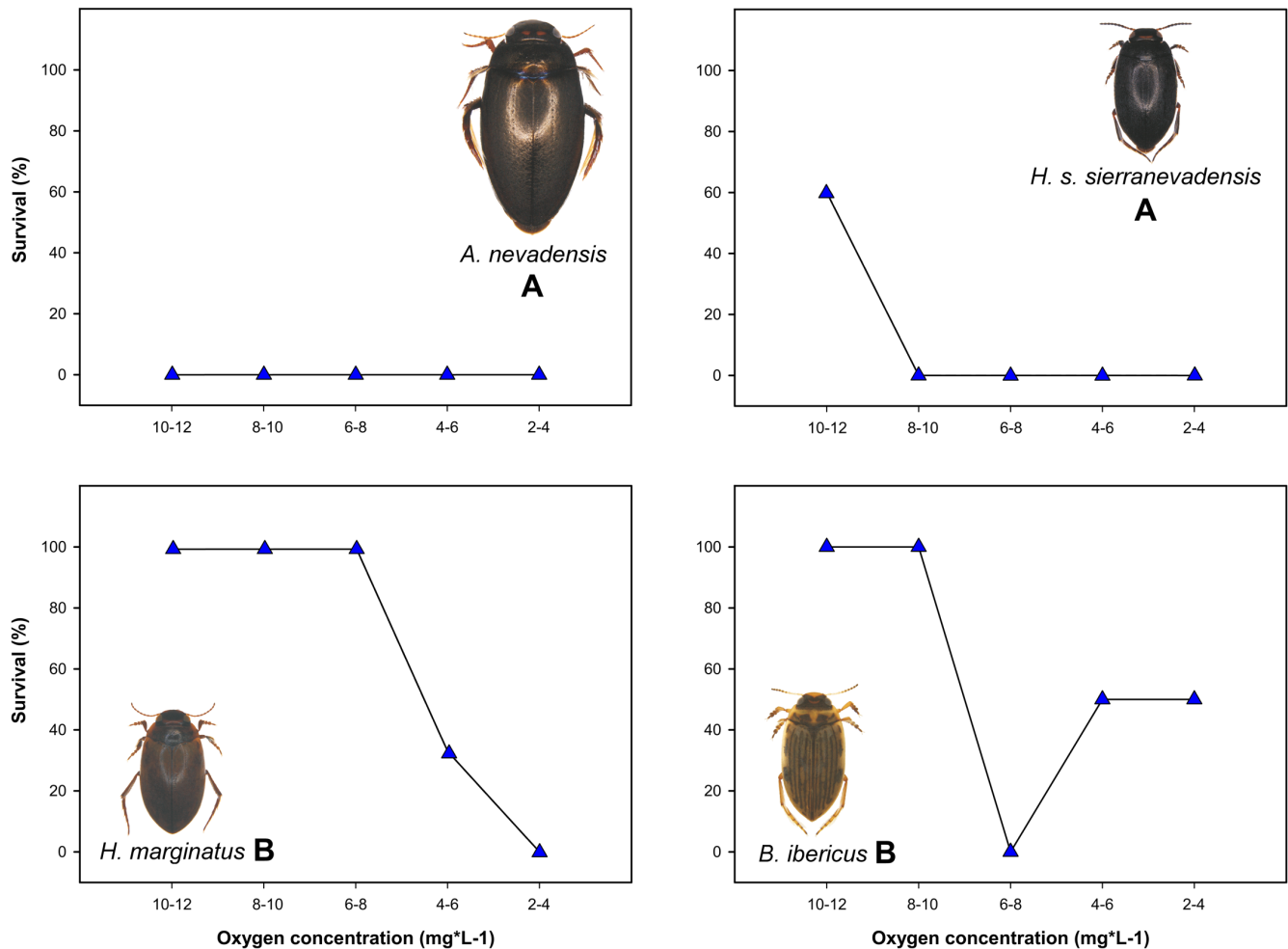


FIGURE 4 Survival curves at decreased oxygen concentration intervals of four of the studied species. Capital letters represent differences between species, determined by Fisher's *post hoc* LSD *t* test with Bonferroni correction.

preventing lowland species from inhabiting alpine systems, leading to the altitudinal segregation of diving beetles from lowlands to high-altitude mountain ranges. However, further studies are needed to confirm this assumption.

4.2 | Overwintering strategies

Aquatic life stages of insects are less exposed to freezing and sub-zero temperatures than terrestrial ones as a result of the thermal buffering of water, especially when they take refuge in the deepest part of the water body during the winter (Boehrer & Schultze, 2008). However, the oxygen concentration beneath the surface decreases with depth in oligotrophic alpine lakes (Rempfer et al., 2010; Rogora et al., 2018), so many species compensate for this by moving to microhabitats such as pond edges, which have higher oxygen concentrations than elsewhere (Brittain & Nagell, 1981). This way, they reduce the exposure to freezing by finding safe refugia in the hyporheic zone (Danks, 2007; Lencioni & Spitale, 2015).

Adults of the studied diving beetle species might represent an exceptional case, as all of them need to take up oxygen in the air, so

overwintering in water under the ice may pose a challenge to meet respiratory requirements. However, underwater respiration has been described previously in other small diving beetle species that also have surface respiration, such as several species from the genera *Hygrotus*, *Deronectes*, *Nebrioporus*, *Oreodytes*, *Stictotarsus*, *Porhydrus*, *Graptodytes* and *Paroster* (Jones et al., 2019; Madsen, 2012; Verberk et al., 2018). These taxa use a kind of specialised plastron where air fluxes from the water to the trachea system through respiratory pores on the elytron surface, which allows the species to stay submerged for several weeks (Madsen, 2012). The pore respiration mode in lentic waters has been interpreted as an advantage to reduce the risk of falling victim to pelagic predators (Madsen, 2012). In alpine ponds, it also could represent an adaptation to overwinter underwater in the adult stage. Submersion tolerance trials indicate that all of the species studied herein, with the exception of *A. nevadensis*, might be able to overwinter underwater without visiting the surface to replenish their air bubbles. *Hydroporus sabaudus sierranevadensis*, *H. marginatus* and *B. ibericus* showed a LLO₅₀/LLO₁₀₀ of 10.67/10.20, 5.70/5.55 and 4.12/3.21 mg/L, respectively, common values in oligotrophic lakes in the Alps (between 6 and 11 mg/L above the first 20–30 m during winter; Rempfer et al., 2010; Rogora et al., 2018), which are similar in altitude and geology to the

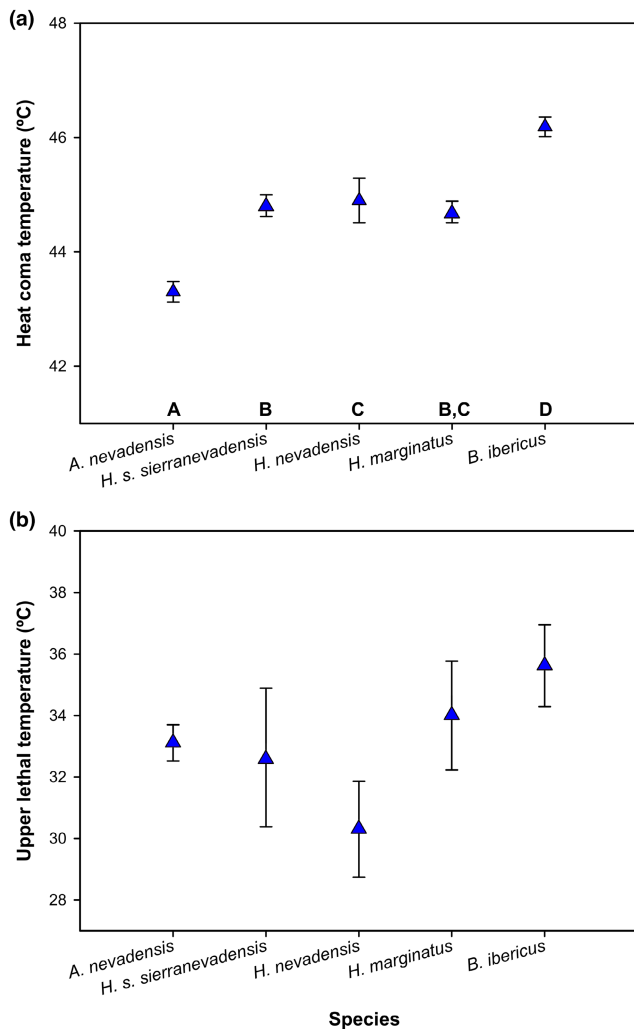


FIGURE 5 (a) Heat coma temperatures (mean \pm SE) and (b) upper lethal temperatures (mean \pm SE) of the five studied species measured at a heating rate of 1°C/min and 3°C/day, respectively. Capital letters represent differences between species, determined by Fisher's *post hoc* LSD *t*-test with Bonferroni correction.

Sierra Nevada alpine lakes. These results support the idea that these species may overwinter as adults (as well as larvae) in water under the ice layer, probably reducing activity and metabolic rate to enhance survival (Guppy & Withers, 1999; Lencioni, 2004).

However, submersion tolerance trials indicated that *A. nevadensis* is a submersion non-tolerant species, as all specimens died at high oxygen concentrations of about 100% saturation, and therefore this species could be considered exclusively as a surface breather. Other *Agabus* species such as *A. guttatus* also have been reported as submersion non-tolerant species (Madsen, 2012), using surface respiration exclusively. Therefore, results seem to point out that this species could winter underwater solely as larvae, which is supported by two extra observations: (1) mainly last-stage larvae of *A. nevadensis* are found at the beginning of the ice-free season (unpublished data) and (2) in an explorative macroinvertebrate sampling under the ice layer carried out for the first time in Sierra Nevada on 25 November 2021 in “La Caldera” lake, only the larvae of *A. nevadensis* were found (Figure 6),

together with active chironomid larvae that constitute their main prey organisms. However, we cannot discount the possibility that *A. nevadensis* could overwinter as adults using habitat selection strategies (Lencioni, 2004). For example, the adults of this species might be able to escape from the water column before ice sealing and migrate to land shelters under rocks/mud, protected from freezing air conditions insulated by overlying snow, where they overwinter by supercooling (Danks, 2007), as described in Gerrids and some caddisflies (Berté & Pritchard, 1983; Spence & Anderson, 1994). In such explorative sampling, adults of *B. ibericus* also were found, supporting the capacity of this species to overwinter underwater in adult stage (see above). Increasing the sampling effort in coming winters will be necessary to disentangle these interesting aspects.

4.3 | Heat tolerance

Whilst ULT did not significantly differ among the studied species, they showed significant differences in HCTs, ranging from 43.3°C to 46.2°C for *A. nevadensis* and *B. ibericus*, respectively. These values match those reported for other aquatic diving beetles from warmer lowlands (e.g., from about 43°C to 46°C for *Nebrioporus* spp. and from about 42°C to 46°C for *Agabus* spp.; Calosi et al., 2008; Hidalgo-Galiana et al., 2014; Sánchez-Fernández et al., 2010). This is congruent with the general pattern in ectotherms showing that as organisms adapt to cooler habitats, selection on upper thermal tolerance is relaxed (Thomas et al., 2001). Meanwhile, adaptation to lower thermal tolerance is intensified, leading to increased breadths of thermal tolerance as upper thermal tolerance is conserved (Araújo et al., 2013; Hoffmann et al., 2013; Kellermann et al., 2012; Lancaster et al., 2015).

A previous study using upper thermal limits to assess the vulnerability to climate change of *A. nevadensis* and *H. s. sierranevadensis* in Sierra Nevada (Pallarés et al., 2020) reported that both species show heat-tolerance limits above the predicted maximum temperatures in their current localities, according to climate projections for the next decades. Such a result suggests that these taxa will have the physiological capacity to deal with warming conditions *in situ*, although their climatically suitable area (estimated by ecological modelling) would be reduced. Considering that the five studied species presented similar heat-tolerance patterns and occur in the same climatic area in Sierra Nevada (mainly the cryoro-Mediterranean climatic belt), this future scenario can be expected for all of the studied taxa. However, biological pressures could be considered as a potential factor threatening the alpine diving beetle assemblages in a climate-warming scenario. Previous studies suggest that aquatic beetles from lowlands are not freeze-tolerant species, preventing them from colonising colder alpine areas in current climate conditions (Calosi et al., 2008; Sánchez-Fernández et al., 2010; Hidalgo-Galiana et al., 2014). In future scenarios where alpine areas would present milder temperature conditions, lowland species could colonise alpine lakes where the studied species currently occur, competing for the resources and potentially modifying these unique



FIGURE 6 Macroinvertebrate sampling carried out in “La Caldera” lake on 25 November 2021. (a) F. Picazo sampling under the ice layer. (b) Larvae of *Agabus nevadensis* (above) and adult of *Boreonectes ibericus* (below) found during sampling. Photo credits: Eulogio Corral (a) and J.A. Carbonell (b).

and fragile aquatic macroinvertebrate communities. Several studies point out that many species from lowlands have already begun migrating upslope, tracking their unsuitable thermal regimes (e.g., Walther et al., 2002). The colonisation of high-mountain regions by lowland species may be an important threat for singular alpine communities, as biological interactions structuring insect communities including predation, disease loads and competition for available resources (Schowalter, 2016) can be affected, which is likely to lead to shifts in the food-web interactions (Rosenblatt et al., 2019) and potential extinction of local species. For example, warming in an arctic lake modified the resident chironomid community, apparently through new competitive interactions with warm-adapted species (Quinlan et al., 2005). Thus, our study sets the basis for further research exploring the lower thermal limits and freeze tolerance of diving beetles from nearby lowland areas, as well as the potential biotic interaction among lowland and alpine beetles, which are needed to complete the knowledge on the potential threats that alpine diving beetle assemblages from Sierra Nevada would face in a climate warming context.

5 | CONCLUSIONS AND FUTURE DIRECTIONS

Our study provides the first comprehensive description of cold tolerance and overwinter strategies of alpine dytiscid beetles. We show that most of the studied species can be considered as moderately freeze-tolerant, as their SCPs are higher than their LLTs. Moreover, all of them can tolerate ice enclosure, suggesting that

they have protective mechanisms to avoid mechanical damage and cellular injuries from ice exposure. All species except for *A. nevadensis* also showed tolerance to submersion in the adult stage, which is a key adaptation to overwintering underwater and reducing risk from freezing conditions in the air.

The studied species did not differ in their upper thermal limits, which were similar to those of non-alpine species, suggesting that increasing average temperatures by climate warming will not be the most important threat for their populations in Sierra Nevada (see Pallarés et al., 2020). However, new biotic interactions with potential colonisation of aquatic beetles from lowlands in a warmer climate scenario may put populations of the studied species at risk (Shah et al., 2020).

AUTHORS CONTRIBUTIONS

Conceptualisation: J. A. Carbonell, S. Pallarés, J. Velasco, A. Millán, F. Picazo, P. Abellán. Developing methods: J. A. Carbonell, S. Pallarés, F. Picazo, P. Abellán. Data analysis: J. A. Carbonell, S. Pallarés. Preparation of figures and tables: J. A. Carbonell. Conducting the research, data interpretation, writing: J. A. Carbonell, S. Pallarés, J. Velasco, A. Millán, F. Picazo, P. Abellán.

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CONFLICT OF INTEREST STATEMENT

The authors declare no competing interest.

DATA AVAILABILITY STATEMENT

Data from the experiments supporting this article will be available in a public repository upon publication.

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SUPPORTING INFORMATION

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

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