

# Reduced salinities compromise the thermal tolerance of hypersaline specialist diving beetles

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**Abstract.** Inland saline waters are globally threatened habitats that harbour unique assemblages of specialist invertebrates. In many Mediterranean regions, irrigation associated with intensive agriculture is lowering the salinity of these habitats, resulting in the loss of their specialist biota, although the mechanisms by which reductions in salinity lead to species loss are poorly understood. In the present study, the effects of reduced salinity on the temperature tolerance and thermal acclimatory abilities of two related species of hypersaline water beetles, *Nebrioporus baeticus* (Schaum) and *Nebrioporus ceresyi* (Aubé), are explored. Both upper (UTL) and lower thermal limits (LTL) are assessed, and both salinity and temperature are found to influence the thermal biology of *Nebrioporus*. Mean UTLs are greater in individuals of both species acclimated at high salinities, with salinity appearing to be more important than acclimation temperature in determining UTL. In both taxa, the lowest mean LTLs are recorded in individuals acclimated at the highest salinities and lowest temperatures; temperature-dependent acclimation is only reported after exposure to relatively high salinities. The data show that salinity influences the thermal tolerance and acclimatory ability of these hypersaline beetles, and that lowered salinity compromises the ability of adult *Nebrioporus* to cope with both heat and cold. Such an effect may partly explain why specialist species are lost from hypersaline habitats subject to salinity reductions, and suggests that ongoing reduction in salinity may compromise the ability of such specialist taxa to cope with rapid climate change.

**Key words.** Climate change, inland saline waters, salinity tolerance, thermal physiology.

## Introduction

The Mediterranean basin is a region of special biogeographic interest, being regarded as one of Europe's biodiversity hot spots (Médail & Quèzel, 1999). The region contains a wide variety of inland aquatic habitats, including saline waters, which can be both running and standing, and these typically occur in coastal environments and Miocene gypsiferous marl-rich basins (Hrbek & Meyer, 2003; Velasco *et al.*, 2006).

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Inland saline waters are characterized by hydrological extremes that result in repeated wet and dry cycles, which, together with high salt concentrations, result in unique assemblages of specialist species (Williams *et al.*, 1990; Rutherford & Kefford, 2005). Several species characteristic of Mediterranean hypersaline systems are of concern with respect to their conservation, having recently been proposed for inclusion in International Red Lists (Sánchez-Fernández *et al.*, 2008).

To date, the criteria that are used to assess species' conservation priorities are almost exclusively ecological, on the assumption that protected species will be able to mount biological responses to climate change, if afforded protection

(Thomas *et al.*, 2004). It is clear, however, that those taxa that are unable to shift their geographical ranges during periods of rapid climate change are at risk of extinction if they are unable to respond either physiologically (Davis & Shaw, 2001; Calosi *et al.*, 2008a; Calosi *et al.*, 2010) or behaviourally (Kearney *et al.*, 2009). Consequently, an understanding of species' physiological performances can help predict their vulnerability to current and future global change scenarios (Stillman, 2003; Deutsch *et al.*, 2008; Tewksbury *et al.*, 2008; Gaston *et al.*, 2009; Helmuth, 2009), and thus enhance species conservation (Kearney & Porter, 2004; Wikelski & Cooke, 2006).

Temperature is long recognized as one of the most important factors influencing species' abundance and distribution because it underpins metabolic activity and life-history processes (Willott & Hassall, 1998), and temperature tolerance is linked to geographical range extent and position in a number of groups (Brattstrom, 1968; Calosi *et al.*, 2008a, b; Calosi *et al.*, 2010). Despite this, other environmental factors must play an important role and may act additively, synergistically or even antagonistically with temperature to determine range extents and limits (Spicer & Gaston, 1999; Gaston, 2003), and thermal tolerances themselves are influenced by other features of the environment that individuals experience (Chown & Nicolson, 2004). In addition to shifts in temperature as a result of global climate change, inland water bodies throughout the world are adversely affected by a range of anthropogenic activities, including the extensive use of fertilizers in agriculture, and changes in hydrology (Polunin, 2008), all of which result in changed environmental conditions for aquatic organisms. To predict organismal responses to global change, there is an urgent need for studies that examine the effects of multiple stressors on organismal physiology (Chown & Gaston, 2008; Pörtner & Farrell, 2008; Widdicombe & Spicer, 2008; Williams *et al.*, 2008).

In marine systems, many studies demonstrate that salinity influences the thermal physiology of a range of animal taxa; for example, Crustacea (Kir & Kumlu, 2008), bivalves (Rajagopal *et al.*, 2005) and fish (Sardella *et al.*, 2008a, b). To date, however, no data are available on the effects of changes in salinity on the thermal biology of organisms living in inland saline systems. Given that much of the biota of inland saline waters originates from within primarily freshwater clades (Ward, 2002) and that these habitats often show extreme fluctuations in salinity (Ueda *et al.*, 2000; Moreno *et al.*, 2009), the responses may differ from those observed in marine taxa. In addition, these habitats are under increasing threat from local anthropogenic influences in their catchments, which modify salinity regimes considerably (Carreño *et al.*, 2008; Gutiérrez-Cánovas *et al.*, 2009).

Although the effects of increasing salinity on freshwater ecosystems and inland salt lakes are reported extensively (Williams, 2001; Jellison *et al.*, 2008), the impact of reductions in salinity on hypersaline systems is much less well-documented; indeed, a recent review of global threats to aquatic ecosystems (Polunin, 2008) does not even refer to the problem. Despite this, a number of studies show that increases in freshwater inflow have a dramatic effect on the communities of hypersaline waters (Velasco *et al.*, 2006; Gutierrez-Cánovas

*et al.*, 2009), giving rise to an increase in diversity (through an influx of generalist taxa) but a reduction in abundance or elimination of hypersaline specialists (Velasco *et al.*, 2006). Although the mechanisms driving these community changes are poorly understood, this reduction in salinity has become an increasingly common phenomenon in the Mediterranean basin as a result of recent changes in agricultural practice (Sánchez-Fernández *et al.*, 2004). Given that salinity is known to affect the thermal physiology of many marine organisms, this dilution process may also have subtle, physiological effects on inland hypersaline specialists. These effects may not lead to the immediate elimination of species from sites experiencing moderate freshwater inflow but may influence species' abilities to respond to future shifts in temperature, although data on individual species' responses are currently lacking.

The present study investigates how salinity influences the thermal biology of two closely-related species of diving beetle that are typical of inland hypersaline systems: *Nebrioporus baeticus* (Schaum) and *Nebrioporus ceresyi* (Aubé) (Coleoptera, Dytiscidae). Of these, *N. baeticus* is endemic to south-eastern Spain and is found in lotic hypersaline waters or ramblas, whereas *N. ceresyi* is a circum-Mediterranean species of standing waters such as salt pans, particularly those located in lowland areas near to the coast (Fery *et al.*, 1996). The two species are closely related, belonging to a clade of hypersaline specialists within *Nebrioporus*, for which the majority of species inhabit lotic freshwaters (Toledo, 2009). The effects of acclimation to different salinities and temperatures on heat and cold tolerance are examined, alongside a consideration of whether the ongoing dilution of hypersaline systems with fresh water could compromise the ability of their specialist inhabitants to survive in a changing climate, providing the first such data for inland saltwater taxa.

## Materials and methods

### *Insect collection, maintenance and experiment preparation*

Adults of *Nebrioporus baeticus* and *N. ceresyi* were collected during August 2007 using a D-framed pond net (500 µm mesh; 20 × 25 cm). Sampling sites were located in south-eastern Spain (Region of Murcia): Rambla del Reventón (37°38'N, 1°22'W) for *N. baeticus* and Calblanque (37°36'N, 0°44'W) for *N. ceresyi*.

After collection, individuals were transported to the laboratory in 1-L plastic containers filled with damp aquatic vegetation, kept within thermally insulated bags (Thermos®; Thermos LLC, Rolling Meadows, Illinois) to minimize thermal variation. Once in the laboratory, specimens were maintained in 5-L aquaria (maximum of 20 individuals per tank) containing aerated artificial pond water (pH 7.5; ASTM, 1980) under an LD 12 : 12 h photocycle, and fed live chironomid larvae *ad libitum*. Each species was divided into 15 equal groups across five salinities (1, 6, 12, 35 and 60 g L<sup>-1</sup>) and three temperatures (14.3, 19.3 and 24.3 °C), where they were kept for 7 days (Hoffmann & Watson, 1993; Klok & Chown, 2003; Terblanche & Chown, 2006; Calosi *et al.*, 2008a, b). Saline solutions were

prepared by dissolving Instant Ocean® (Aquarium Systems, France) in double-distilled water. Temperatures and salinities were chosen as being representative of the habitat range where species are present and to simulate the effects of lowered salinity, which have been observed in some of these systems (Velasco *et al.*, 2006). These species are typical from waters with salinity above 20 g L<sup>-1</sup> (*N. baeticus*: mean salinity 30.01 g L<sup>-1</sup>, range 0.2–280.0 g L<sup>-1</sup>; *N. ceresyi*: mean salinity 34.1 g L<sup>-1</sup>, range 0.7–188.3 g L<sup>-1</sup>). Mean ± SE field temperatures are 21.8 ± 5.9 °C for *N. baeticus* and 20.6 ± 5.4 °C for *N. ceresyi* (D. Sánchez Fernández, P. Arribas, J. Velasco and A. Millán, personal observations).

After acclimation, individuals from each of the 15 treatments were further randomly assigned to two equal subgroups: one was used to measure upper thermal limits (UTL) and the other measured lower thermal limits (LTL). Finally, individuals were weighed (±0.001 g) using a Sartorius 1204 MP2 balance (Sartorius Ltd, U.K.).

### Experimental procedure

Experiments to determine upper and lower thermal limits commenced at the temperature at which individuals of a given sub-group had been acclimated. A total of 313 individuals were used: *N. baeticus* ( $n = 155$ ) and *N. ceresyi* ( $n = 158$ ). Tests were carried out in air, employing a dynamic method and using a ramping program (±1 °C min), with a computer-controlled water bath [Grant LTC 6-30, using Grant Coolwise software; Grant Instruments (Cambridge) Ltd, U.K.]. The experimental ramping rate and equilibration temperature can influence the outcome of thermal tolerance tests (Terblanche *et al.*, 2007; Chown *et al.*, 2009) and, consequently, selecting ecologically realistic ramping rates is difficult when comprehensive environmental data are lacking. Therefore, an identical ramping rate was employed to allow comparisons amongst treatments and taxa (Lutterschmidt & Hutchison, 1997a, b; Calosi *et al.*, 2008a, b). Individuals were introduced, one per well (diameter 12 mm, depth 18 mm), into a generic 24-well plastic culture plate (Corning Ltd, U.K.), whose external base was painted white with Tipp-Ex® to allow easy visualization of temperature-related responses. A maximum of 12 individuals was tested at any one time, and two investigators worked together to ensure accuracy when recording thermal limits. Holes were made in the sides and bottom of the plates (but not the actual wells) to allow for maximum circulation of water (used for UTL experiments) or 70% ethylene glycol solution (used for LTL experiments) around the wells. The actual temperature within each well was measured directly using a calibrated digital thermometer (Omega® HH11; Omega Engineering Inc., Stamford, Connecticut) equipped with an Omega® precision fine-wire thermocouple (type K–diameter/gauge 0.010 Teflon). Individuals were removed from their acclimation aquaria, quickly and carefully dried on absorbent paper, and then placed in a clean, dry well. To avoid escape, plates were covered with a lid between additions of individuals. Once the experiment started, the lid was removed to avoid the build-up of water vapour, which can affect thermal tolerance (Pörtner, 2001).

### Determination of upper and lower thermal tolerance

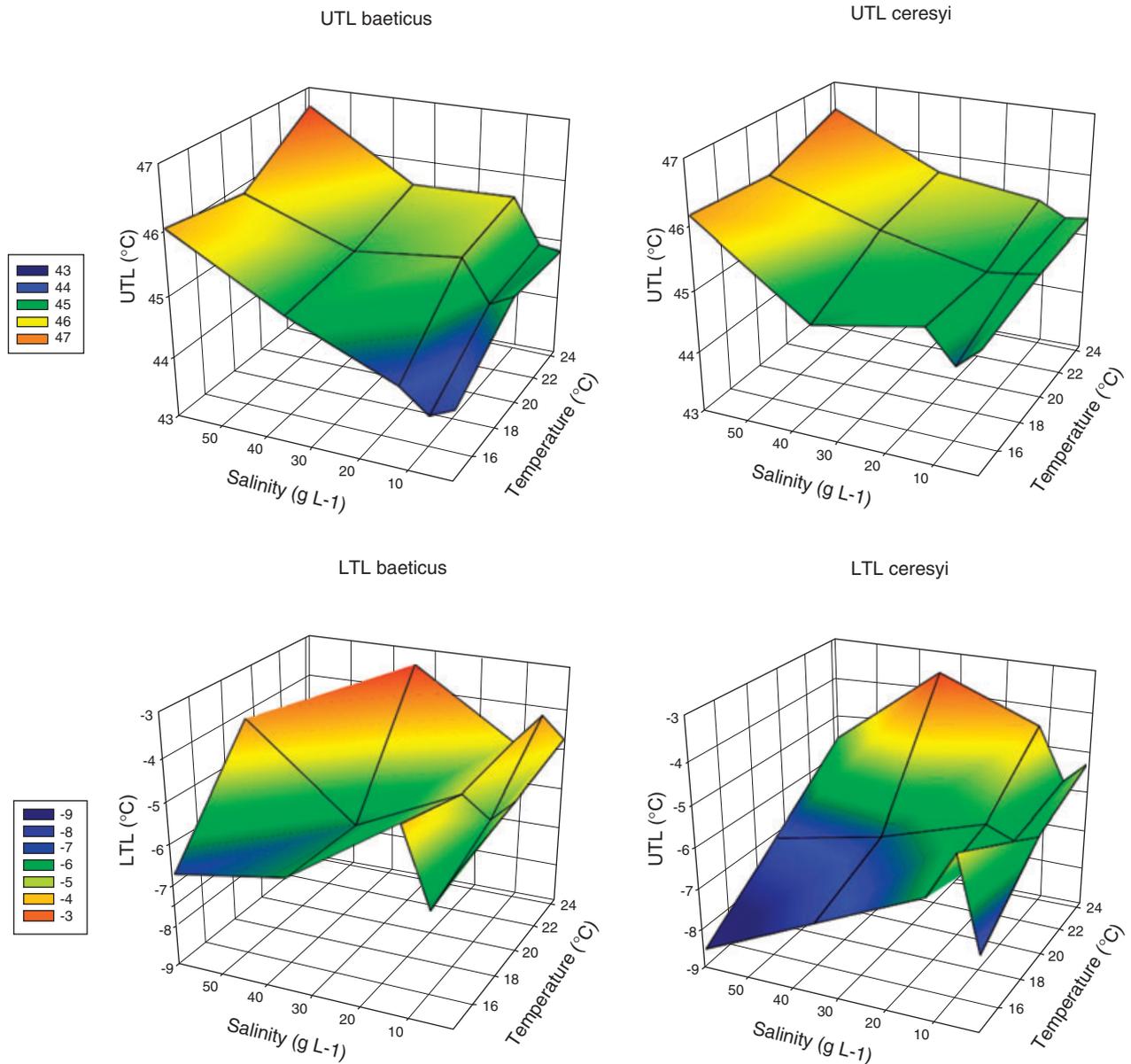
Although a number of potential end-points were identified, for both tolerance to heat and cold, in preliminary experiments, lethal points were more repeatedly identifiable for UTL and LTL, as already documented for other diving beetle species (Calosi *et al.*, 2008a, b). Death was readily identifiable in upper thermal tolerance experiments, with this comprising the point at which all movement of antennae and palpi ceased. Such cessation of movement was preceded by spasmodic movement of legs, antennae and palps, and therefore represents heat coma (Chown & Terblanche, 2007; Hazell *et al.*, 2010). Beetles never revived after the cessation of movement in UTL experiments. Defining lower lethal limits proved more difficult than for upper ones because individuals exhibiting total paralysis (and apparently dead) would revive and recover full or partial locomotory abilities shortly after the end of the exposure period. Consequently, paralysed individuals were instantly warmed to 30 °C for 3 s, and returned to the experiment if they recovered. This operation was first performed at 2 °C below the temperature at which total paralysis was initially observed, and was then repeated at regular intervals of 2 °C of cooling, until beetles no longer recovered and death was recorded (Calosi *et al.*, 2008b).

### Statistical analysis

Data met the assumptions of normality for both UTL and LTL (Shapiro–Wilks test, minimum  $W = 0.954$ ,  $P = 0.06$ ) but did not meet assumptions for homoscedasticity of variance ( $P = 0.001$ ). Despite this, and because there were 15 treatments with 313 individuals in total, an analysis of variance approach was employed (Underwood, 1997). Multifactorial analysis of variance was used with untransformed data to investigate the effects of salinity and acclimation temperature (with mass as a covariate) on both UTL and LTL, separately for each species. Post-hoc Bonferroni tests were employed to identify significant differences amongst mean UTLs or LTLs at given temperatures or salinities of acclimation. All statistical analyses were conducted using SPSS, version 15.0 (SPSS Inc., Chicago, Illinois).

### Results

Overall, levels of tolerance to heat (UTLs) were higher in individuals acclimated at high temperatures and high salinities (Fig. 1), and higher tolerances to cold (LTLs) were observed in individuals acclimated at higher salinities and lower temperatures (Fig. 1). *Nebrioporus ceresyi* and *N. baeticus* had comparable UTLs, whereas *N. ceresyi* showed a higher tolerance to cold (LTLs), making the thermal range of this species broader ( $P < 0.05$ ; Fig. 1). No individuals died during acclimation, across the entire range of salinities and temperatures employed.



**Fig. 1.** Variation in upper thermal limit (UTL) and lower thermal limit (LTL) of *Nebrioporus baeticus* and *Nebrioporus ceresyi* acclimated to different combinations of salinity (1, 6, 12, 35 and 60 g L<sup>-1</sup>) and temperature (14.3, 19.3 and 24.3 °C).

#### Upper thermal limits

As stated above, *N. baeticus* and *N. ceresyi* have comparable mean UTLs. The minimum mean UTL for both *N. baeticus* and *N. ceresyi* was observed in individuals acclimated at a salinity of 6 g L<sup>-1</sup> and 14.3 °C (Fig. 1). On the other hand, maximum mean UTLs for both species were measured in individuals acclimated at 60 g L<sup>-1</sup> and 24.3 °C.

Overall similar responses to acclimation were observed for the two species examined, with temperature and salinity observed to exert an additive positive effect on mean UTL. For both species, a significant positive relationship between

UTL and both salinity (maximum  $P < 0.001$ ; Fig. 2 and Table 1) and acclimation temperature (maximum  $P = 0.003$ ; Fig. 2 and Table 1) was found, whereas the interaction between temperature and salinity was not statistically significant ( $P > 0.05$ ; Fig. 2 and Table 1). Body mass positively affected mean UTL in *N. ceresyi* ( $P = 0.009$ ; Table 1) but not in *N. baeticus* ( $P > 0.05$ ; Table 1). Of particular interest, in both species, mean UTLs of individuals that had been maintained at 14.3 °C were significantly lower than those of beetles kept at 24.3 °C, although neither of these values differed significantly from the mean UTL of beetles acclimated at 19.3 °C (Fig. 2A, C). Otherwise, individuals acclimated at 60 g L<sup>-1</sup> had significantly higher UTLs than those kept at lower

**Table 1.** Influence of acclimation salinity (S), acclimation temperature (T) and body mass on the upper thermal limit (UTL) of *Nebrioporus baeticus*; the upper thermal limit of *Nebrioporus ceresyi*; the lower thermal limit (LTL) of *N. baeticus*; and the lower thermal limit of *N. ceresyi*.

Source	SS	d.f.	F	P
<b>Upper thermal limit</b>				
<i>Nebrioporus baeticus</i>				
Full model	43.78	15	4.81	<0.001
Intercept	1317.50	1	2169.08	<0.001
Body mass	2.28	1	3.76	0.052
S	10.03	2	8.26	0.001
T	19.03	4	7.83	<0.001
S × T	3.47	8	0.71	0.678
Error	39.48	65		
<i>Nebrioporus ceresyi</i>				
Full model	23.28	15	5.80	<0.001
Intercept	3598.37	1	13450.11	<0.001
Body mass	1.98	1	7.38	0.009
S	10.64	4	9.94	<0.001
T	3.47	2	6.49	0.003
S × T	1.56	8	0.73	0.664
Error	17.12	64		
<b>Lower thermal limit</b>				
<i>Nebrioporus baeticus</i>				
Full model	67.21	15	2.25	0.014
Intercept	15.00	1	7.55	0.008
Body mass	0.87	1	0.44	0.510
S	2.48	4	0.31	0.869
T	19.53	2	4.91	0.011
S × T	39.50	8	2.48	0.022
Error	115.26	58		
<i>Nebrioporus ceresyi</i>				
Full model	123.46	15	4.06	<0.001
Intercept	54.02	1	26.63	<0.001
Body mass	0.00	1	0.00	0.973
S	26.53	4	3.27	0.017
T	50.31	2	12.40	<0.001
S × T	33.98	8	2.09	0.049
Error	125.79	62		

SS, sum of squares; d.f, degrees of freedom.

salinities (1–35 g L<sup>-1</sup>) (Fig. 2B, D). Beyond the positive additive effect observed here, salinity acclimation appears to have a higher influence on mean UTL (+1.6 °C in *N. baeticus* and +1.2 °C in *N. ceresyi*) than temperature acclimation (+0.9 °C in *N. baeticus* and +0.8 °C in *N. ceresyi*).

#### Lower thermal limits

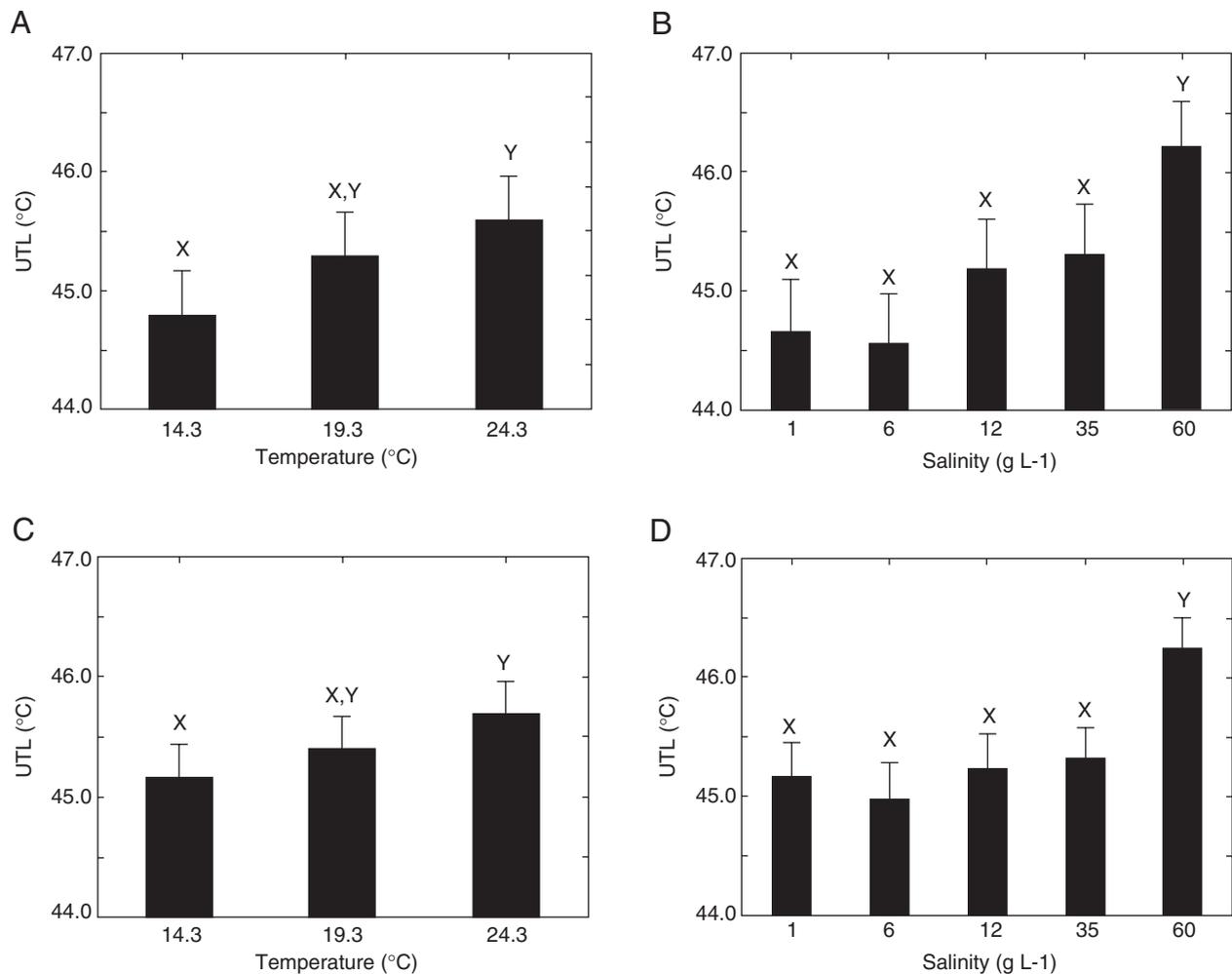
*Nebrioporus ceresyi* showed, in general, a higher tolerance to cold than *N. baeticus* ( $P < 0.05$ ; Fig. 1). The maximum mean LTL for both was observed in individuals acclimated at 24.3 °C and 35 g L<sup>-1</sup>, whereas the minimum mean LTL for both species was observed after acclimation at 14.3 °C and 60 g L<sup>-1</sup> (Fig. 1). Body mass was not significantly related to LTL in either species (Table 1).

A significant interaction between salinity and acclimation temperature was observed for LTL in both species (maximum

$P = 0.05$ ; Fig. 3 and Table 1). In general, treatments with higher salinity and lower acclimation temperatures had higher tolerance to cold. In both species, the mean LTLs measured for animals acclimated at 14.3, 19.3 and 24.3 °C did not differ significantly from one another at salinities of 1, 6 and 12 g L<sup>-1</sup>. By contrast, at salinities of 35 and 60 g L<sup>-1</sup>, the lowest means LTLs were recorded in individuals acclimated at the lowest temperature employed (14.3 °C; Fig. 3A, B), with the exception of *N. baeticus* at 35 g L<sup>-1</sup>, where the lowest mean LTL was recorded in individuals acclimated at 19.3 °C. In both species, mean LTLs of individuals maintained at 35 g L<sup>-1</sup> and 14.3 °C and 19.3 °C did not differ significantly ( $P > 0.05$ ; Fig. 3A, B), although they were significantly lower than the mean LTL of individuals acclimated at 24.3 °C ( $P < 0.05$ ; Fig. 3A, B). In the case of *N. baeticus* maintained at 60 g L<sup>-1</sup>, the mean LTL of individuals acclimated at 14.3 °C was significantly lower than that of those kept at 19.3 °C ( $P < 0.05$ ; Fig. 3A), although neither of these values differed significantly from the mean LTL of beetles acclimated at 24.3 °C ( $P > 0.05$ ; Fig. 3A). By contrast, in the case of *N. ceresyi* that had been kept at this salinity, the mean LTL of individuals acclimated at 14.3 °C was significantly lower than beetles that had been maintained at 24.3 °C ( $P < 0.05$ ; Fig. 3B). Finally, mean LTLs measured at a given acclimation temperature did not differ significantly across the entire range of salinities tested in *N. baeticus*, whereas, in *N. ceresyi*, the mean LTL measured in beetles acclimated at 14.3 °C and 6 g L<sup>-1</sup> was significantly higher than the mean LTL of individuals maintained at 60 g L<sup>-1</sup> ( $P < 0.05$ ; Fig. 3B).

#### Discussion

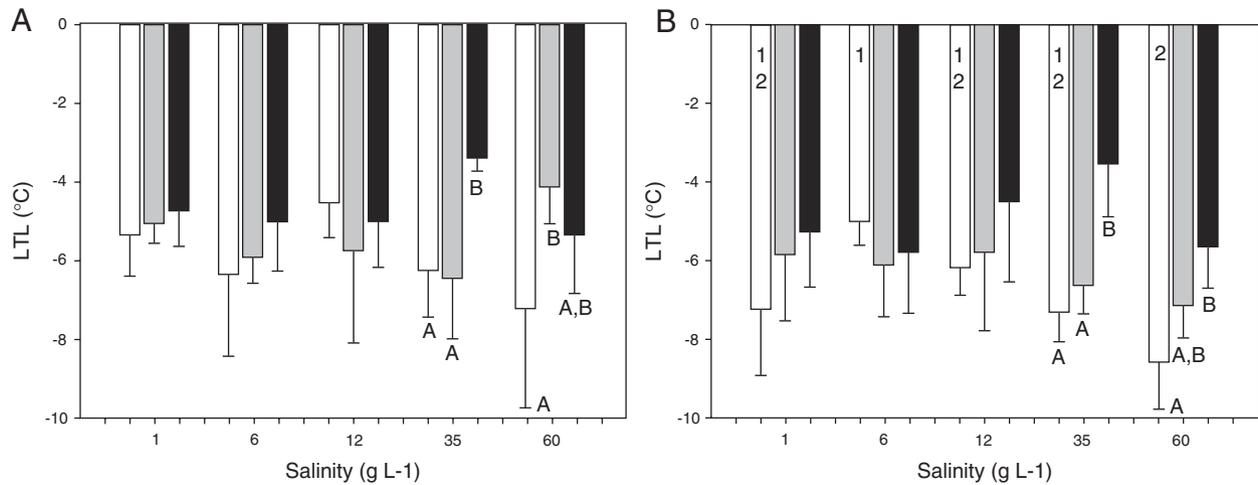
Both temperature and salinity influence the thermal biology of adult *Nebrioporus* diving beetles. In particular, mean UTLs are higher in individuals of both species acclimated to relatively high salinities and high temperature because these two environmental parameters exert a positive additive effect on tolerance to heat. However, it appears that salinity plays a more important role than acclimation temperature in determining the heat tolerance of these saline water beetles, at least within the ranges examined in the present study for temperature (14.3 and 24.3 °C) and salinity (1–60 g L<sup>-1</sup>). An absence of thermal acclimation of UTL is reported in a number of insects associated with extreme environments, such as larvae of the soldier fly *Stratiomys japonica* (Garbuz *et al.*, 2008) and the larvae of an Antarctic midge *Belgica antarctica* (Rinehart *et al.*, 2006). The results of the present study are also consistent with the findings of Calosi *et al.* (2008a, b), who report limited acclimation to heat in a number of European dytiscids. In terms of LTL, a significant interaction between temperature and salinity is observed for both species examined, indicating the presence of a synergistic effect of these two environmental factors in determining tolerance to cold. In particular, temperature-dependent acclimation of LTL in both species occurs only in individuals acclimated to higher salinities (35–60 g L<sup>-1</sup>), and the lowest LTLs are recorded after acclimation at the highest salinities (35–60 g L<sup>-1</sup>) and



**Fig. 2.** Histograms of mean  $\pm$  SE upper thermal limit (UTL) ( $^{\circ}$ C) in individuals of *Nebrioporus baeticus* (A, B) and *Nebrioporus ceresyi* (C, D) acclimated to different temperatures (14.3, 19.3 or 24.3  $^{\circ}$ C) and salinities (1, 6, 12, 35 and 60 g L<sup>-1</sup>). Significantly different mean UTLs ( $P < 0.05$ ) are indicated by different letters above the histograms (according to Bonferroni tests). Because there was no significant interaction between the effects of acclimation salinity and temperature, the effects of these factors are shown separately.

the lowest temperature (14.3  $^{\circ}$ C) employed. A number of insects (including some diving beetles) are able to adjust their physiological performance (including thermal limits) after a period of acclimation (Worland & Convey, 2001; Klok & Chown, 2003; Terblanche *et al.*, 2005; Calosi *et al.*, 2008a), allowing them the potential to express a wider thermal tolerance range. However, the present study is the first to demonstrate a significant effect of salinity on such an thermal ability in aquatic insects. Of the two species under investigation in the present study, *N. ceresyi*, which is the most widespread geographically, has the greatest thermal range, largely through its greater tolerance to cold. Such a finding is consistent with the environmental variability hypothesis (Gaston, 2003; Calosi *et al.*, 2008b; Calosi *et al.*, 2010), where widespread species are expected to show greater physiological tolerances than their more restricted relatives.

The differences in thermal tolerance found in the present study, within and between species, do appear likely to be relevant to their population persistence, particularly when considering the ecology of these insects in more detail. Air temperatures in the regions occupied by these taxa (as measured by weather stations) regularly approach, or even exceed, their thermal limits as estimated in the present study. Values of 47  $^{\circ}$ C are reported for Murcia in 1995 and values of -10  $^{\circ}$ C are reported for Guadalajara in 2000 (Instituto Nacional de Meteorología, 2005), comprising two weather stations located close to sampling sites where these species occur. Although these are aquatic animals, and maximum and minimum water temperatures in streams and lagoons are generally less extreme than those in air (e.g.  $\leq 30$   $^{\circ}$ C in water versus  $\leq 45$   $^{\circ}$ C in air during July and August in Fortuna, Murcia), these beetles do spend some of their life cycle on land. The pupal stage and the early stage of adult dytiscids is



**Fig. 3.** Histograms of mean  $\pm$  SE lower thermal limit (LTL) ( $^{\circ}$ C) in individuals of (A) *Nebrioporus baeticus* and (B) *Nebrioporus ceresyi* acclimated to different temperatures [14.3 (white), 19.3 (grey) or 24.3  $^{\circ}$ C (black)] and salinities (1, 6, 12, 35 and 60 g L $^{-1}$ ). Significantly different mean LTLs ( $P < 0.05$ ) measured at different acclimation temperatures within the same salinity treatment are indicated by different letters below the histograms, whereas significantly different mean LTLs ( $P < 0.05$ ) measured at different acclimation salinities within the same temperature treatment are indicated by different numbers inside the histograms (according to Bonferroni tests).

spent on land in small burrows beside the water (e.g. Galewski, 1971) and individuals will be exposed to greater temperature fluctuations than when they are submerged. In *Nebrioporus*, this stage takes place in Spring and early Summer in the region, when air temperatures can exceed 40  $^{\circ}$ C. In addition, inland saline waters are characterized by repeated wet and dry cycles, which can be unpredictable in occurrence (Moreno *et al.*, 2009), and, in times of drought, the sites occupied by these species do sometimes lack surface water. Members of the group can sometimes be collected from the water during winter, although some individuals may also overwinter on land, as is observed in a number of other dytiscids (Galewski, 1971), meaning that they will experience sub-zero temperatures.

The physiological mechanisms through which changes in salinity influence the thermal limits of hypersaline *Nebrioporus* species remain unknown, although a number of relevant processes have been identified in other organisms that allow for some speculation. *Culex* and *Culiseta* mosquito larvae show some degree of osmoconformity to changes in salinity (Garrett & Bradley, 1984), meaning that the osmotic concentration of their haemolymph matches that of the external medium outside a given salinity range. If this applies also to the beetles examined in the present study when exposed to salinities greater than 35 g L $^{-1}$ , the increase in haemolymph osmolality could represent a potential mechanism for their elevated cold hardiness. However, such a passive increase in osmotic concentration after exposure to elevated salinities would not directly explain the observed increases in the upper thermal tolerance. Extracellular spaces may become hypertonic in individuals kept at salinities above 35 g L $^{-1}$ , which, amongst other things, may induce an increase in the intracellular production of heat shock proteins (Feder & Hofmann, 1999; Diamant *et al.*, 2001; Santos *et al.*, 2003). Such an effect is documented in a range of organisms, including *Stratiomys*

soldier flies (Garbuz *et al.*, 2008), mosquito larvae (Benoit *et al.*, 2010) and the fish *Gillichthys mirabilis* (Kultz, 1996). Other possibilities include changes in the structure and density of mitochondria, which extend thermal tolerance windows, as observed in Mozambique tilapia *Oreochromis mossambicus* (Sardella *et al.*, 2008a). Furthermore, if individuals kept at salinities above 35 g L $^{-1}$  respond with hypometabolism, this may result in an improvement in individual thermal tolerance (Klok *et al.*, 2004; Pörtner *et al.*, 2006).

In addition to the effects of ongoing climatic change, which are projected to result in increased temperatures in most Mediterranean areas (IPCC, 2007), the specialist inhabitants of inland saline waters are threatened by a dilution process, which is on the increase as irrigated agriculture continues to intensify in these regions. The present study shows that, for these beetles at least, reductions in the mean salinity of their habitats, although not lethal, are likely to compromise their abilities to withstand both heat and cold, and could partly explain why such specialist taxa are lost when salinities fall in these systems. For the majority of acclimation treatments employed in the present study, individuals of both species have greater tolerance to heat when maintained in relatively high salinities, with UTLs being lower in beetles that had been kept in low salinities (1–12 g L $^{-1}$ ). Increased freshwater input to inland saline waters, through agricultural run-off, lowers the modal salinity in these habitats (Velasco *et al.*, 2006; Carreño *et al.*, 2008), and the results of the present study suggest that such changes will compromise the ability of these beetles to tolerate higher temperatures in the future, making them particularly vulnerable in a warming climate. In terms of cold tolerance, although salinity does not generally appear to significantly influence the LTL of a beetles at a given acclimation temperature, animals only show an acclimation response to temperature after exposure at the two highest salinities. As

with UTL, it would appear that lowered salinities compromise the responses of these taxa to cold, which may partly explain their inability to persist in the face of reduced salinity.

In conclusion, as well as being threatened by habitat loss, the specialist inhabitants of these ecosystems (Abellán *et al.*, 2007; Sánchez-Fernández *et al.*, 2008) may be compromised physiologically by anthropogenic changes to the salinity regimes of their habitats. These changes may affect their ability to persist in individual localities, and have important consequences for the way in which they deal with global climate change.

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