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Lethal and sublethal behavioural responses of saline water beetles to acute heat and osmotic stress

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Abstract. 1. As species' physiological breadth determines their potential to deal with environmental changes, and influences individuals' survival and the persistence of populations, information about lethal and sublethal responses could be fundamental for conservation purposes.

2. We used a standard experimental approach to explore mortality and behavioural avoidance responses (i.e. flight and emersion from the water) to a combination of acute heat and osmotic stress on six species of saline water beetles (belonging to *Enochrus*, *Nebrioporus*, and *Ochthebius* genera).

3. Heat stress affected survival and behavioural responses in all of the species, whereas osmotic stress and the interaction between both stressors only showed significant effects for the *Ochthebius* genus. Behavioural and survival patterns were highly interrelated across the stress gradients. The *Enochrus* and *Nebrioporus* studied species showed maximum avoidance activity at 35–40 °C, and a short (< 30 min) exposure to 45 °C was lethal. *Ochthebius* species were the most heat tolerant and displayed increasing behavioural responses with increasing temperature. In the *Nebrioporus* and *Ochthebius* genera, the species occupying lotic, more environmentally stable habitats, showed greater mortality, and avoidance responses were higher or initiated at lower stress thresholds than lentic species. In contrast, both *Enochrus* species displayed a similar mortality, and the lentic species *E. bicolor* emerged and flew more than the lotic *E. falcarius,* in concordance with its higher dispersal capacity.

4. Avoidance responses could provide interesting information about species' physiological amplitudes as a complement to lethal responses. The lotic species here studied showed narrower physiological amplitude (i.e. *N. baeticus* and *O. glaber*) or lower dispersal ability (i.e. *E. falcarius*) than their lentic relatives; both traits could result in a higher vulnerability of lotic species to thermal habitat changes.

Key words. Behavioural avoidance responses, global change, habitat stability, heat stress, osmotic stress, physiological breadth, saline habitats, stress tolerance, water beetles.

Introduction

Understanding the ways in which organisms deal with and respond to environmental changes is of considerable importance in determining past and present processes affecting species (Chown, 2001). Species' physiology defines the breadth of fundamental niches (Gaston, 2003) and so, has been identified as relevant when forecasting the effects of habitat modification on species and population viability (e.g. Tewksbury *et al*., 2008; Gaston *et al*., 2009; Helmuth, 2009), particularly in the current context of global warming and stressed biodiversity loss (Deutsch *et al*., 2008; Bozinovic *et al*., 2011). Recent studies have shown that laboratory-determined species' physiological amplitudes are a good approximation to species fitness under natural changes in their habitats (Gaston & Spicer, 2001; Deutsch *et al*., 2008; Barnes *et al*., 2010). As a result, many studies examining the effects

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of stressors on species' physiology for conservation purposes and predicting future trends under global warming scenarios have emerged (e.g. Swanson *et al*., 2000; Homan *et al*., 2003; Pandolfo *et al*., 2010; Sánchez-Fernández *et al*., 2010).

Together with lethal responses, behavioural adjustments are fundamental in defining species' physiological boundaries and can substantially influence organisms' survival and the persistence of local populations (Huey, 1991; Marais & Chown, 2008; Angilletta, 2009). Organisms employ diverse strategies to avoid stress (i.e. avoidance responses), such as moving to other areas through dispersal, or on a smaller scale, to more favourable microclimates within their current habitats (Massot *et al*., 2008; Feder, 2010). These avoidance responses are initiated when organism fitness has deteriorated, and reflect the sublethal stress limits that organisms can tolerate. Despite their informative potential, few studies have included behavioural traits to assess stress tolerance of species (but see Hazell *et al*., 2010) and as a result, data on the relationship between survival patterns and behavioural avoidance responses under stress are still lacking for many organisms.

One of the main environmental stressors for species is temperature, which has long been recognised as one of the most important dimensions of species' niche, as it underpins metabolic activity and life-history processes (Willott & Hassall, 1998), especially for ectotherms (e.g. Bale, 2002; Hoffmann *et al*., 2003; Chown & Nicolson, 2004). Indeed, insect responses to temperature extremes over short periods may be an important driver of population dynamics and, consequently, species' abundance and geographic distribution over longer timescales (Chown & Terblanche, 2007; Hoffmann, 2010). In addition to temperature, other stressors can simultaneously affect species and may result in synergistic or even antagonistic effects (Gaston, 2003; Terblanche *et al*., 2011). Salinity has been identified as one of the main factors constraining inland aquatic communities (Williams *et al*., 1990; Pinder *et al*., 2005; Rutherford & Kefford, 2005). Recent studies have demonstrated that salinity also affects thermal amplitude of a wide range of organisms, mainly marine (e.g. Kir & Kumlu, 2008; Sardella *et al*., 2008), but also for inland water bodies taxa (e.g. Sánchez-Fernández *et al*., 2010). Experimental approaches combining temperature with other environmental stressors (e.g. salinity for aquatic organisms) are highly significant for evaluating the effect of their interactions on organisms' responses, especially in the context of global warming (Pörtner & Farrell, 2008; Williams *et al*., 2008).

Here we use a standard experimental approach to explore the physiological tolerance (lethal and sublethal responses) of saline water beetles to heat and osmotic stress, by measuring mortality and two common behavioural avoidance responses displayed by aquatic beetles (i.e. flight and emersion from the water). Locomotion performance is ecologically relevant for insects' survival under extreme environmental stressors (Clusella-Trullas *et al*., 2010), and flight is the main escape reaction and seems to be promoted by increases in air temperature (Zalom *et al*., 1980; Velasco & Millán, 1998). Emersion from the water is also a typical response that reduces stress and provides support for flight.

Species that inhabit inland saline waters are an interesting group to explore stress responses for two main reasons. First, Mediterranean saline water bodies present naturally stressful conditions that comprise high levels of salinity and water temperature. In addition to 'natural' stress, climate change predictions forecast increased temperatures and reduced precipitation in the Mediterranean area (IPCC, 2007), which, together with an increase in the frequency and severity of extreme events (Easterling *et al*., 2000), would likely result in intensified heat and salinity stress for organisms that inhabit inland saline waters. Second, saline water fauna offers an ideal group to compare stress responses between related species that occupy habitats with contrasting environmental stability. The climatic variability hypothesis (Janzen, 1967) establishes that climatic stability in the tropics compared to higher latitudes favours organisms with narrow physiological tolerance amplitude. At a habitat scale, lentic (standing) water bodies experience greater daily and seasonal temperature and salinity fluctuations than lotic (running) waters (Álvarez-Cobelas *et al*., 2005; Florencio *et al*., 2009) and so, species in less stable lentic water bodies are forced to develop higher colonisation capacities as well as broader fundamental niches (sensu Brown, 1984) compared with their lotic relatives (Ribera, 2008). As a result, the capacity to deal with acute stress and species sensitivity to environmental changes could be mediated by habitat specialisation.

The aim of this study was to compare physiological amplitude through lethal and sublethal behavioural avoidance responses in three pairs of congeneric species of Iberian saline water beetles, with different habitat occupation (lotic–lentic), under acute heat and osmotic stress, as an approximation to their potential to deal with environmental changes in their habitats. It was expected that: (i) the combination of high temperature and salinity would reduce survival and affect the capacity of water beetles to perform behavioural avoidance responses; (ii) patterns of behavioural avoidance responses and mortality would be interrelated across the stress gradient; (iii) lotic species would have lower physiological amplitude than lentic (i.e. higher mortality and avoidance activity and/or lower stress thresholds for avoidance responses); and so (iv) lotic species would be more susceptible to environmental changes than lentic ones.

Material and methods

Target species

Coleoptera is one of the most common and richest insect orders in inland saline waters (Millán *et al*., 2011). The most representative families of water beetles inhabiting saline habitats are Hydraenidae, Hydrophilidae (suborder Polyphaga), and Dytiscidae (suborder Adephaga). The present study focused on three pairs of congeneric beetle species typical of inland meso- and hypersaline systems with contrasting habitat occupation patterns and geographic range size. They are included in three genera: *Nebrioporus* [*N. ceresyi* (Aubé, 1836) and

N. baeticus (Schaum, 1864); family Dytiscidae], *Enochrus* [*E. bicolor* (Fabricius, 1792) and *E. falcarius* Hebauer, 1991; family Hydrophilidae], and *Ochthebius* (*O. notabilis* Rosenhauer, 1856 and *O. glaber* Montes & Soler, 1988; family Hydraenidae).

Nebrioporus ceresyi is a circum-Mediterranean species that occupies standing waters such as wetlands and salt pans, particularly those located in lowland areas near the coast. Conversely, *N. baeticus* is endemic to southeastern Spain, and is found in lotic hypersaline streams usually far from the coast (Fery *et al*., 1996; Toledo, 2009).

Enochrus bicolor inhabits lentic saline systems (wetlands and salt pans) and it is found across Europe, northern Africa, and Asia east to Mongolia (Schödl, 1998; Hansen, 2004). Its related species, *E. falcarius,* has a narrower distribution and occupies saline streams in the southern Iberian Peninsula, Tunisia, and Sicily (Schödl, 1998; Hansen, 2004) as well as Morocco (A. Millán *et al*., pers. obs.). In fact, a recent study has revealed that this species, as currently understood, actually comprises a complex of different lineages, each with restricted, disjointed distributions across the Mediterranean area (Arribas *et al*., 2012a). Here we studied the Iberian taxon of this species complex ('*E. falcarius* IP' *sensu* Arribas *et al*., 2012a, here *E. falcarius* for simplicity).

Ochthebius notabilis is found in saline lagoons across the Iberian Peninsula and northern Africa, whereas *O. glaber* is endemic to the southern Iberian Peninsula and is restricted to running waters (Abellán *et al*., 2009).

Experimental design

Survival and behavioural avoidance responses to acute heat and salinity stress were evaluated in the three pairs of sister species selected by employing a static protocol (Lutterschmidt & Hutchison, 1997), which allowed the comparison of specimens' physiological amplitudes between related species to be made.

Approximately 400 individuals of each *Enochrus* and *Nebrioporus* species and 600 of *Ochthebius* were collected from different areas (one locality per species) in southeastern Spain (see Table 1 for collection locations). Specimens were maintained under laboratory conditions for 1 week in aquaria with filtered water from the collection sites, artificial aeration and periodic feeding (chironomid larvae for predator species, *Nebrioporus*; *Ruppia maritima* for herbivorous species, *Enochrus*; and biofilm for *Ochthebius*). After this week, the specimens were maintained for 24 h without feeding in an environmental chamber (SANYO MLR-351, Sanyo Electric Co., Ltd., Moriguchi City, Osaka, Japan) at a constant temperature (20 °C), LD 12 : 12 h cycle and light intensity of 15 μmol m^{-2} s^{-1}.

Finally, 10 specimens were randomly assigned to each of the 12 (for *Enochrus* and *Nebrioporus* species) or 20 (for *Ochthebius* species) combined conductivity and temperature treatments, which were replicated three times for each species. Conductivities were chosen according to the environmental gradient where these species appear: 20, 50, 80 mS cm^{-1} to *Enochrus* and *Nebrioporus* species and 20, 50, 80, 180, 240 mS $cm^{-1}$to *Ochthebius* species (Velasco *et al*., 2006). Saline solutions were prepared by dissolving marine salt (Ocean Fish, Prodac®) in distilled water. Tested temperatures represent a gradient from habitual temperatures in the natural habitat of the species (20, 35 °C) to extreme temperatures (40, 45 °C) that are close to the sublethal and upper lethal limits recorded for these species in previous studies (Sánchez-Fernández *et al*., 2010; Arribas *et al*., 2012a,b). The inland saline water bodies of the Iberian Peninsula that the studied species inhabit are characterised by extreme and large seasonal and daily variations in environmental conditions (Velasco *et al*., 2006; Millán *et al*., 2011; Gutiérrez-Cánovas *et al*., 2012). For example, in the Rambla Salada stream (southeastern Spain), the observed daily water temperature amplitude could commonly reach 10–12 °C and up to 18 °C, and water temperatures of 35 °C and heating rates of approximately 1 °C 2 h^{-1} are frequent during the summer (J. Velasco, unpublished).

Each experimental aquarium contained 100 ml of solution and an artificial stone partially emerged to help individuals emerge and fly to avoid stressful conditions. Aquaria were introduced into a temperature-controlled water bath (Precisterm 6000141, J. P Selecta, Barcelona, Spain) (i.e. ± 1 °C). Each set of individuals was removed from the acclimation aquaria and immediately exposed to the assigned treatment for 30 min. During this exposure period, behavioural responses and mortality were recorded. The number of individuals on the stone in each aquarium was recorded every 2 min to determine emersion response. Specimens that flew or were dead were counted and removed at 2-min intervals. However, due to the small size of individuals from the *Ochthebius* species, it was impossible to determine the exact time of the specimens' death,

Table 1. Species' natural habitat information and collection sites data (geographical coordinates and mean conductivity).

Species	Habitat occupancy	Conductivity range (mS cm^{-1})	Sample location	Latitude	Longitude	Mean conductivity of the locality (mS cm^{-1})
N. ceresyi	Lentic	2–128	Laguna Cotorrillo, Murcia	37.82516	−0.76196	60
N. baeticus	Lotic	2–160	Río Chícamo, Murcia	38.21753	−1.05113	19
E. bicolor	Lentic	4–103	Laguna del Mojón Blanco, Albacete	38.47530	−1.25582	65
E. falcarius	Lotic	7–160	Rambla Salada, Murcia	38.16993	−1.12565	70
O. notabilis	Lentic	50–220	Estrecho de la Salineta, Alicante	38.43459	−0.78006	140
O. glaber	Lotic	20–250	Rambla de Librilla, Murcia	37.90656	−1.37102	180

Table 2. Effect of temperature and conductivity on overall response variables for *Enochrus, Nebrioporus*, and *Ochthebius* species.

Effect	Pillai's trace	*F*-value	d.f.	*P*-value
Enochrus				
Temperature	1.597	18.223	9	<0.001
Conductivity	0.065	0.523	6	0.789
Species	0.361	8.654	3	<0.001
Temperature × Conductivity	0.312	0.929	18	0.545
Temperature × Species	0.377	2.302	9	0.019
Conductivity × Species	0.182	1.572	6	0.164
Temperature × Conductivity × Species	0.321	0.957	18	0.512
Nebrioporus				
Temperature	1.909	27.981	9	<0.001
Conductivity	0.042	0.335	6	0.917
Species	0.406	10.460	3	<0.001
Temperature × Conductivity	0.199	0.568	18	0.918
Temperature × Species	0.622	4.185	9	<0.001
Conductivity × Species	0.008	0.060	6	0.999
Temperature × Conductivity × Species	0.236	0.683	18	0.079
Ochthebius				
Temperature	1.100	15.442	9	<0.001
Conductivity	0.815	7.460	12	<0.001
Species	0.524	28.637	3	<0.001
Temperature × Conductivity	0.895	2.835	36	<0.001
Temperature × Species	0.604	6.718	9	<0.001
Conductivity × Species	0.527	4.259	12	<0.001
Temperature × Conductivity × Species	0.796	2.408	36	<0.001

d.f., degrees of freedom.

and total mortality was recorded at the end of the experiment for *O. glaber* and *O. notabilis*.

Mortality was expressed as the percentage of individuals that died during 30 min of acute exposure. For behavioural responses, in the case of *Enochrus* and *Nebrioporus* species, percentage of emersions and flights in each treatment was expressed in relation to the number of alive individuals present in the aquaria at the moment of recording (i.e. every 2 min). For *Ochthebius* species, as dynamic mortality data were not available, behavioural responses were expressed as the mean percentage of individuals that emerged or flew (respectively) during the experimental time divided by the number of surviving individuals after exposure.

Data analysis

Multifactorial MANOVA analyses were performed using the Pillai's trace test to assess the global effect of temperature, conductivity, and species on overall response variables within each genus. Univariate analyses of variance (ANOVAS) were also conducted to determine the effects of each factor and interactions independently on each variable. Mortality percentages were arcsine transformed before the analyses.

Because homocedasticity and normality of raw data and generalised linear model residuals were not satisfied in some cases, a more conservative approach was employed by reducing the signification level ($P \leq 0.01$) and using *post-hoc* analyses with Bonferroni correction to identify significant differences among treatments (Underwood, 1997; Rutherford, 2001). All statistical analyses were conducted using SPSS for Windows (Rel. 15.0.1. 2006, SPSS Inc., Chicago, Illinois).

Results

Effects of temperature and conductivity on response variables

Multivariate tests showed global significant differences in response variables between temperature levels in all pairs of species examined (Table 2). Similar results were found in the ANOVAS of each response variable (see Table 3 for mortality; Table 4 for emersion; and Table 5 for flight). In general, both behavioural responses and mortality increased with increasing temperatures, although in *Enochrus* and *Nebrioporus* species the most extreme temperature significantly increased mortality and reduced behavioural responses.

The effect of conductivity was only significant for the *Ochthebius* species (Table 2) for all of the response variables (Tables 3–5). The interaction of temperature × conductivity also showed significant effects for these species (Table 2) and the response patterns across heat and osmotic stress differed between the two congeneric species (see below).

Lethal responses: mortality

Both *Enochrus* species showed similar tolerance to acute heat stress (see Species and Temperature × Species interaction in Table 3). *Enochrus bicolor* and *E. falcarius* displayed high

Table 3. Effect of temperature and conductivity on mortality for *Enochrus*, *Nebrioporus*, and *Ochthebius* species.

	Dependent variable: mortality			
Effect	SS	d.f.	*F*-value	*P*-value
Enochrus				
Full model	18.487	23	40.640	<0.001
Intercept	7.482	1	378.316	<0.001
Temperature	18.172	3	306.265	<0.001
Conductivity	0.049	2	1.236	0.300
Species	0.000	1	0.019	0.891
Temperature × Conductivity	0.183	6	1.539	0.186
Temperature × Species	0.023	3	0.389	0.761
Conductivity × Species	0.014	2	0.350	0.706
Temperature × Conductivity × Species	0.046	6	0.387	0.884
Error	0.949	48	–	–
Nebrioporus				
Full model	32 189.498	23	15.393	<0.001
Intercept	20.518	1	1188.512	<0.001
Temperature	28.149	3	543.505	<0.001
Conductivity	0.020	2	0.577	0.565
Species	0.198	1	11.460	0.001
Temperature × Conductivity	0.013	6	0.122	0.993
Temperature × Species	0.807	3	15.577	<0.001
Conductivity × Species	0.000	2	0.007	0.993
Temperature × Conductivity × Species	0.108	6	1.047	0.408
Error	0.829	48	–	–
Ochthebius				
Full model	15.435	39	24.074	<0.001
Intercept	9.886	1	601.357	<0.001
Temperature	9.913	3	201.007	<0.001
Conductivity	0.853	4	12.965	<0.001
Species	1.386	1	84.340	<0.001
Temperature × Conductivity	0.874	12	4.432	<0.001
Temperature × Species	1.084	3	21.982	<0.001
Conductivity × Species	0.466	4	7.082	<0.001
Temperature × Conductivity × Species	0.858	12	4.351	<0.001
Error	1.315	80	–	–

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

survival at all temperature levels tested, except at 45 °C, where after 30 min of exposure most individuals died (Fig. 1a).

Similarly, for both *Nebrioporus* species the increased temperature reduced the specimens' survival, and most individuals died at 45 °C (Fig. 1b). Total mortality of the lotic species *N. baeticus* was significantly higher than for the lentic *N. ceresyi* (Species in Table 3), and this difference was especially great at 40 °C ($P < 0.001$ in the *post-hoc* test for species difference at 40 °C; Fig. 1b).

Ochthebius species displayed varying tolerances to both stressors (Temperature × Conductivity × Species in Table 3). Mortality of the lotic species *O. glaber* was higher than in the lentic *O. notabilis* in all of the stress treatments (Species in Table 3, Fig. 1c,d). In *O. glaber*, mortality increased progressively with temperature. At higher temperatures (40–45 °C), the mortality of *O. glaber* was also significantly greater at the most extreme conductivity level (240 mS cm^{-1}) (Fig. 1c). However, in *O. notabilis,* mortality was low or null at 20, 35, and 40 °C; only 45 °C significantly reduced survival and no significant differences in mortality were observed among conductivity levels (see Conductivity *post-hoc* tests for each species in Fig. 1c,d).

Sublethal behavioural responses

Emersion. The lentic species *E. bicolor* emerged more than the lotic *E. falcarius* (see Species in Table 4). However, *Enochrus* species showed no significant differences in emersion activity pattern across temperature treatments (Temperature × Species in Table 4), i.e. emersion increased from 20 to 40 °C, when the maximum emersion response was reached by both species (Fig. 2a).

No significant differences either in emersion response magnitude or patterns across temperature treatments were found between lotic and lentic *Nebrioporus* species (Species and Temperature × Species in Table 4). Thus, 40 °C was the critical thermal threshold where maximum emersion activity was observed for both species, after which no further emersion was recorded (Fig. 2b).

Similarly, no significant differences in magnitude of emersion response were detected between the *Ochthebius*

Table 4. Effect of temperature and conductivity on emersion response for *Enochrus*, *Nebrioporus*, and *Ochthebius* species.

Effect	Dependent variable: emersion			
	SS	d.f.	*F*-value	*P*-value
Enochrus				
Full model	37 775.999	23	4.504	<0.001
Intercept	104 761.476	1	287.253	<0.001
Temperature	30 534.568	3	27.908	<0.001
Conductivity	313.644	2	0.430	0.653
Species	2702.195	1	7.409	0.009
Temperature × Conductivity	810.362	6	0.370	0.894
Temperature × Species	2429.023	3	2.220	0.098
Conductivity × Species	809.732	2	1.110	0.338
Temperature × Conductivity × Species	176.76	6	0.081	0.998
Error	17 505.627	48	–	–
Nebrioporus				
Full model	32 189.498	23	15.393	<0.001
Intercept	21 606.420	1	237.638	<0.001
Temperature	31 030.047	3	113.761	<0.001
Conductivity	63.235	2	0.348	0.708
Species	108.586	1	1.194	0.280
Temperature × Conductivity	456.951	6	0.838	0.547
Temperature × Species	497.775	3	1.825	0.155
Conductivity × Species	2.028	2	0.011	0.989
Temperature × Conductivity × Species	30.876	6	0.057	0.999
Error	4364.243	48	–	–
Ochthebius				
Full model	51 983.679	39	6.748	<0.001
Intercept	67 635.235	1	342.389	<0.001
Temperature	32 140.818	3	54.235	<0.001
Conductivity	6787.594	4	8.590	<0.001
Species	1306.765	1	6.615	0.012
Temperature × Conductivity	7640.230	12	3.223	0.001
Temperature × Species	348.966	3	0.589	0.624
Conductivity × Species	1171.376	4	1.482	0.215
Temperature × Conductivity × Species	2587.929	12	1.092	0.379
Error	15 803.137	80	–	–

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

species (Species in Table 4) and a similar response pattern across temperature and conductivity treatments was displayed by both species (Temperature × Conductivity × Species in Table 4). The number of emersions increased with increasing temperature, reaching the maximum response at 45 °C (Fig. 2c,d), and decreased significantly at high conductivities. The combination of the highest temperatures (40–45 °C) and conductivity (240 mS cm^{-1}) caused a significant reduction in emersion response (Conductivity × Temperature in Table 4, Fig. 2c,d).

Flight. Between *Enochrus* species, the lentic *E. bicolor* showed a higher flight response than the lotic *E. falcarius* (see Species in Table 5, Fig. 3a) but flight activity patterns were similar between both species and across all the temperature range (Temperature and Species × Temperature in Table 5, Fig. 3a).

In *Nebrioporus* species, the lotic *N. baeticus* flew more than the lentic *N. ceresyi* at all temperature levels (Species in Table 5). The response pattern did not significantly differ between both species; the highest flight activity was displayed at 35–40 °C and minimum response was shown at 20 and 45 °C (Temperature and Species × Temperature in Table 5, Fig. 3b).

Flight response had a similar magnitude between both *Ochthebius* species (Species in Table 5). However, response patterns across temperature and conductivity treatments differed between the lotic and the lentic species (Temperature × Conductivity × Species in Table 5). Flight activity increased with increasing heat stress in both species but *O. glaber* reached the maximum response at 45 °C and *O. notabilis* at 40 °C (Fig. 3c,d). The effect of conductivity on flight was only significant for the lentic species *O. notabilis*, which showed the greatest flight activity at the higher conductivities (180 and 240 mS cm^{-1}) (see Conductivity *post-hoc* tests for each species in Fig. 3c,d). The Temperature × Conductivity interaction differed between species (Table 5). At the most extreme temperatures (40–45 °C), the lotic species *O. glaber* showed a significant decrease in flight response at 240 mS cm^{-1}(Fig. 3c). In contrast, at the highest

Table 5. Effect of temperature and conductivity on flight response for *Enochrus*, *Nebrioporus*, and *Ochthebius* species.

Effect	Dependent variable: flight			
	SS	d.f.	*F*-value	*P*-value
Enochrus				
Full model	138.181	23	1.1774	0.047
Intercept	192.263	1	56.765	<0.001
Temperature	32.743	3	3.222	0.031
Conductivity	2.596	2	0.383	0.684
Species	26.957	1	7.962	0.007
Temperature × Conductivity	8.524	6	0.419	0.862
Temperature × Species	23.795	3	2.342	0.085
Conductivity × Species	7.509	2	1.109	0.338
Temperature × Conductivity × Species	36.047	6	1.774	0.125
Error	162.576	48	–	–
Nebrioporus				
Full model	194.797	23	2.293	0.008
Intercept	110.767	1	29.986	<0.001
Temperature	63.057	3	5.690	0.002
Conductivity	1.014	2	0.137	0.872
Species	49.030	1	13.273	0.001
Temperature × Conductivity	22.756	6	1.027	0.420
Temperature × Species	29.103	3	2.626	0.061
Conductivity × Species	1.257	2	0.170	0.844
Temperature × Conductivity × Species	28.579	6	1.289	0.280
Error	177.311	48	–	–
Ochthebius				
Full model	224.753	39	4.228	<0.001
Intercept	377.240	1	276.754	<0.001
Temperature	73.485	3	17.970	<0.001
Conductivity	20.944	4	3.841	0.007
Species	0.147	1	0.108	0.744
Temperature × Conductivity	35.477	12	2.169	0.021
Temperature × Species	19.307	3	4.721	0.004
Conductivity × Species	30.157	4	5.531	0.001
Temperature × Conductivity × Species	45.236	12	2.766	0.003
Error	711.040	80	–	–

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

temperatures (40 and 45 °C) the lentic *O. notabilis* showed the maximum flight response at the highest conductivities (180 and 240 mS cm^{-1}) (Fig. 3c,d).

Discussion

Does the combination of high temperature and salinity stress affect lethal and sublethal responses in saline water beetles?

In our acute stress experiments, a reduced effect of salinity and its interaction with temperature on survival and behavioural avoidance responses was observed. Only for the *Ochthebius* species, the combination of high conductivities and extreme temperatures had a synergistic effect, reducing the emersion response of both species, and reducing flight activity and specimens survival of the less tolerant species *O. glaber*. In this case, the interactions between both factors appeared to be more important near the tolerance limits, as other authors have stated in regards to a coral species (Coles & Jokiel, 1978). Osmoregulatory mechanisms could be impaired at extreme temperatures, which could explain the severe fitness loss observed in the individuals of *O. glaber* exposed to high temperatures and salinities.

An acute exposure to osmotic stress did not affect survival or behavioural responses on the *Nebrioporus* and *Enochrus* species studied here. However, recent work has documented the effect of chronic exposure to salinity on the lethal thermal limits for *N. baeticus*, *N. ceresyi* (Sánchez-Fernández *et al.*, 2010), and *E. falcarius* (Arribas *et al.*, 2012a); the upper thermal limits of these species are higher in individuals acclimated to relatively high salinities and temperatures. Thus, the effect of salinity and the interaction of Temperature × Salinity on these species might be highly mediated by exposure time, in agreement with many studies that have found that lethal and sublethal responses could differ depending on the duration of exposure to a determined stressor (e.g. Reynaldi & Liess, 2005; Terblanche *et al.*, 2008; Nel *et al.*, 2011). In general, the physiology and behavioural regulation ability of the studied saline species was affected more immediately and strongly by heat shock than acute osmotic stress. To date, similar studies

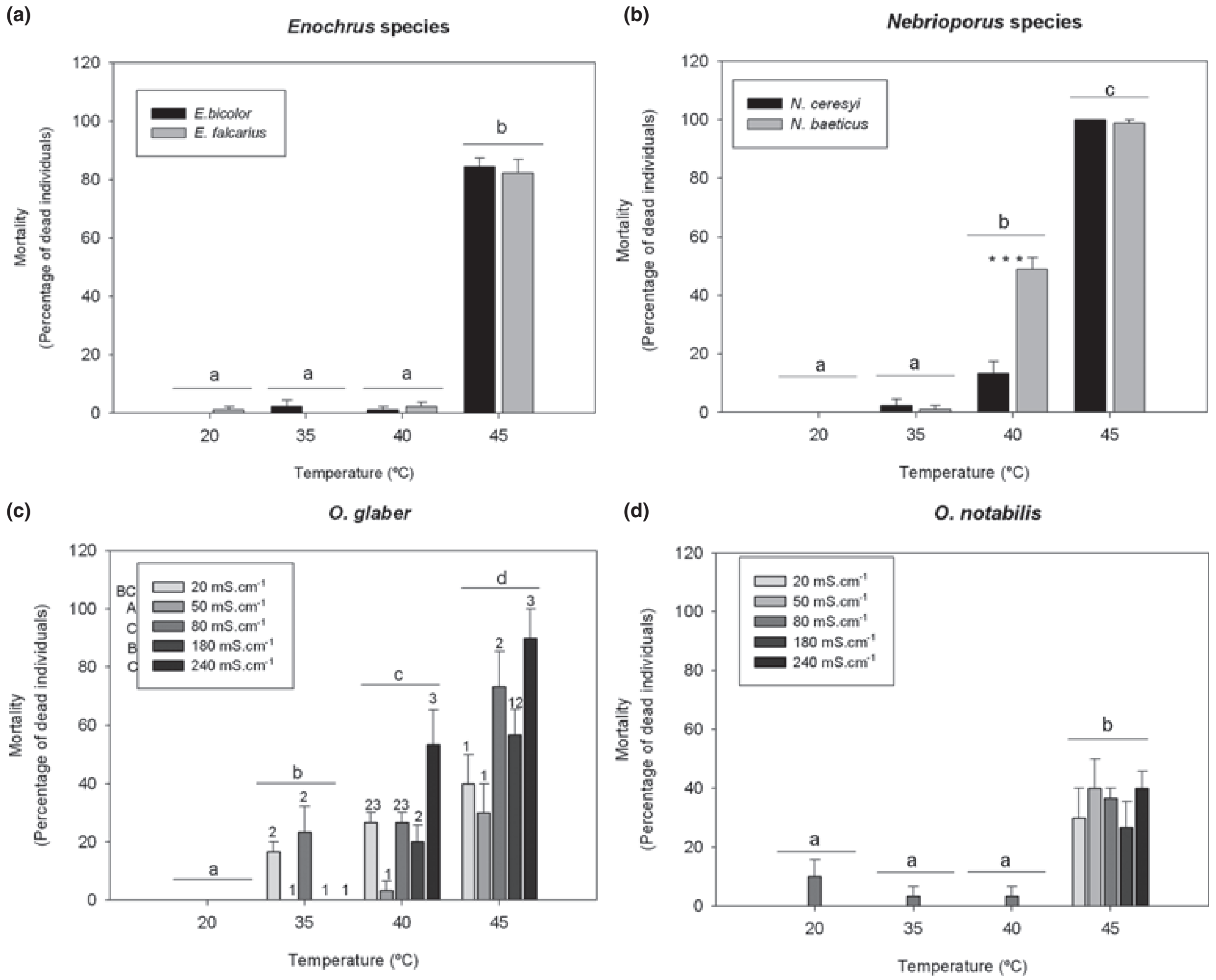

Fig. 1. Mean ± SE mortality of each species. Significant differences determined by *post-hoc* analysis employing Bonferroni correction are indicated as follows: by capital letters in the legend for conductivity levels, by lower case above the bars for temperature levels, by numbers above the bars for conductivity levels within the same temperature level, and by asterisks above the bars for differences between species within the same treatment.

evaluating the effect of acute exposure to temperature and salinity on aquatic organisms' survival and sublethal responses are scarce, so it is difficult to discern if the pattern found in these species is common.

Are species' behavioural and lethal responses interrelated?

As a general pattern in all of the studied species, avoidance responses increased in magnitude as stress levels intensified, within a range of low to moderate heat and salinity stress below their tolerance limits. However, when stress levels approached these limits, behavioural thermoregulation was impaired possibly owing to the failure of physiological mechanisms regulating temperature and salinity tolerance.

In *Enochrus* and *Nebrioporus* species, low mortality and increasing emersion and flight activity were recorded between 20 and 40 °C. At 45 °C only a few individuals survived, and behavioural responses were significantly reduced due to the irreversible physiological damage caused by the extreme heat stress.

Ochthebius species were the most heat tolerant and in general displayed more intense behavioural activity. The higher tolerance to temperature observed in these species is congruent with the extreme hypersaline habitats they inhabit (Velasco *et al*., 2006; Abellán *et al*., 2009; Millán *et al*., 2011). Osmoregulation mechanisms could enhance their thermal resistance through the accumulation of osmolytes in the haemolymph, and the consequent reduction of protein denaturation during heat stress (Harada *et al*., 2011), which would provide these species a cross-tolerance to salinity and heat. This cross-tolerance between different stressors has been studied in some terrestrial insects (e.g. Tauber *et al*., 1986; Bayley *et al*., 2001; Bubliy *et al*., 2012). However, there have been no studies on the mechanism of such cross-tolerance in saline species.

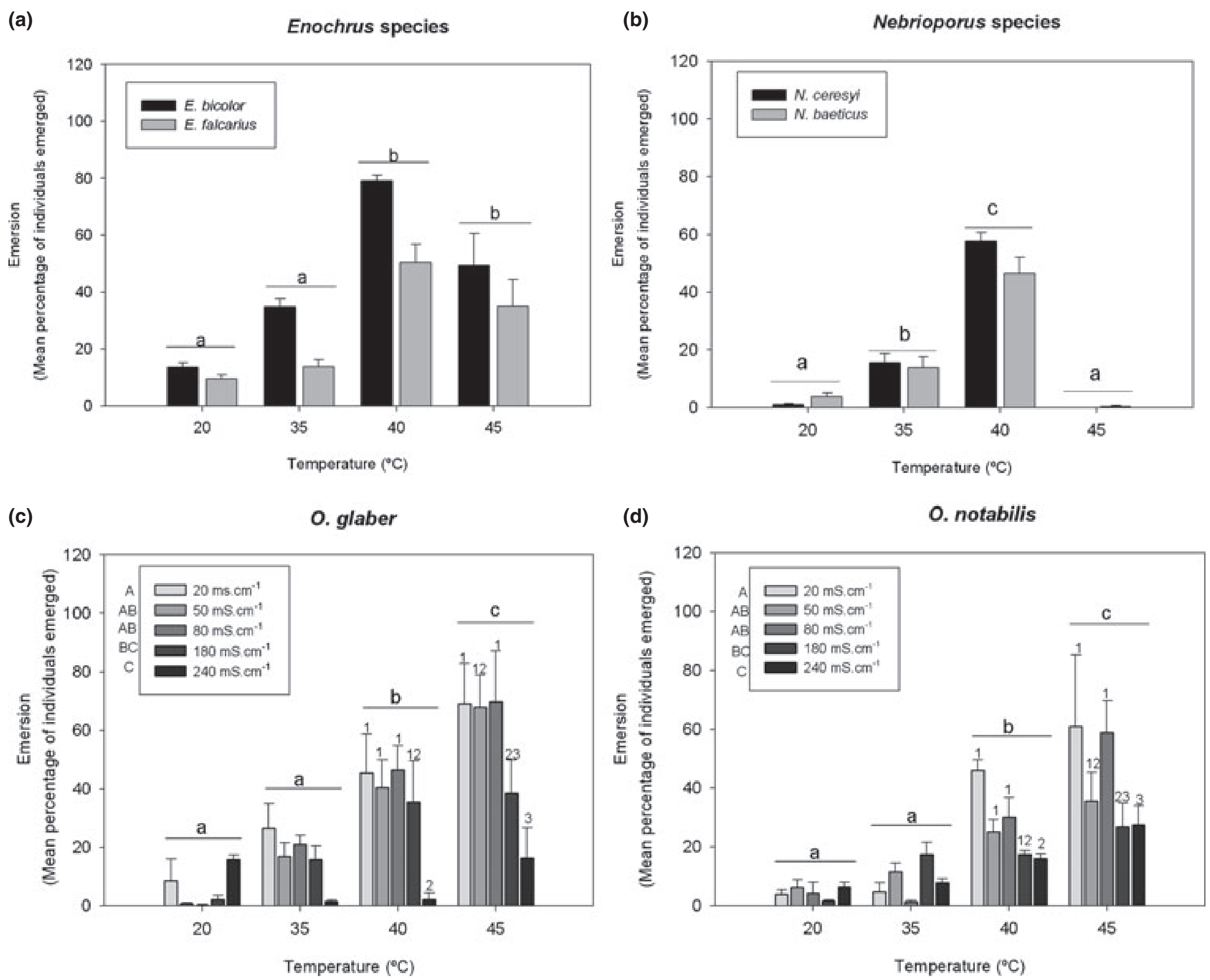

Fig. 2. Mean ± SE emersion of each species. Significant differences determined by *post-hoc* analysis employing Bonferroni correction are indicated as follows: by capital letters in the legend for conductivity levels, by lower case above the bars for temperature levels, by numbers above the bars for conductivity levels within the same temperature level, and by asterisks above the bars for differences between species within the same treatment.

The ability to develop behavioural responses is highly determined by individual's physiological tolerance (Wijnhoven *et al*., 2002), a pattern that has been observed in our results. Furthermore, behavioural adjustments modify the environmental conditions that an organism experiences, and therefore influence its fitness and short-term physiological performance (Huey, 1991). Consequently, a proper evaluation of the physiological amplitudes of species should include not only measures of survival limits, but also other sublethal responses.

Are the lotic species more sensitive to stress than lentic species?

As expected, in two of the three studied species pairs (i.e. *Nebrioporus* and *Ochhtebius* species), those occupying lotic, more environmentally stable habitats, were more sensitive to heat stress.

Within the *Nebrioporus* species, the lotic *N. baeticus* was less tolerant to heat stress than the lentic *N. ceresyi.* This result is in concordance with data obtained from lethal thermal limit experiments by Sánchez-Fernández *et al*. (2010), where *N. ceresyi* showed greater thermal range than *N. baeticus*. Both species displayed maximum behavioural responses at the same temperature thresholds (35–40 °C), although *N. baeticus*, the less tolerant species, showed higher flight activity than *N. ceresyi*.

Parallel differences in stress responses were found within the *Ochthebius* species. *Ochthebius glaber*, which inhabits lotic water bodies, showed greater mortality and initiated avoidance responses at lower stress thresholds than *O. notabilis*, which occupies lentic habitats with greater thermal and saline variability (Abellán *et al*., 2007, 2009). At the most extreme heat level, *O. glaber* flew more than *O. notabilis* in lower salinities, while at the most extreme conductivity (240 mS cm^{-1}) *O. notabilis* showed more flight and emersion activity in concordance with its greater salinity and heat tolerance.

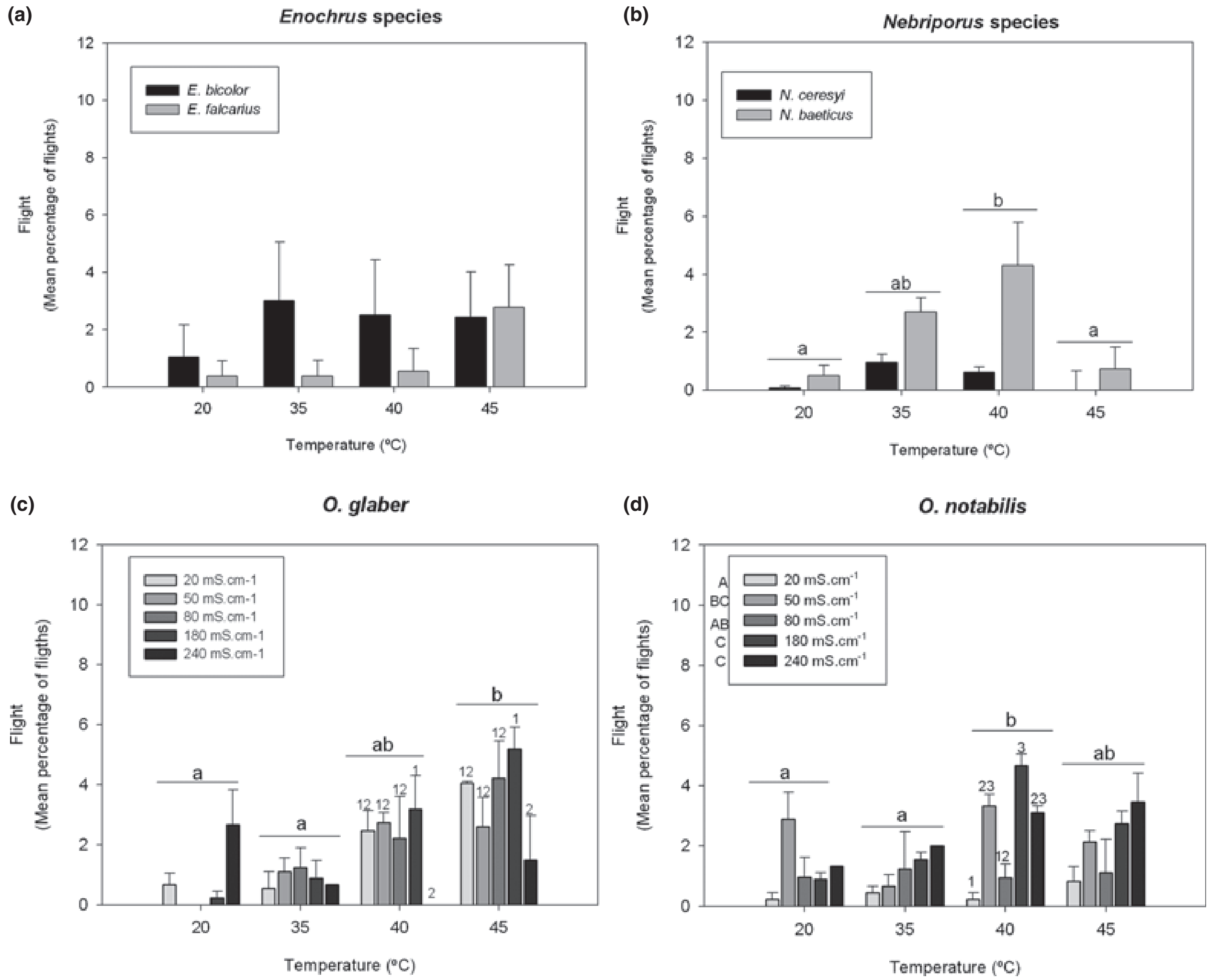

Fig. 3. Mean ± SE total flight of each species. Significant differences determined by *post-hoc* analysis employing Bonferroni correction are indicated as follows: by capital letters in the legend for conductivity levels, by lower case above the bars for temperature levels, by numbers above the bars for conductivity levels within the same temperature level, and by asterisks above the bars for differences between species within the same treatment.

Contrary to the pattern observed in the *Nebrioporus* and *Ochthebius* species, both *Enochrus* species displayed similar tolerance to heat stress. However, *E. bicolor*, the lentic species, exhibited higher emersion and flight activity than *E. falcarius*. These results are in agreement with those obtained by Arribas *et al*. (2012a), where dispersal capacity, rather than physiological tolerances, was identified as driving biogeographical differences between lentic and lotic species in the *E. bicolor* group (including *E. bicolor* and *E. falcarius*).

Our results suggest that tolerance to environmental changes in the studied species could be mediated by habitat stability. Differences in the environmental stability of lentic and lotic habitats could promote the evolution of different stress response strategies among species in each kind of habitat (Ribera, 2008). Thus, species adapted to less stable lentic habitats would have developed higher colonisation capabilities that would be mediated by both improved physiological tolerances (e.g. *Nebrioporus* and *Octhebius* species) or dispersal abilities (e.g. *Enochrus* species) compared to their lotic counterparts.

Which species within each genus could be more susceptible to climate change on the basis of these lethal and behavioural responses?

Despite the wide tolerance of saline species to environmental changes (Millán *et al*., 2011), on the basis of the responses studied here, the lotic species *N. baeticus* and *O. glaber* could be more vulnerable than their respective lentic species *N. ceresyi* and *O. notabilis* to a rapid temperature increase. Particularly, *O. glaber,* which is considered to be highly threatened in the Iberian Peninsula (Sánchez-Fernández *et al*., 2008), seems to be the most endangered due to its higher sensitivity to heat and osmotic stress coupled with the high fragmentation of its habitats (hypersaline streams) and low dispersal capacity (Abellán *et al*., 2007, 2009; Arribas *et al*., 2012b). In the case of *Enochrus* species, the lower dispersal ability of *E. falcarius* (Arribas *et al*., 2012a) also points to a higher vulnerability to environmental changes than for the lentic *E. bicolor*.

However, even in the context of rapid environmental changes, variation rates in natural conditions are much slower than those tested here, allowing organisms to acclimate through short-term plasticity (Stillman, 2003). Thus, despite the fact that study of physiological amplitudes provides valuable information about potential species' sensitivity to environmental changes, species' responses in nature could be also affected by phenotypic plasticity, which could enhance survival rates. Although in unpredictable environments such as saline water bodies, acclimation effects would be reduced (Chown & Terblanche, 2007), further studies applying dynamic protocols with more gradual change rates could be key to obtain more realistic estimates of species responses to increasing environmental stress.

In summary, data from this study suggest that specialised aquatic fauna in saline lotic habitats could represent a vulnerable component of arid environment biodiversity (Millán *et al*., 2011). We therefore propose that biomonitoring and extra conservation efforts are focused on these singular habitats.

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