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RESEARCH ARTICLE

Aquatic insects in a multistress environment: cross-tolerance to salinity and desiccation

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ABSTRACT

Exposing organisms to a particular stressor may enhance tolerance to a subsequent stress, when protective mechanisms against the two stressors are shared. Such cross-tolerance is a common adaptive response in dynamic multivariate environments and often indicates potential co-evolution of stress traits. Many aquatic insects in inland saline waters from Mediterranean-climate regions are sequentially challenged with salinity and desiccation stress. Thus, cross-tolerance to these physiologically similar stressors could have been positively selected in insects of these regions. We used adults of the saline water beetles *Enochrus jesusarribasi* (Hydrophilidae) and *Nebrioporus baeticus* (Dytiscidae) to test cross-tolerance responses to desiccation and salinity. In independent laboratory experiments, we evaluated the effects of (i) salinity stress on the subsequent resistance to desiccation and (ii) desiccation stress (rapid and slow dehydration) on the subsequent tolerance to salinity. Survival, water loss and haemolymph osmolality were measured. Exposure to stressful salinity improved water control under subsequent desiccation stress in both species, with a clear cross-tolerance (enhanced performance) in *N. baeticus*. In contrast, general negative effects on performance were found under the inverse stress sequence. The rapid and slow dehydration produced different water loss and haemolymph osmolality dynamics that were reflected in different survival patterns. Our finding of cross-tolerance to salinity and desiccation in ecologically similar species from distant lineages, together with parallel responses between salinity and thermal stress previously found in several aquatic taxa, highlights the central role of adaption to salinity and co-occurring stressors in arid inland waters, having important implications for the species' persistence under climate change.

KEY WORDS: Inland waters, Beetles, Drought, Osmotic stress, Homeostasis, Water balance

INTRODUCTION

The persistence of animal populations in dynamic and multivariate environments greatly depends on their ability to deal with the interactive effects of different stressors occurring simultaneously or sequentially over short time scales (Gunderson et al., 2016). Exposure to a particular stressor might enhance tolerance to a subsequent stress if the physiological protective mechanisms against the two stressors are shared (cross-tolerance, e.g. Elnitsky et al., 2009; Holmstrup et al., 2002) or, conversely, can cause the organisms to be more susceptible to the second stress (cross-susceptibility) (Sinclair et al., 2013; Todgham and Stillman, 2013).

Studies on multiple stressors have received increasing attention for their potential to reveal interesting information, which would be difficult to predict based on single stressor approaches (DeBiasse and Kelly, 2016; Gunderson et al., 2016), as well as to increase our understanding of responses to global change in natural multivariate environments (Hewitt et al., 2016). However, these approaches are still scarce in the literature and are mostly focused on the combined effects of temperature with other factors (e.g. Huth and Place, 2016; Pansch et al., 2012; Todgham et al., 2005).

Inland saline waters in arid and semi-arid regions represent a template for the evolution of mechanisms to deal with multiple sources of stress, especially high and fluctuating temperatures and water salinity levels coupled with seasonal wet and dry periods (Gasith and Resh, 1999; Millán et al., 2011). In temporary saline waterbodies, droughts are often preceded by an increase in salinity as the water level drops (Hershkovitz and Gasith, 2013); therefore, many aquatic organisms living in these environments are sequentially and repeatedly challenged with salinity and desiccation stress. Some aquatic insects face droughts *in situ* in microrefuges (e.g. Stubbington et al., 2016), whereas others with flying adults disperse to more favourable wet habitats (Bilton, 2014; Strachan et al., 2015). During dispersal, aquatic insects may experience important water losses associated with exposure to the desiccating aerial medium and flight activity (Dudley, 2000). The persistence of these species greatly depends on the ability of adults to deal with such osmotic and dehydration stress, especially for those with no desiccation-resistant stages (eggs or larvae), like most true water beetles (*sensu* Jäch and Balke, 2008) (Millán et al., 2014).

Both salinity and desiccation lead to dehydration and osmotic stress, which is a critical problem at the cellular level (Bradley, 2009; Cohen, 2012; Evans, 2008). Therefore, salinity and desiccation stress in insects trigger common physiological mechanisms, mainly aimed at increasing water content (e.g. drinking from the medium), avoiding its loss (e.g. control of cuticle permeability) and maintaining ionic homeostasis (e.g. activity of Malpighian tubules and specialized parts of the hindgut) (Bradley, 2009; Dow and Davies, 2006; Gibbs and Rajpurohit, 2010; Larsen et al., 2014). However, the efficiency of these mechanisms in terms of water and ionic balance under sequential exposure to both stressors, as frequently occurs in nature, has not been studied in saline aquatic insects. If the stressors occur close enough in time, so that exposure to the second stressor takes place while the physiological response to the first stressor is still being mounted, interactive effects that result in cross-tolerance or cross-susceptibility are likely to occur (Gunderson et al., 2016).

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List of abbreviations

CD	control desiccation
M_0	initial mass
M_d	fresh mass after desiccation pre-treatment
M_s	fresh mass after salinity pre-treatment
OS	optimum salinity
RD	rapid desiccation
SD	slow desiccation
SLS	sublethal salinity
WC_0	initial water content
WC_d	water content after desiccation pre-treatment
WC_s	water content after salinity pre-treatment
WLR	water loss rate (mean, maximum, final)

Cross-tolerance is not necessarily adaptive *per se* (i.e. it might be the consequence of general responses to stressors that are not experienced together in nature), but in many cases it appears to be under selection in response to synchronous or sequential stressors (e.g. Chen and Stillman, 2012; Kumlu et al., 2010; Sánchez-Fernández et al., 2010; Todgham et al., 2005). Therefore, identifying cross-tolerance responses could offer significant information on the evolutionary history of interactions among stressors (Bubliy et al., 2012; Sinclair et al., 2013). In a recent study on a lineage of water beetles (*Enochrus* species of the subgenus *Lumetus*), Arribas et al. (2014) found that transitions from fresh to saline waters occurred in periods of global aridification and showed a positive correlation between the salinity and aridity of species' habitats. From these results, the authors hypothesized a correlated evolution of salinity and desiccation tolerance in this group, potentially as an exaptation process due to linked physiological mechanisms to deal with the two stressors. The positive association found between desiccation resistance and salinity tolerance across species of this genus (Pallarés et al., 2016) also points in that direction. In light of this potential evolutionary link between salinity and desiccation tolerance and their common physiological basis, cross-tolerance might have been selected in species living in saline inland waters where salinity and desiccation stress co-occur.

Here, we tested cross-tolerance to salinity and desiccation in two Iberian water beetle species with a clear habitat preference for saline waters: *Enochrus jesusarribasi* and *Nebrioporus baeticus*. They belong to two representative coleopteran families in inland waters (Hydrophilidae and Dytiscidae) that have colonized and diversified across aquatic habitats independently (Hunt et al., 2007). We examined survival and two key measures of osmoregulation and water conservation capacity (water loss and haemolymph osmolality) under controlled-sequential exposure to salinity and desiccation in the laboratory. We predicted that the activation of mechanisms for ionic and water control during exposure to stressful but non-lethal levels of either salinity or desiccation would help specimens to deal with further osmotic-dehydration stress, improving performance under a subsequent exposure to the other stressor.

Our results on the comparative physiology of two ecologically similar species from different lineages of water beetles show the importance of the adaptation to both stressors in inland saline waters and provide new insights into the processes of colonization and diversification in these systems. This information could also be highly relevant to understanding how aquatic insects respond to the ongoing aridification in Mediterranean inland aquatic ecosystems, where more extreme and prolonged droughts and increased salinity levels are predicted (Bonada and Resh, 2013; Filipe et al., 2013; Sala et al., 2000).

MATERIALS AND METHODS

Target species, specimen collection and maintenance

Adult specimens of the water beetle species *Enochrus jesusarribasi* Arribas and Millán 2013 (suborder Polyphaga, family Hydrophilidae) and *Nebrioporus baeticus* (Schaum 1864) (suborder Adephaga, family Dytiscidae) were used as models for this cross-tolerance study. Both species have been proposed as 'vulnerable' because of their strong habitat specificity and endemic character, restricted to inland saline streams in southeastern Spain (Arribas et al., 2013; Millán et al., 2014; Sánchez-Fernández et al., 2008).

These species have been shown to be effective euryhaline osmoregulators in laboratory assays (Céspedes et al., 2013; Pallarés et al., 2015) and are mainly found in meso-hypersaline waters in nature constituting high-abundance populations (Velasco et al., 2006). Dispersal by flying in the adult stage is the main strategy for coping with seasonal droughts in these species, whose larvae and eggs are desiccation sensitive.

Adult beetles were collected in two intermittent saline streams located in Murcia (SE Spain): Rambla Salada (*E. jesusarribasi*) and Río Chícamo (*N. baeticus*). Specimens were held in the laboratory at 20°C with substratum and water taken from the collection sites, at conductivities of 65 and 12 mS cm^{-1} for *E. jesusarribasi* and *N. baeticus*, respectively, until they were used for the experiments. During this time, beetles were fed with macrophytes (*E. jesusarribasi*) or chironomid larvae (*N. baeticus*).

Cross-tolerance experiments

We conducted two independent experiments to assess the effects of (i) exposure to stressful salinity on the subsequent resistance to desiccation and (ii) exposure to desiccation stress on the subsequent tolerance to salinity. The stress treatments represented sublethal conditions, which were adapted to the specific tolerance ranges of each species (see specific conditions in Fig. 1) according to pilot trials and previous studies (Arribas et al., 2012b; Céspedes et al., 2013; Pallarés et al., 2015, 2016; Sánchez-Fernández et al., 2010). Pilot trials were also conducted to determine the number of replicates (i.e. specimens) needed to ensure adequate power to detect the effect of the stressors.

The experiments were conducted at a constant temperature (20°C) and light:dark cycle (12 h:12 h) in an environmental chamber with humidity control (CLIMACELL-404, MMM Medcenter Einrichtungen GmbH, Germany). At the end of each experiment, dry mass (M_{dry}) of all the tested specimens was measured (after drying at 50°C for 48 h) with an electronic high-precision balance (±0.00001 g) and beetles were sexed by examination of genitalia.

Effect of salinity on desiccation resistance

Groups of 30–40 specimens of each species were randomly assigned to the following pre-treatments, for 1 week (Fig. 1): (i) optimum salinity (OS), i.e. the most frequent salinity levels of each species' habitat or (ii) a higher sublethal salinity (SLS). Solutions were prepared by dissolving the appropriate amount of marine salt (Ocean Fish, Prodac, Citadella Pd, Italy) in distilled water. Food was provided for the first 5 days and removed 48 h prior to desiccation exposure. After the salinity pre-treatments, surviving specimens of each salinity that showed no sign of critical stress (i.e. were able to normally move) were used to obtain fresh mass (M_s; mg) and their survival and water loss under desiccation were investigated. For this, specimens were gently dried on blotting paper, held for 10 min at room temperature until the cuticle surface was totally dry and then individually placed in open glass vials and subjected to 40±5% relative humidity (RH) in an environmental chamber. Survival was checked and specimens were

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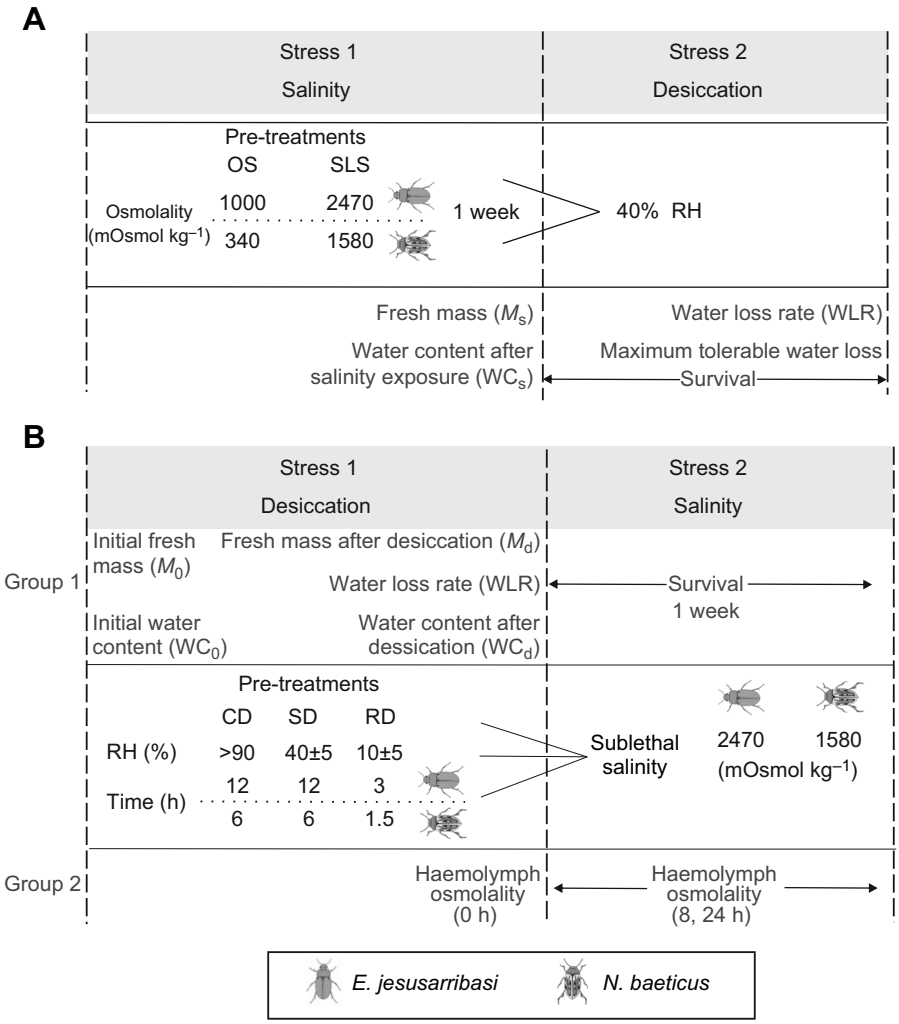

Fig. 1. Experimental conditions and variables obtained for each experimental step. (A) Effect of salinity on desiccation resistance. (B) Effect of desiccation on salinity tolerance. Experimental conditions [relative humidity (RH) osmolality and exposure time] that differ between the species are indicated with species symbols. OS, optimum salinity; SLS, sublethal salinity; CD, control desiccation; SD, slow desiccation; RD, rapid desiccation.

re-weighed every 2 h. Using such fresh mass measures and M_{dry} of each specimen (see above), we estimated their water content after salinity pre-treatment (WC_s; mg), the rate of total water loss (i.e. cuticular, respiratory and excretory) under desiccation (WLR; mg h^{-1}) and the final water content remaining at death (WC_f; mg).

Kaplan–Meier survivorship curves (Altman, 1992) were used to compare survival to desiccation between salinity pre-treatments, specifying right censored data for those individuals that were alive at the end of the experiment. Differences in mean survival between optimum and stressful salinity pre-treatments were tested using the log-rank (Mantel–Cox) test (e.g. Folguera et al., 2011; Kefford et al., 2012). Generalized linear models (GLMs) were used to test for differences between the two salinity pre-treatments in WC_s and WLR under the subsequent desiccation. Because the change in WLR was not linear over desiccation exposure, this variable was estimated for each individual as (i) the mean of the rates measured every 2 h (WLR_{mean}), (ii) the maximum rate (WLR_{max}) and (iii) the final rate (WLR_f; i.e. the rate measured at the interval prior to dead). To correct the analyses for the individual variation in mass and water status and to account for potential sex-specific differences in the response variables (see Chown and Nicolson, 2004; Le Lagadec et al., 1998), initial M_s and sex were included as covariates, plus WC_s in the analysis of WLRs. In this case, as M_s and WC_s are highly correlated, their effects were evaluated separately to avoid statistical problems of collinearity between predictors.

Effect of desiccation on salinity tolerance

Groups of 50 specimens of each species were randomly assigned to the following pre-treatments (specific exposure times for each treatment and species are shown in Fig. 1): (i) non-desiccation control (CD; at RH>90%), (ii) rapid desiccation (RD; at 10% RH) or (iii) slow desiccation (SD; at 40% RH). For the SD pre-treatment, specimens were placed in individual open glass tubes in the environmental chamber set at 40±5% RH. For the RD pre-treatment, 2 g of silica gel were added to individual glass tubes and separated from the specimen using a piece of foam. The tubes were covered with Parafilm to maintain a low RH (10±5%) (e.g. Bazinet et al., 2010; Lyons et al., 2014). In the control, the open glass tubes were introduced into a 7 l plastic aquarium with deionized water in the base (approximately 2 cm) enclosed with plastic film, reaching RH levels close to saturation (i.e. >90%).

Food was removed from the maintenance aquaria 48 h prior to desiccation and withheld for the duration of the experiments. All specimens were initially weighed (M_0) and after desiccation, survival and ability to move were checked, discarding individuals that showed movement difficulties. A subgroup of 20–30 specimens were re-weighed (fresh mass after desiccation, M_d) and used for estimation of WC_0 and WC_d (before and after the desiccation pre-treatment, respectively) as well as WLR. These same specimens were subsequently used for the survival assay under stressful high salinity. For this purpose, specimens were placed in individual

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Table 1. GLM results on the differences in water content between salinity pre-treatments, and influence of initial body mass (M_s) and sex

Species	Predictors	Slope (mean±s.e.m.)	d.f.	*F*-statistic	Explained deviance (%)
E. jesusarribasi	Intercept	−0.415±0.153**			
	Pre-treatment: SLS	0.185±0.043***	1	327.203***	96.8
	M_s	0.721±0.042***	1	1461.570***	
	Sex (male)	−0.019±0.041	1	0.213	
N. baeticus	Intercept	1.158±0.328***			
	Pre-treatment: SLS	0.083±0.073	1	22.271***	76.6
	M_s	0.563±0.050***	1	127.984***	
	Sex (male)	−0.057±0.071	1	0.629	

SLS, sublethal salinity. **P<0.01; ***P<0.001.

plastic containers with 40 ml of the specific SLS solution for each species (Fig. 1). Survival was checked every hour for the first 12 h and subsequently at 12 h intervals, for 1 week.

Survival during the salinity exposure was compared among the three desiccation pre-treatments following the procedure explained for the effect of salinity (see above). In this case, interval censored data were specified in Kaplan–Meier curves for those deaths registered between 12 h intervals. WLR and WC_d were compared among pre-treatments using GLMs and Bonferroni *post hoc* pairwise comparisons in order to quantify the magnitude of desiccation stress and determine how it affected survival under the subsequent salinity exposure. M_0, sex and WC_0 were included as covariates.

In parallel, another subgroup of 18–24 specimens per species exposed to the same three desiccation pre-treatments was used to obtain haemolymph samples immediately after desiccation (time 0) and after 8 and 24 h of salinity exposure (N=6–8 specimens per species per time) (Fig. 1). Haemolymph extraction was conducted following the procedures described in Pallarés et al. (2015) and osmolality was obtained using a calibrated nanolitre osmometer (Otago Osmometers, Dunedin, New Zealand) (see details of the same measurement procedure in Williams et al., 2004). Some haemolymph samples were discarded because the volume was insufficient or they showed a dark colour indicating potential oxidation, which resulted in a small sample size for some groups (n<5). Therefore, non-parametric tests (Kruskall–Wallis and Dunn's *post hoc* multiple comparison test) were used to compare the osmotic concentration of haemolymph after desiccation among treatments and its temporal variation during salinity exposure.

The number of replicates in statistical analyses was equal to the number of tested specimens in individual vials (desiccation exposure) or containers (salinity exposure). Gaussian distribution and identity link function were assumed in all GLMs. Models were validated by graphical inspection of residuals versus fitted values to verify homogeneity and *Q*–*Q* plots of the residuals for normality (Zuur et al., 2009). All analyses were implemented in R v.3.2.2 using the packages stats, phia and survival.

RESULTS

All the measurements from our experiments as well as the variables estimated for analyses (as both percentages and absolute units in the case of WC and WLR) are supplied in Tables S1–S3.

Effect of salinity stress on desiccation resistance

Most of the individuals exposed to the sublethal salinities survived (around 70% in both species) and showed no signs of critical stress (i.e. were able to move normally).

Specimens' performance under desiccation did not differ between salinity pre-treatments in *E. jesusarribasi* (log rank test: χ^2=2.2, P=0.135), although survivorship curves showed a tendency of higher survival in individuals from the SLS pre-treatment than those from the OS pre-treatment (Fig. 2). The mean body water content of specimens after salinity pre-treatments (WC_s) was significantly higher in the sublethal (5.66±0.14 mg) than in the OS pre-treatment group (4.92±0.14 mg; Table 1). WLR tended to decrease during exposure to desiccation (Fig. S1). Mean WLR did not differ between salinity pre-treatments (0.113±0.004 and 0.117±0.006 mg h^{-1} in the SLS and OS groups, respectively). WLR_{max} and WLR_{final} did not differ either (Table 2; Table S4). The mean water content at death (WC_f) was 2.58±0.07 mg (i.e. approximately 52% of WC_s).

A clear cross-tolerance response was observed in *N. baeticus*; individuals exposed to SLS showed higher survival than those exposed to OS (χ^2=6.5, P=0.011; Fig. 2). Similar to *E. jesusarribasi*, WC_s after the SLS exposure (5.17±0.10 mg) was higher than that following OS exposure (4.83±0.08 mg; Table 1). The change in WLR with exposure time was not linear, reaching a maximum at 4 h in both pre-treatments (Fig. S1). In agreement with survival patterns, WLR_{mean} and WLR_f during desiccation were

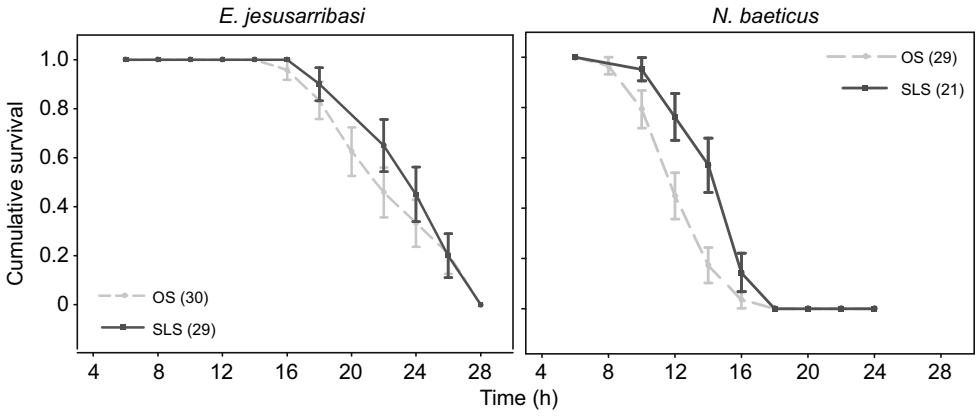

Fig. 2. Kaplan–Meir survivorship curves for exposure to desiccation following salinity pre-treatment. Each data point represents survival probability (mean± s.e.m.). Numbers in parentheses indicate the number of specimens in each group. OS, optimum salinity; SLS, sublethal salinity.

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Table 2. GLM results on the differences in mean, maximum and final water loss rate under desiccation between salinity pre-treatments, and influence of initial water content and sex

Species	Variable	Predictors	Slope (mean±s.e.m.)	d.f.	*F*-statistic	Explained deviance (%)
E. jesusarribasi	WLR_{mean}	Intercept	0.033±0.026			19.2
		Pre-treatment: SLS	−0.016±0.008	1	0.196	
		WC_s	0.017±0.005***	1	13.598***	
		Sex (male)	−0.004±0.008	1	0.217	
	WLR_{max}	Intercept	0.019±0.025			27.6
		Pre-treatment: SLS	−0.012±0.008	1	0.056	
		WC_s	0.020±0.005***	1	21.115***	
		Sex (male)	−0.006±0.007	1	0.450	
	WLR_f	Intercept	0.041±0.026			16.8
		Pre-treatment: SLS	−0.016±0.008	1	0.308	
		WC_s	0.016±0.005**	1	11.509**	
		Sex (male)	0.003±0.008	1	0.175	
N. baeticus	WLR_{mean}	Intercept	0.239±0.081**			13.3
		Pre-treatment: SLS	−0.038±0.016*	1	5.590*	
		WC_s	0.007±0.017	1	0.061	
		Sex (male)	−0.017±0.015	1	1.215	
	WLR_{max}	Intercept	0.174±0.138			4.7
		Pre-treatment: SLS	−0.014±0.028	1	0.028	
		WC_s	0.034±0.029	1	1.085	
		Sex (male)	−0.028±0.026	1	1.137	
	WLR_f	Intercept	0.239±0.081**			13.3
		Pre-treatment: SLS	−0.038±0.016*	1	5.590*	
		WC_s	0.007±0.017	1	0.061	
		Sex (male)	−0.017±0.015	1	1.215	

SLS, sublethal salinity; WLR, water loss rate [mean, final (f) and maximum]; WC_s, initial water content. *P<0.05; **P<0.01; ***P<0.001.

slightly but significantly lower in individuals from the SLS pre-treatment group (WLR_{mean}: 0.226±0.012 mg h^{-1}) when compared with those from the OS group (WLR_{mean}: 0.262±0.069 mg h^{-1}). However, WLR_{max} did not differ between treatments (Table 2; Table S4). The mean water content at death (WC_f) was 2.53± 0.06 mg (i.e. approximately 59% of WC_s).

Effect of desiccation stress on salinity tolerance

Desiccation pre-treatments did not cause significant mortality in either species; only a few specimens of *N. baeticus* (<10% of the exposed individuals) died during the SD pre-treatment.

In *E. jesusarribasi*, survival under stressful salinity conditions after SD exposure showed a rapid decline when compared with that of individuals exposed to RD or not desiccated (CD) (log rank test CD versus SD: χ^2=14.4, P<0.001; Fig. 3). Although differences in mean survival between the RD and CD pre-treatments were not significant (log rank test: χ^2=1.2, P=0.277), Kaplan–Meier survivorship curves showed a better performance in individuals previously subjected to RD during the first 12 h of salinity exposure. However, after 72 h this pre-treatment showed a higher mortality than the control (Fig. 3). The highest WLRs were recorded in the RD group (in accordance with the nature of this treatment), but specimens lost a significantly higher amount of water (lower WC_d) in the longer SD pre-treatment group (Table 3, Fig. 4; Table S5). Haemolymph osmolality differed among treatments (χ^2=10.1, P=0.006) and exposure time (χ^2=7.5, P=0.023). Specimens' osmotic concentration at time 0 (i.e. after the pre-treatment) in the RD pre-treatment group was higher than that in both the CD and the SD group (P<0.05 in Dunn's *post hoc* comparison), but remained stable during salinity exposure. In contrast, in specimens from the SD pre-treatment and the CD group, haemolymph osmolality significantly increased during salinity exposure (P<0.05) (Fig. 5).

In *N. baeticus*, the specimens exposed to the RD and SD pre-treatments showed lower survival to salinity than those from the CD group (log rank test CD versus SD: χ^2=4.1, P=0.043; CD versus RD: χ^2=6.5, P=0.011). Such a decline in performance occurred in both treatment groups after 12 h of exposure to salinity (Fig. 3). The RD pre-treatment produced a significantly higher

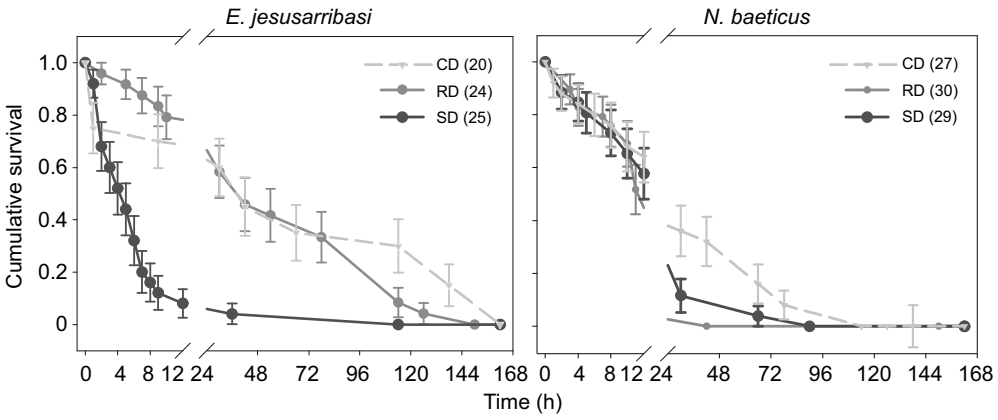

Fig. 3. Kaplan–Meir survivorship curves for exposure to salinity following desiccation pre-treatment. Each data point represents survival probability (mean±s.e.m.). Numbers in parentheses indicate the number of specimens in each group. CD, control desiccation; SD, slow desiccation; RD, rapid desiccation.

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Table 3. GLM results on variation in WLR and final water content between desiccation pre-treatments and influence of initial water content and sex

Species	Variable	Predictors	Slope (mean±s.e.m.)	d.f.	*F*-statistic	Explained deviance (%)
E. jesusarribasi	WLR	Intercept	−0.098±0.065			
		Pre-treatment: RD	0.247±0.021***	2	65.682***	
		Pre-treatment: SD	0.126±0.022***			70.0
		WC_d	0.026±0.011*	1	6.446*	
		Sex (male)	0.002±0.019	1	0.016	
	WC_d	Intercept	0.672±0.297*			
		Pre-treatment: RD	−0.384±0.097***	2	97.713***	
		Pre-treatment: SD	−1.518±0.099***			88.8
		WC_d	0.771±0.050***	1	273.278***	
		Sex (male)	0.041±0.086	1	0.225	
N. baeticus	WLR	Intercept	−0.267±0.094**			
		Pre-treatment: RD	0.318±0.031***	2	44.856***	
		Pre-treatment: SD	0.166±0.030***			59.9
		WC_d	0.085±0.018***	1	22.498***	
		Sex (male)	−0.002±0.024	1	0.006	
	WC_d	Intercept	0.875±0.387			
		Pre-treatment: RD	−0.663±0.126***	2	39.651***	
		Pre-treatment: SD	−0.827±0.124***			68.4
		WC_0	0.671±0.074***	1	82.868***	
		Sex (male)	−0.052±0.101	1	0.265	

RD, rapid desiccation; SD, slow desiccation; WC_d, water content after desiccation; WC_0, initial water content.
Significance levels: *P<0.05; **P<0.01; ***P<0.001.

WLR than the SD and CD pre-treatments, but the WC_d was similar between the two desiccation pre-treatment groups (Table 3, Fig. 4; Table S5). Haemolymph osmolality followed a similar temporal variation pattern in all treatments, remaining stable during salinity exposure (χ^2=4.5, P=0.106) but differed in magnitude among treatments (χ^2=13.4, P=0.001). Specimens previously exposed to SD showed a higher osmotic concentration than those from the CD and RD pre-treatments (P<0.001 in Dunn's *post hoc* comparisons) (Fig. 5).

DISCUSSION

We found similar interactive effects of salinity and desiccation stressors in two species from main representative coleopteran families inhabiting saline inland waters. Exposure to stressful salinity had beneficial effects on the regulation of water balance under a subsequent desiccation stress. In contrast, a negative synergistic effect on performance was found when the order of exposure to the stressors was inverted. These results are clear evidence of the mechanistic links between tolerance to these co-occurring stressors in water beetles, which could have played a key role in the colonization of these systems and may have important implications in the context of climate change.

Physiological mechanisms linking tolerance to salinity and desiccation

In our first experiment, specimens of the two studied species showed higher body water content after exposure to the stressful salinities than those held at their respective optimum salinities, and also reduced water loss under the subsequent desiccation exposure in the case of *N. baeticus*. Such adjustments contributed to extended survival time in *N. baeticus* and had a smaller, but still positive, effect on the performance of *E. jesusarribasi*.

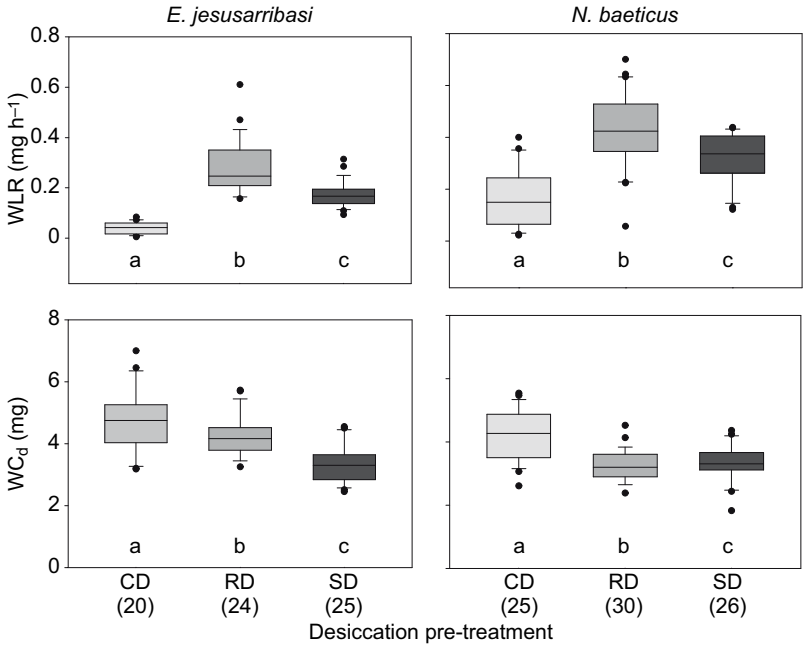

Fig. 4. WLR and WC_d following each desiccation pre-treatment. WLR, water loss rate; WC_d, water content after desiccation pre-treatment. Box length represents the interquartile range (IQR) of the data and whiskers are 1.5 times the IQR. Data outside this range are represented as points. Letters indicate significant differences between treatments (Bonferroni *post hoc* comparisons, P<0.05) and numbers in parentheses indicate the number of specimens in each group.

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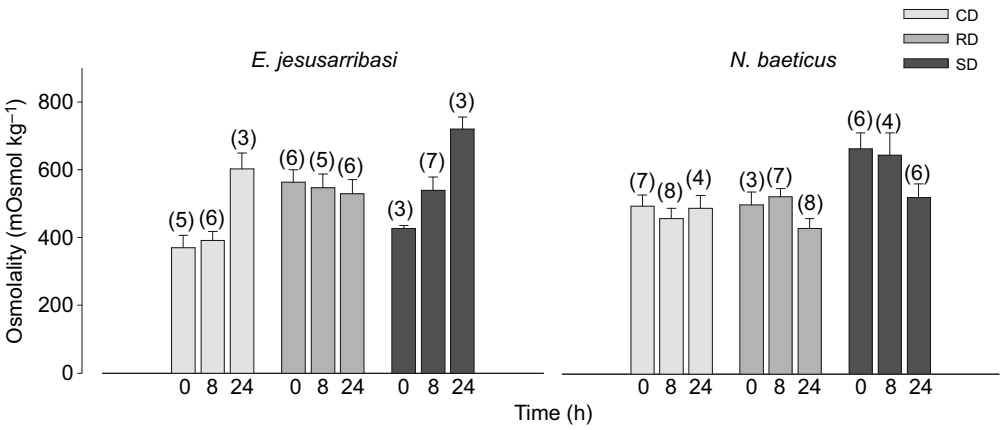

Fig. 5. Haemolymph osmolality measured immediately after desiccation (time 0) and after 8 and 24 h of exposure to sublethal salinity. Salinity was 2470 and 1580 mOsmol kg^{-1} for *E. jesusarribasi* and *N. baeticus,* respectively. Bars represent means±s.e.m. and numbers in parentheses indicate the number of samples in each group. CD, control desiccation; SD, slow desiccation; RD, rapid desiccation.

Although knowledge on the specific mechanisms of osmoregulation in aquatic beetles is still too poor to provide a mechanistic explanation for the observed cross-tolerance responses, different non-mutually exclusive processes might underlie this pattern. The specimens could have increased their drinking rates during exposure to hyperosmotic conditions in order to compensate for water loss by osmosis, a common behaviour in other saline aquatic insects such as mosquito larvae (e.g. Bradley and Phillips, 1975; Patrick and Bradley, 2000). This could account for the increase in water content and subsequent higher desiccation resistance observed here, but would also have the obvious collateral effect of ingestion of a substantial amount of salts from the medium. However, the species studied here have been shown to be able to osmoregulate over a wide hyperosmotic range including the stressful salinities tested here (Pallarés et al., 2015). In Coleoptera, excretion of salts and water reabsorption are mainly achieved through the activity of Malpighian tubules and specialized parts of the hindgut, such as the rectal pads (Crowson, 1981; Elliott and King, 1985; Machin and O'Donnell, 1991; Ramsay, 1964). The pre-activation of these osmoregulatory organs and tissues to maintain water and osmotic balance during salinity exposure probably contributed to minimize water loss during the subsequent desiccation exposure. The control of cuticle permeability is also one of the main mechanisms used to prevent water loss in terrestrial insects and has been shown to be a phenotypically plastic trait (e.g. Stinziano et al., 2015; Terblanche et al., 2010), although its role in aquatic insects has been less well explored (e.g. Alarie et al., 1998; Jacob and Hanssen, 1986). In the case of the species studied here, which use plastron or air bubbles for aquatic respiration, the cuticle surface exposed to water is reduced during water immersion. In consequence, modulation of cuticle permeability could make a smaller contribution to the control of water loss in the aquatic than in the aerial medium and therefore would have a relatively minor influence in the cross-tolerance between salinity and desiccation. Further studies on the relative contributions of these different mechanisms to the maintenance of water and ionic balance would improve understanding of the physiological basis of the cross-tolerance pattern found here.

In the second experiment, contrary to our expectations, exposure to either slow desiccation at a moderate relative humidity or rapid extreme desiccation reduced performance under a subsequent salinity stress in the two species. However, it cannot be discarded that less severe conditions than those tested here could elicit cross-tolerance to salinity, especially in *E. jesusarribasi*, which showed a short-term survival improvement after rapid desiccation exposure.

Homeostasis of the extracellular fluid is highly plastic in insects; different types of dehydration (fast versus slow) can induce different homeostatic processes (Beyenbach, 2016) and molecular responses (e.g. Lopez-Martinez et al., 2009). This seems to be the case in *E. jesusarribasi*, which showed clearly different responses after the two desiccation pre-treatments tested here in relation to the WLR and total water loss. After a slow but intense desiccation, this species maintained its haemolymph osmolality close to the values measured under control conditions and around the typical osmotic concentration of osmoregulatory insects, i.e. 300–400 mOsmol kg^{-1} (Bradley, 2009). Such an ability to display strict osmotic regulation under extreme conditions of dehydration has been observed in desert beetles (e.g. Naidu and Hattingh, 1988; Naidu, 2001) and *Drosophila* (Albers and Bradley, 2004). Nevertheless, osmolality increased rapidly when beetles were transferred to the hyperosmotic medium (second stress), probably as a consequence of a large intake of saline water to compensate for the large quantity of water previously lost and a disruption of osmoregulatory mechanisms. In contrast, when *E. jesusarribasi* was subjected to rapid dehydration, specimens showed a high haemolymph osmolality, suggesting that the osmotic concentration could have been sacrificed in this case in order to preserve extracellular and intracellular fluid volume under desiccation (Beyenbach, 2016). Although this water conservation strategy is apparently less energetically costly than active osmoregulation (Evans, 2008; Peña-Villalobos et al., 2016), maintaining high haemolymph concentrations was detrimental under the subsequent salinity stress in the long term for this species. These differences in osmoregulatory responses between the different desiccation conditions were not so evident in *N. baeticus*, probably because this species experienced similar water loss under the two desiccation pre-treatments, resulting in a similar decline of performance under the subsequent salinity stress.

Taken together, the results of our two experiments show that the effects on fitness of the combination of salinity and desiccation differ drastically depending on the order of the stress sequence (i.e. which stress precedes the other) as well as on their intensity and duration. Plastic osmoregulatory and water balance responses have both costs and benefits, and these are determined by the time scale and magnitude of variation in environmental conditions (e.g. Kleynhans et al., 2014; Todgham et al., 2005).

Ecological and evolutionary implications of linked salinity and desiccation tolerance

To our knowledge, only one case of cross-tolerance between salinity and desiccation has previously been reported in insects, specifically in larvae of the Antarctic midge *Belgica antarctica* (Elnitsky et al., 2009). However, there is evidence of cross-tolerance between salinity and thermal tolerance in diverse saline-tolerant taxa in inland waters, as for the branchiopod *Daphnia pulex* (Chen and Stillman, 2012), the water boatmen *Tricocorixa verticalis* and *Sigara lateralis* (Coccia et al., 2013), as well as several water beetle species, including those studied here (Arribas et al., 2012b; Botella-Cruz et al., 2016; Sánchez-Fernández et al., 2010). The finding of these patterns in ecologically similar species from different lineages highlights the ecological relevance of the cross-tolerance phenomenon (Hochachka and Somero, 2002; Kültz, 2005) and the central role of adaption to salinity and co-occurring stressors in arid inland waters.

During drought events in saline water, drying is often preceded by an increase in salinity levels. Acclimatization to such increasing salinities might allow insect populations showing cross-tolerance to enhance their resistance to the high temperatures and dehydration stress that they face during dispersal to wet refuges. This would also imply that adults from generations that emerge and develop in different seasons (i.e. spring–summer versus autumn–winter) could show different desiccation and thermal resistance in relation to the seasonal salinity levels of their habitat (e.g. Kalra and Parkash, 2016).

Considering the predicted intensification of droughts and water salinization across the Mediterranean region (Bonada and Resh, 2013; Filipe et al., 2013; Sala et al., 2000), cross-tolerance to salinity and desiccation could provide a significant physiological advantage for saline species to deal with such changes over related freshwater species in the same climatic area. However, as the combined effects of these stressors greatly depend on the intensity and relative timing of each stressor and also considering that most of the fauna in saline inland waters already inhabit conditions that are close to their physiological limits (Arribas et al., 2012a), persistent droughts may strongly limit the potential for salinity acclimation of these endemic species (Arribas et al., 2012b; Sánchez-Fernández et al., 2010), compromising their persistence in their current localities.

Salinity and desiccation play essential roles in the distribution and diversification of aquatic lineages. Tolerance to these stressors could have co-evolved in water beetle lineages as an exaptation process (Arribas et al., 2014; Pallarés et al., 2016). However, the exaptation hypothesis assumes that salinity and desiccation tolerance are mechanistically linked, something which had not previously been demonstrated. The cross-tolerance found here provides a solid, experimentally based trace of a potential parallel evolution of these traits in water beetles, offering a new frame to interpret diversification in inland waters. Under this scenario, global aridification events, which are broadly recognized as drivers of diversification in aquatic taxa (e.g. Pinceel et al., 2013; Dorn et al., 2014), could have been particularly relevant in the case of saline environments as one of the main forces for the colonization and further diversification in these systems (Arribas et al., 2014). Further studies on the temporal sequence of evolution of desiccation resistance and salinity tolerance in aquatic lineages could provide important insights for understanding the role of these mechanisms in driving evolution in inland waters.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

All authors conceived the idea and revised the manuscript; J.V., P.A., M.B.-C. and S.P. designed the experiments; J.V., A.M., M.B.-C. and S.P. conducted specimen collection in field; S.P. and M.B.-C. performed the experiments and statistical analyses; S.P. wrote the manuscript and the final version was revised and approved by all authors.

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Supplementary information

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