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Ecological and Evolutionary Physiology of Aquatic Beetles: Coping with Multiple Stressors in Inland Saline Waters.

Respuestas Ecofisiológicas y Evolutivas de Escarabajos Acuáticos frente a Múltiples Factores de Estrés en Medios Salinos.

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Ecological and evolutionary physiology of aquatic beetles: coping with multiple stressors in inland saline waters.

Respuestas ecofisiológicas y evolutivas de escarabajos acuáticos frente a múltiples factores de estrés en medios salinos.

Dissertation submitted by

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A mis padres y a Abraham, por dar lo mejor
de sí para que yo sacara lo mejor de mí.

“The most erroneous stories are those we think we know best
- and therefore never scrutinize or question.”

(Stephen J. Gould)

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BIOSKETCH AND FUNDING

Charca de Cuatro Cerros, Ciudad Real (Spain). Author: Andrés Millán

BIOSKETCH

I was born in 1987 in Madrid, but soon escaped from the big city and moved to San Javier (Murcia). Thus, I was lucky to grow near the sea and in contact with the natural environment.

During my undergraduate student period, I enjoyed several scholarships for research initiation and collaboration which allowed me to join the Aquatic Ecology research group as a collaborator student. I helped in several tasks that the group was carrying out like processing samples for the study of streams metabolism and laboratory experiments for physiological research with water beetles. Along this collaboration period, I discovered that I really enjoyed field and lab work and decided to take the leap into the scientific world. I developed my degree thesis “Effect of thermal and salinity stress in aquatic beetles” supervised by Josefa Velasco and Andrés Millán, which was presented in the national congress CONAMA10 in Madrid. I graduated in 2010 being awarded with an “Extraordinary Graduation Award” and the “Rector Antonio Soler Award” from the University of Murcia.

In 2011 I achieved a M.Sc. in Biodiversity management in Mediterranean ecosystems and joined the Doctorate Program “Biodiversity and Environmental Management” that led to the present thesis, funded by a predoctoral grant from the University of Murcia. As part of the practical training of the master degree, I collaborated in the Department of Zoology and Physical Anthropology of the University of Murcia, in the research group of Animal Phylogeny and Evolution under the supervision of the Professor José Galián. I learned molecular techniques and tested real-time PCR protocols for the study of gene expression.

I started my PhD thesis in 2012. The thesis objectives were framed within two research projects of the Aquatic Ecology group from the Spanish Ministry of Science and Education: “Integrating ecophysiological and evolutionary aspects to understand past, present and future distribution of iberian saline water beetles.” (2010-2013) and “The evolution of habitat transitions in aquatic Coleoptera” (2014-2017). As a PhD student, I enjoyed several visitor research fellowships, which greatly contributed to my scientific formation and the development of the present thesis. In 2013, I visited the Environmental Proteomics Lab in California State Polytechnic University (San Luis

Obispo), headed by Dr. Lars Tomanek. I learned proteomic techniques to study the proteomic response to osmotic stress in water beetles. In 2014 I visited the University of Plymouth (UK) under the supervision of Dr. David T. Bilton (Marine Biology and Ecology Research Centre, School of Marine Science and Engineering), where I developed part of the desiccation experiments that contributed to chapters 4 and 5 of this thesis. In 2015 I visited the Institute of Evolutionary Biology in Barcelona where I carried out the sequencing and phylogenetic analyses of the 5th chapter of the thesis with Dr. Ignacio Ribera (Water and Cave Beetle Evolution Lab).

I have also participated in teaching tasks of the Department of Ecology and Hydrology, specifically in the subjects “Ecología” and “Gestión del Medio Natural”. In the course 2014/2015 I co-supervised the master thesis presented by María Botella (current PhD student in the Aquatic Ecology group) entitled “Salinity acclimation enhances desiccation tolerance in the aquatic beetle *Nebrioporus baeticus* (Dytiscidae) but not the other way”, awarded as the best poster presentation in the 9th International Congress of Comparative Physiology and Biochemistry (Cracow, 2015).

Most of the chapters of this thesis have been presented in international congresses (16th Congress of The Iberian Association of Limnology in Portugal (2012), 10th European Congress of Entomology in UK (2014), 9th International Congress of Comparative Physiology and Biochemistry in Poland (2015) and 33rd Congress of the International Society of Limnology in Italy (2016)) and published in SCI-indexed journals (listed in the “General Introduction” section).

I am currently collaborating with the PhD students María Botella and Adrián Villastrigo in the characterization of the cuticular lipids of water beetles and their role in tolerance to salinity and desiccation. As a result of this and other previous collaborations I have co-authored the following papers:

- CÉSPEDES, V; PALLARÉS, S.; ARRIBAS, P.; MILLÁN, A. & VELASCO, J. 2013. Water beetle tolerance to salinity and anionic composition and its relationship to habitat occupancy. *Journal of Insect Physiology*, 59: 1076-1084.
- BOTELLA-CRUZ, M.; CARBONELL, J.A.; PALLARÉS, S.; MILLÁN, A. & VELASCO, J. 2016 Plasticity of thermal limits in the aquatic saline beetle Enochrus politus (Küster 1849) under changing environmental conditions. *Limnetica*, 35: 131-142

- BOTELLA-CRUZ, M.; VILLASTRIGO, A.; PALLARÉS, S.; LÓPEZ, E.; MILLÁN, A. & VELASCO, J. 2017. Cuticule hydrocarbons in saline aquatic beetles. Under review.

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RESUMEN

(Summary)

Laguna Mojón Blanco, Albacete (Spain). Author: Susana Pallarés

RESUMEN

Las características del hábitat definen los rasgos de las especies que lo ocupan, actuando a escala ecológica y evolutiva (concepto de *habitat templet* de Southwood, 1977). En los ecosistemas acuáticos continentales, el carácter lótico (aguas corrientes) o lenítico (aguas estancas) tiene una importante influencia en los rasgos biológicos de las especies acuáticas. La hipótesis del *habitat constraint* predice que en los medios leníticos, de carácter más inestable y discontinuo (espacial y temporalmente) que los lóticos, las especies han desarrollado una mayor capacidad de colonización (mayor capacidad de dispersión y tolerancia ambiental). Además de la estabilidad del hábitat, el estrés abiótico también constituye un importante filtro ambiental y determina la evolución de los nichos ecológicos fundamentales de las especies. En zonas áridas, la salinidad del agua, la temperatura y la desecación ejercen una presión selectiva particularmente importante sobre las fauna acuática. Estos factores actuando simultánea o secuencialmente pueden tener efectos sinérgicos o antagónicos sobre los organismos, en general poco estudiados a nivel experimental en estos medios.

En la Cuenca Mediterránea, una de las regiones que alberga más biodiversidad del mundo, encontramos una gran variedad de ambientes acuáticos continentales, incluyendo sistemas lóticos y leníticos en un amplio gradiente de temporalidad y salinidad, por lo tanto con características físicas, químicas, hidrológicas y biológicas muy diferentes. Entre esta diversidad, los medios salinos destacan por su alta singularidad ambiental y biológica, pero han sido tradicionalmente menos estudiados que los de agua dulce. Se trata de ambientes con un alto grado de estrés natural, acentuado actualmente por las presiones humanas y el cambio climático, que albergan un limitado número de especies comparados con los medios dulciacuícolas, generalmente más diversos. Sin embargo, precisamente por su adaptación a unas condiciones ambientales tan extremas, la fauna de estos medios tiene un gran interés desde un punto de vista ecológico y evolutivo. Junto a la salinidad, la desecación y las altas temperaturas (comunes durante las sequías estivales) son los factores más determinantes para las comunidades de los medios salinos mediterráneos. Por tanto, estos ambientes y sus especies constituyen el sistema ideal para estudiar cómo múltiples factores de estrés configuran los procesos fisiológicos, ecológicos y evolutivos asociados con la colonización de ambientes extremos.

Entre los macroinvertebrados que habitan los sistemas acuáticos, los coleópteros son uno de los grupos más interesantes y con un mayor potencial para abordar dichas cuestiones. Se trata de uno de los órdenes de insectos más diversos en el medio acuático; han ocupado todos los tipos de hábitats continentales gracias a una excepcional diversidad de adaptaciones fisiológicas, morfológicas y de comportamiento. En los linajes de escarabajos acuáticos es común que se dé una marcada especificidad de hábitat, existiendo especies filogenéticamente muy próximas con una clara preferencia por medios lóticos o leníticos y rangos específicos de salinidad, unas características ideales para estudios comparativos. Sin embargo, es sorprendente el importante desconocimiento sobre los mecanismos fisiológicos y procesos evolutivos que han resultado en la extraordinaria diversificación de este grupo en los medios acuáticos continentales. Esta falta de conocimiento es particularmente significativa para las especies salinas, cuyo estudio puede proporcionar información muy valiosa y de interés general en los campos de la fisiología del estrés y la biología evolutiva.

En esta tesis, especies congenéricas de escarabajos acuáticos con distintas preferencias de hábitat se usaron como modelos en estudios ecofisiológicos para explorar experimentalmente: 1) diversos componentes de la capacidad de colonización (tolerancia ambiental) en relación con la inestabilidad del hábitat y el estrés del mismo, definido por la interacción de múltiples factores, en el marco teórico del *habitat templet* y 2) los mecanismos de osmorregulación por los que se ha adquirido la tolerancia a la salinidad, sus interacciones con otros factores de estrés y su origen evolutivo.

En el **capítulo 1** se estudió el patrón de regulación osmótica (osmorregulación *vs*. osmoconformismo) en ocho especies con clara especificidad de hábitat a lo largo del gradiente salino (ambientes de agua dulce, hiposalinos, mesosalinos o hipersalinos) pertenecientes a dos linajes de escarabajos acuáticos (género *Nebrioporus*, familia Dytiscidae y género *Enochrus*, familia Hydrophilidae). Se analizó la concentración osmótica de la hemolinfa en individuos previamente expuestos a diferentes salinidades dentro del rango de tolerancia específico de cada especie. En ambos géneros, todas las especies fueron hiperreguladoras en condiciones hiposmóticas respecto a la concentración de su hemolinfa. Sólo las especies salinas mostraron capacidad de osmorregulación en medios hiperosmóticos, en un rango de osmolalidad del medio externo específico de cada especie y acorde con la salinidad máxima que habitualmente

ocupan en la naturaleza. Así, la ocupación de hábitat en el gradiente de salinidad parece estar mediada en parte por la capacidad de hiporregulación máxima en los dos géneros estudiados. Las especies salinas son fisiológicamente capaces de explotar los ambientes de agua dulce, pero otros factores, probablemente bióticos, deben limitar su ocupación.

En el **capítulo 2** se estudiaron respuestas letales (mortalidad) y subletales (respuesta de escape: emersión y vuelo) frente a la combinación de estrés térmico y salino, en tres pares de especies salinas congenéricas lóticas y leníticas (géneros *Enochrus*, *Nebrioporus* y *Ochthebius*). Individuos de cada especie fueron sometidos a un shock osmótico y térmico agudo (combinando distintos niveles de temperatura y salinidad) registrando la mortalidad, frecuencia de emersión del agua y número de vuelos. La temperatura fue el principal factor determinante de estas respuestas, aunque en las especies de *Ochthebius* la combinación de alta temperatura y alta salinidad tuvo además un efecto sinérgico significativo. Las diferencias entre especies leníticas y lóticas no fueron consistentes en los tres géneros; en *Nebrioporus* y *Ochthebius* las especies lóticas fueron más sensibles al estrés que las leníticas, de acuerdo con lo esperado en base a la mayor inestabilidad de los hábitats leníticos. Sin embargo, en el género *Enochrus* no hubo diferencias. Estos resultados y trabajos previos indican que las especies lóticas podrían ser más vulnerables frente a un aumento de temperatura en su hábitat, por su menor tolerancia térmica (*Nebrioporus* y *Ochthebius*) o menor capacidad de dispersión (*Enochrus*).

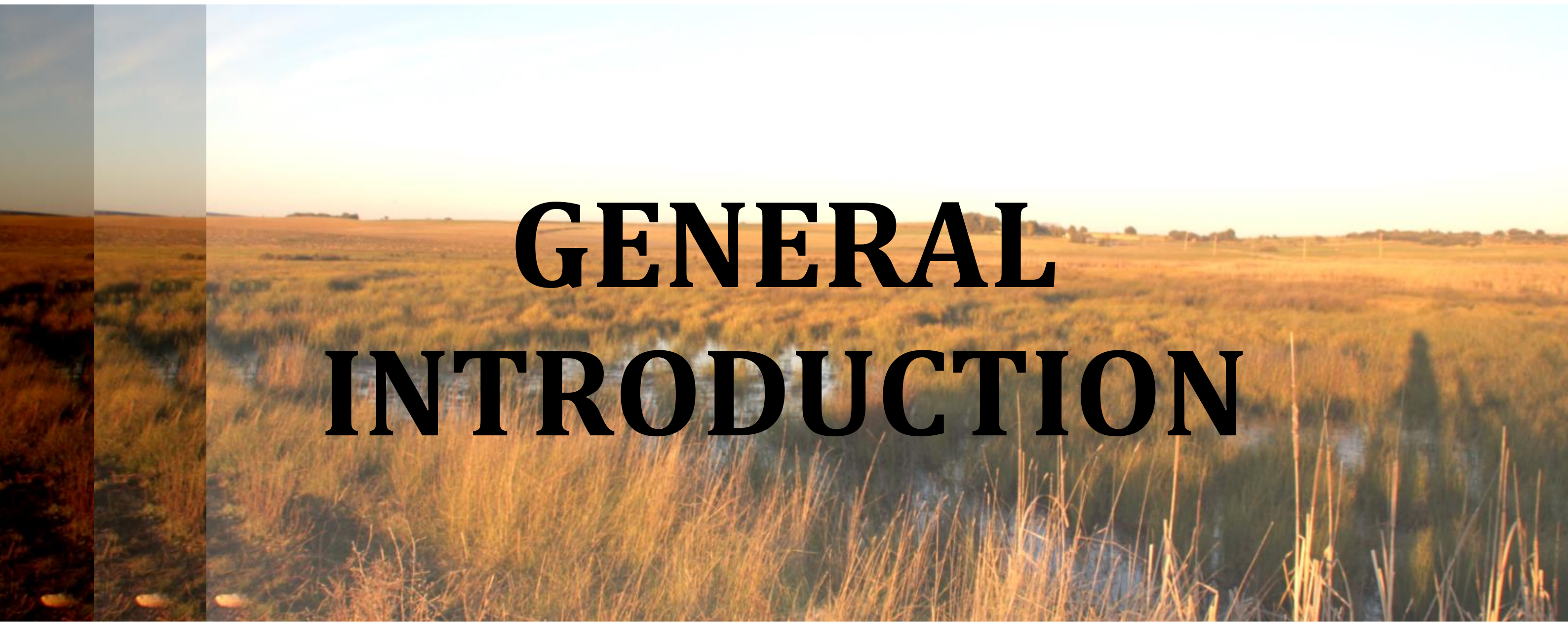
En el **capítulo 3** se analizó la variación intra- e interespecífica de diversos rasgos relacionados con la resistencia a la desecación en cuatro especies del género *Enochrus* con especificidad de hábitat entre ambientes lóticos y leníticos y en un gradiente de salinidad dulce-hipersalino. Las especies meso e hipersalinas fueron más resistentes a la desecación que las de agua dulce e hiposalina, mostrando mayor contenido en agua y menor tasa de pérdida de agua durante una exposición a desecación. No se observó asociación entre resistencia a la desecación y preferencia por medios lóticos y leníticos. A nivel intraespecífico, la variación de las tasas de pérdida de agua entre individuos estuvo positivamente relacionada con el contenido inicial de agua, pero no con el peso fresco ni el contenido en cutícula (peso relativo de la cutícula). Por lo tanto, la cantidad de agua disponible parece regular los mecanismos de control de pérdida de agua en estas especies. La asociación entre resistencia a la desecación y salinidad del hábitat apoya hipótesis previas que sugieren que la colonización de los ambientes salinos pudo ser promovida por el desarrollo de mecanismos de resistencia a la aridez.

La relación entre los mecanismos de resistencia a la desecación y tolerancia a la salinidad se exploró en el **capítulo 4** en dos especies meso-hipersalinas, *Enochrus jesusarribasi* y *Nebrioporus baeticus*. Se realizaron dos experimentos independientes para testar i) si la exposición a una salinidad alta subletal confiere tolerancia cruzada a una posterior desecación y ii) si la exposición a distintas condiciones de desecación subletales confiere tolerancia cruzada a un posterior estrés salino. En las dos especies, los individuos expuestos a la salinidad subletal mostraron un mayor contenido en agua y además, *N. baeticus* tuvo menores tasas de pérdida de agua y mayor supervivencia durante la desecación posterior (tolerancia cruzada). En cambio, cuando la desecación fue el primer estrés al que se expusieron los individuos, la supervivencia frente a un estrés salino posterior disminuyó, en algunos casos drásticamente, respuesta asociada en general a una importante pérdida de agua y a la disrupción de la capacidad de osmorregulación. Las interacciones entre tolerancias a distintos factores de estrés pueden haber sido clave en el proceso de adaptación a las condiciones ambientales extremas de los medios salinos continentales en zonas áridas. Además, estas respuestas pueden ser determinantes para la persistencia de las especies frente al cambio climático en estas áreas, donde se prevén sequías más intensas y prolongadas.

Finalmente, en el **capítulo 5** se combinaron datos ecológicos, experimentales y moleculares para reconstruir la evolución de la resistencia a la desecación, capacidad de osmorregulación y las transiciones de hábitat en el gradiente de salinidad en un linaje de escarabajos acuáticos (subgénero *Lumetus*, género *Enochrus*, familia Hydrophilidae). En base a la asociación fisiológica entre los mecanismos de osmorregulación y de resistencia a la desecación demostrada en el capítulo anterior, se predijo que dichas tolerancias mostrarían una correlación evolutiva y se testaron tres hipótesis sobre su orden relativo de aparición: 1) la capacidad de osmorregulación en aguas salinas (hiporregulación) fue una adaptación secundaria derivada de los mecanismos que evolucionaron originalmente para la resistencia a la desecación, 2) los mecanismos de resistencia a la desecación fueron una adaptación secundaria derivada de los mecanismos de hiporregulación y 3) los mecanismos de osmorregulación y resistencia a la desecación evolucionaron conjuntamente. De acuerdo a la reconstrucción ancestral,

una resistencia a la desecación relativamente alta y cierta capacidad de hiporregulación son características ancestrales del subgénero. La capacidad de control de pérdida de agua frente a la desecación se ha mantenido relativamente estable en la historia evolutiva del linaje. En cambio, la capacidad de hiporregulación ha aumentado significativamente y con tasas de evolución rápidas en algunas ramas de la filogenia, coincidiendo con transiciones a los medios meso e hipersalinos en periodos de aridificación global. Todas las especies que actualmente ocupan medios meso e hipersalinos tienen una alta capacidad de hiporregulación y resistencia a la desecación. Entre las especies de ambientes hiposmóticos (agua dulce – hiposalina), algunas tienen tasas de pérdida de agua comparables a las de las especies más salinas pero limitada capacidad de hiporregulación y otras muestran una resistencia a la desecación y capacidad de hiporregulación similares a la de las especies más salinas. Esto apunta a una secuencia evolutiva en la que la resistencia a la desecación ancestral del linaje proporcionó la base fisiológica para el desarrollo de una capacidad de osmorregulación más eficiente, que permitió a algunas especies colonizar los ambientes más extremos (meso e hipersalinos). La mayoría de nuestros resultados son consistentes con la primera hipótesis, aunque a la escala evolutiva abordada no se observó que la resistencia a la desecación preceda a la capacidad de hiporregulación en la filogenia.

GENERAL INTRODUCTION



Charcas junto a laguna de Pétrola, Albacete (Spain). Author: Susana Pallarés

GENERAL INTRODUCTION

Habitat filters shaping the life history strategies of aquatic species

The *habitat templet* concept (Southwood, 1977, 1988) conceives habitat characteristics (in terms of its variability in space and time) as a template that constraints species´ biological, ecological and physiological traits. On an ecological scale, spatial and temporal variation associated with a given environment, filters out unsuccessful strategies from the pool of colonists, controlling community composition (Scarsbrook & Towsend, 1993; Poff, 1997). On an evolutionary timescale, the selective forces maintained over those specimens able to surviving and reproducing there, modulate the evolution of species traits. In the context of inland aquatic ecosystems, the identification of the spatial and temporal axes of such templet for macroinvertebrates is a revisited, yet still pending topic (e.g. Williams, 1985, Hildrew & Townsend, 1987; Townsend, 1989; Towsend & Hildrew, 1994; Poff & Ward, 1990; Herbtst, 2001).

One of the dimensions of this general framework has led to the *habitat constraint* hypothesis, which predicts how habitat type (standing *vs*. running waters) may influence the macroecological and macroevolutionary patterns of freshwater invertebrates (Ribera & Vogler, 2000; Ribera, 2008). In inland waters, one of the most influential habitat constraints is the divide between lentic (standing) and lotic (running) waters, which differ not only in terms of water flow, but also in a wide range of associated physical and biological characteristics. Rivers and streams, which are always associated with a particular hydrological network, are expected to persist over longer geological periods, and to exhibit greater spatial and temporal continuity than small standing water bodies that tend to disappear due to sediment filling. Therefore, the species in more ephemeral and unstable lentic habitats are predicted to have developed better colonisation abilities than lotic ones, with consequences at different hierarchical levels (from individual to lineages). For example, lotic species have shown poorer dispersal ability (Arribas *et al.*, 2012a), a lower inter-population gene flow and higher phylogeographical structure (Marten *et al.*, 2006; Abellán *et al.*, 2009), narrower geographical ranges (e.g. Ribera & Vogler, 2000; Hof *et al.*, 2006; Sánchez-Fernández *et al.*, 2012) and higher evolutionary turnover (Ribera *et al.*, 2003; Abellán *et al.*, 2009) than lentic ones. Therefore, they could be more sensitive to global change, as they will have more difficulty in tracking rapid changes in a discontinuous habitat matrix than lentic species (Hof *et al.*, 2012; Sánchez-Fernández *et al.*, 2012).

A species' colonisation potential depends on its dispersal ability (i.e. the ability to move *sensu* Bilton *et al.*, 2001) (Rundle *et al*., 2002), but also on its ability to establish successfully at the new site, i.e. to tolerate the new abiotic and biotic conditions (Shurin, 2000; Shurin *et al.*, 2000). Therefore, in the *habitat constraint* context, both the dispersive and physiological components of colonisation could be expected to be associated with habitat stability, i.e. species that live in less stable lentic habitats are expected to not only be better dispersers, but to also have broader physiological tolerances than lotic species. In the aquatic environment, the association between physiological tolerances and habitat stability has been explored mainly for marine organisms (e.g. Stillman, 2002; Harley et al., 2003; Tomanek, 2010) but scarcely in continental water fauna (but see Arribas *et al*, 2012a).

In addition to habitat stability, abiotic stress also constitutes an important habitat filter for species occurrence (Odum, 1985; Weiher & Keddy, 1995; Gutiérrez-Cánovas *et al.*, 2013) and is a powerful driver of the diversification process (Parsons, 2005; Dunson & Travis, 1991). Among the multiple abiotic factors that affect organisms' physiology, ecology and evolution, temperature, salinity and desiccation are expected to exert the strongest selective pressures over inland water fauna. Temperature is one of the most important dimensions of a species' niche (Spicer & Gaston, 1999), as it underpins metabolic activity and life-history processes (Willott & Hassall, 1998). Indeed the critical role of temperature in shaping habitat occupation and species distribution is unquestionable (Ward & Stanford, 1982). Strong interannual, seasonal and diel thermal variations occur in inland waters of arid or semi-arid regions, particularly in hydrological disconnected shallow waters where extreme water temperatures (> 30ºC), which approach or exceed species' upper thermal limits, are common in summer (e.g. Leigh *et al.*, 2015). Finally, for many aquatic insects, temperature strongly drives the success of dispersal and reproduction events in which specimens leave the aquatic environment and are exposed directly to aerial conditions.

Salinity is one of the major sources of stress for organisms, because salts at concentrations over physiological limits can disrupt metabolism and water balance (Kefford *et al.*, 2002). Therefore, physiological tolerance to salinity determines habitat occupation, abundance and broad scale distribution of organisms (Williams *et al*., 1990; Millán *et al.*, 2011; Pétillon *et al.*, 2011). In fact, salinity and water chemistry have traditionally been considered fundamental components of the *habitat templet* axes for defining habitat types and physiological strategies in inland waters (Williams, 1985; Herbst, 2001). Similarly to salinity, desiccation also alters the water and ionic balance of internal fluids, both critical for cellular functioning (Evans, 2008). Reduction in water level and recurrent exposure to air are two of the most important factors that structure aquatic communities in climatic regions subjected to seasonal drought (Boulton, 2003; Fritz & Dodds, 2004). The patterns of habitat or microhabitat choice displayed by some aquatic species are linked to desiccation resistance (e.g. Wissinger *et al*., 2003; Yoder *et al*., 2014) and, together with thermal tolerance, desiccation resistance is essential for the dispersal to other habitat paths for many species (Strachan *et al.*, 2015).

In natural environments, the complex and often non-linear interactions between multiple stressors need to be taken into account to make inferences about organisms' physiological responses (McBryan *et al*., 2013; Whitehead, 2013). The combination of simultaneous or sequential changes in several biotic or abiotic factors can have different effects on specimens other than the isolated effects of each individual factor (Koussoroplis *et al*., 2016). Indeed, recent meta-analyses have shown that non-additive effects of multiple stressors (i.e. antagonistic or synergistic) are more common than additive responses in freshwater, marine and terrestrial communities (Darling & Côté, 2008; Harvey *et al*., 2013; Jackson *et al*., 2016). For this reason, the interactive effects of multiple stressors have recently received growing attention in the literature and are considered crucial to generate more realistic inferences about the effects of global change on biodiversity (DeBiasse and Kelly, 2016; Gunderson *et al*., 2016; Hewitt *et al*., 2016). However, most studies on stress responses have recurrently considered single environmental factors, and are, to a large extent, restricted to the effects of temperature on specimens' performance. In particular, empirical evidence of the effects of multiple stressors on freshwater species is still very limited (Jackson *et al*. 2016).

Based on the *habitat templet* concepts from Southwood (1977, 1988), Williams (1985) and Herbst (2001), and also considering the multivariate nature of inland water systems in arid and semiarid regions, this thesis proposes a theoretical framework within which the biological and physiological traits of aquatic insects can be predicted. This *habitat templet* could be characterised by two main axes: one defined by habitat stability, with lotic-permanent and lentic-temporary habitats at the opposite ends of the spectrum, and other by a multistress gradient of the major abiotic stressors in these systems and their interactions (Fig. 1). Aquatic insects' colonisation capacity (and all the linked traits) are expected to increase with both habitat instability and the stress level, by a greater dispersal ability and higher tolerances to multiple stressors. Nonetheless, this theoretical framework also needs to consider the historical and phylogenetic constraints on the match between organism and habitat conditions.

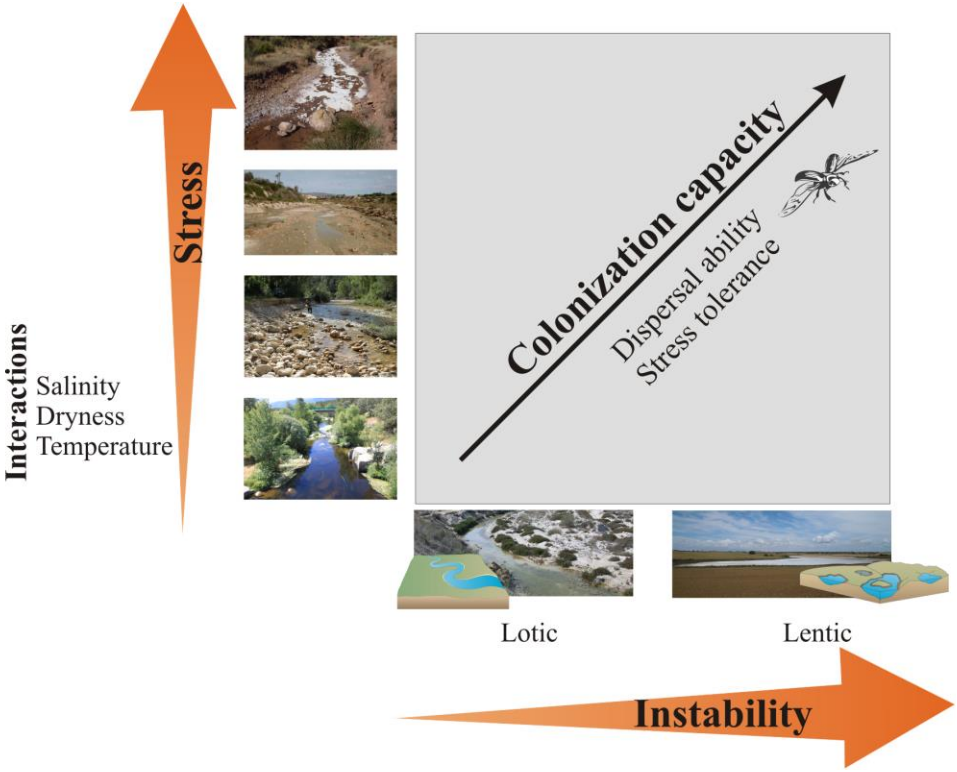


Figure 1. *Habitat templet* model for aquatic insects in inland aquatic systems of arid and semiarid areas.

To date, most approaches to the *habitat templet* concept for inland waters have been focused on identifiying general patterns, while mechanistic approaches to the evolutionary and physiological processes that lie behind such patterns are scarce. There is, therefore, a need to examine a large set of phenomena from a mechanistic perspective to understand how aquatic habitat features interact with species traits determining diversification and distribution patterns. Further research is particularly needed on the physiological and behavioural traits that determine the differences in colonisation capacity between species and the interactive effects of multiple stressors on such traits. A more integrative approach to the ecology and evolution of species requires a comprehensive understanding of physiology and behaviour (Spicer & Gaston, 1999; Gaston *et al.*, 2009; Kearney & Porter, 2009; Bozinovic *et al.*, 2011). Comparative physiology provides powerful approaches to address these issues (Somero, 2011). Indeed, the consideration of the mechanistic links between traits of organisms and their environment is a current trend in the field of niche modelling, as it could improve predictions on species responses to climate change (e.g. Arribas *et al.*, 2012b; Foden *et al.*, 2013; Sunday *et al.*, 2014; Sánchez-Fernández *et al.*, 2016).

Mediterranean inland saline waters: life in extreme environments

The Mediterranean region is one of the world’s most biodiverse places and hosts a wide variety of ecologically unique inland aquatic habitats (Alvarez-Cobelas *et al.*, 2005). Unfortunately, it is also one of the areas most impacted by human development and is strongly threatened by the effects of climate change (Zacharias & Zamparas, 2010; Bonada & Resh, 2013; Filipe *et al.*, 2013). In recent decades, several climatic alterations have been observed including higher annual average air temperatures, a drop in annual average precipitation, hydrologic alterations and an increase in the frequency, intensity and duration of extreme events, such as floods, droughts and fires (Filipe *et al.*, 2013; IPCC, 2013). A realistic assessment of global change effects on Mediterranean biodiversity requires an understanding of species’ physiological and behavioural responses to cope with such increasingly stressful conditions.

The variety of inland waters in the Mediterranean region encompasses all the possible habitat types along the environmental stability and stress gradients, i.e. lotic and lentic systems, from perennial to temporary, with differing water chemical compositions and within a wide salinity range from fresh to hypersaline waters (Fig. 2). Among these different habitats, saline waters are especially singular given their particular physicochemical and biological characteristics (Herbst, 2001; Williams, 2002; Millán *et al.*, 2011), but have been traditionally less studied than freshwater systems. Natural saline waters are relatively common in arid and semiarid areas, but are very rare in a European context. Because of their extreme environmental conditions and fragmented nature, these systems are characterised by a high degree of rare and/or endemic species. The strong selective pressure exerted by salinity limits the number of species that are able to colonise saline waters and those able to inhabit these systems present unique biological adaptations.

In addition to the effect of salinity, multiple sources of stress converge in saline waters, as they are generally located in small watersheds with low mean precipitations and high temperatures and most are intermittent (in both time and space) (Millán *et al.*, 2011). Some saline waters in arid areas reach salinity concentrations up to six times seawater values. Salinity levels are usually higher in summer due to evaporation, and some shallow waterbodies or reaches of intermittent streams can remain completely dry for several months (Velasco *et al.*, 2006). Therefore, inland saline waters provide an ideal reference system for studies integrating multiple stressors to examine the evolutionary and physiological processes associated with colonisation of extreme habitats.

Life in extreme environments is associated with the evolution of novel traits that improve species' ability to cope with stress. Understanding such mechanisms, their origin and evolutionary consequences are central questions in evolutionary biology. As mentioned above, the species that live in inland saline waters have to deal with multiple physiological challenges - mainly the osmotic stress imposed by water salinity and exposure to desiccation and high temperatures when their habitats dry out, and all this in a changing and usually unpredictable environment. A variety of physiological, morphological and behavioural adaptations have evolved among macroinvertebrates to successfully withstand such harsh environmental conditions. Some saline-tolerant insects have developed extraordinary osmoregulatory capacities that allow them to maintain their internal concentration within narrow ranges regardless of the osmotic concentration of the external media (e.g. Tones & Hammer, 1975; Herbst *et al*., 1988). Others concentrate organic osmolytes intracellularly and/or extracellularly to equilibrate the osmotic gradient with the external medium (osmoconformity strategy, e.g. Garret & Bradley, 1987; Patrick & Bradley, 2000). Some invertebrate groups have developed profound physiological adaptations to resist drying *in situ* through using microrefuges or diverse forms of resting stages (e.g. diapausing larvae or desiccation-resistant eggs) (Robson *et al*., 2011; Strachan *et al*., 2015). Insects with flying adults, such as Coleoptera and Hemiptera, can disperse to other habitat paths using a resilient strategy that permits population recovery by recolonisation when the flow resumes (Williams, 2006). Regarding high temperatures, there are few identified mechanisms by which ectotherms can survive extreme heat stress. Some taxa minimise heat exposure to by avoidance behaviour (e.g. Wharton, 1983; Cloudsley-Thompson, 1990). The plasticity of thermal limits allow others to increase their thermotolerance to some extent (e.g. Sánchez-Fernández *et al*., 2010) and up-regulation of heat shock proteins is associated usually with tolerance to extreme temperatures (e.g. Garbuz *et al*., 2008). However, for some of the main components of saline waters communities, such as aquatic Coleoptera, these mechanisms have not yet been studied in detail.

Aquatic beetles as the study group

Beetles are the most speciose group of animals on a planetary scale (Misof *et al*., 2014; Ribera & Beutel, 2014; Mckenna *et al*., 2015; Smith & Marcot, 2015) and are found in almost all kinds of habitats. They are one of the most diverse insect orders in inland waters and, together with Diptera and Hemiptera, are one of the few that have successfully colonised saline waters (Millán *et al*., 2011). Hence, water beetles represent a great contribution to aquatic biodiversity in the Mediterranean region, which hosts a large proportion of endemic beetle species (Sánchez-Fernández *et al*., 2008; Millán *et al*. 2014).

Transition to the aquatic environment from terrestrial ancestors has occurred multiple times independently along the evolution of Coleoptera, resulting in exceptionally

Figure 2. Diversity of inland aquatic habitats in the Mediterranean region. From the upper left to the bottom right corner: freshwater river (Río Tiétar, Toledo, Spain), freshwater pond (La Posadilla, Ciudad Real, Spain), saline stream (Rambla de Minglanilla, Cuenca, Spain), saline pond (Pétrola, Albacete, Spain). Authors: Félix Picazo & Susana Pallarés.

diverse behavioural and morphological adaptations to very different aquatic environments in this group (Jäch & Balke, 2008). Salinity tolerance is thought to have independently arisen in different families (e.g. Hydraenidae, Hydrophilidae and Dytiscidae), genera and lineages, from freshwater ancestors (e.g. Arribas *et al*., 2014). Therefore, marked habitat specialisation is common within aquatic beetle lineages, with closely related species living in either lotic or lentic systems and within specific salinity ranges. For this reason, water beetles constitute an ideal study model for ecophysiological and evolutionary comparative studies, particularly to explore the physiological mechanisms that enable salinity tolerance and its evolution. Aquatic beetles also present certain advantages that make them suitable target organisms for experimental work: i) in general they are locally abundant (especially saline ones) and thus easy to collect in large numbers and ii) adults are easy to handle and maintain under laboratory conditions.

However, comparative experimental studies on water beetles are scarce, and these mostly concern freshwater species (e.g. Calosi *et al.*, 2010). Therefore, little is known about the physiological mechanisms that lie behind the diversification success of this group of insects in inland waters in general, and for saline lineages in particular. The present thesis uses water beetles as models for ecophysiological studies that address some questions previously outlined, in order to cover part of such knowledge gap. This information is especially relevant in the context of global change and ongoing aridification in Mediterranean-climate regions.

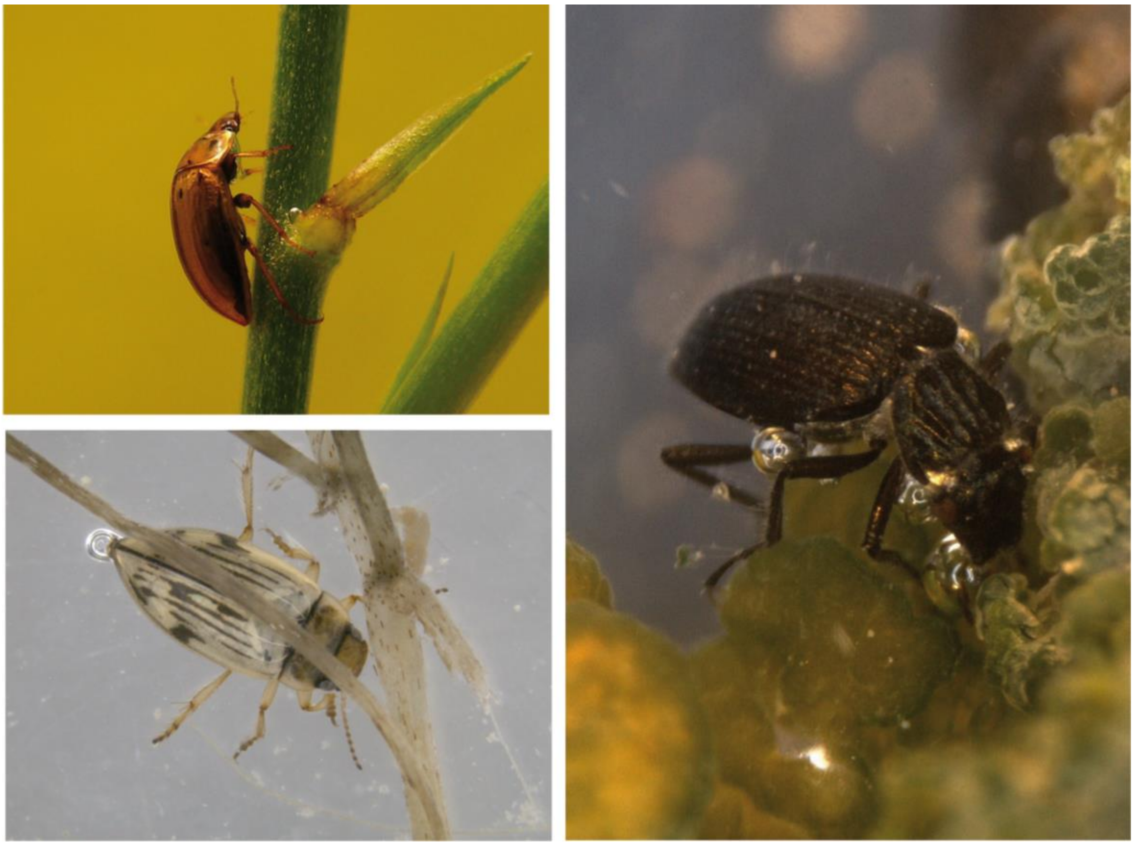


Figure 3. Upper left: *Enochrus jesusarribasi* (Hydrophilidae), lower left: *Nebrioporus baeticus* (Dytiscidae), right: *Ochthebius glaber* (Hydraenidae). Photos: Jesús Arribas, Susana Pallarés & Jose A. Carbonell.

Objectives and thesis outline

This thesis explores several key physiological and behavioural traits of aquatic beetles to cope with the multiple natural stressors in arid inland aquatic ecosystems. Comparative experimental approaches are employed, using congeneric species from different lineages with contrasting habitat preferences to understand how species' ability to deal with natural stressors is associated with habitat occupation. The central interest lies on the mechanisms by which salinity tolerance was acquired (Fig. 4). The thesis is arranged as five chapters that correspond to the following specific objectives:

Chapter 1. Identify the osmotic regulation strategy (osmoregulation *vs.* osmoconformity) of two representative independent lineages of beetles in inland waters that include species with different saline habitat preferences. The osmotic capacity of the species (the difference between the osmotic concentration of the haemolymph and the external medium) is measured to determine whether osmoregulatory capacity may mediate habitat segregation among congeners across the fresh-hypersaline gradient.

Chapter 2. Examine lethal and sublethal behavioural responses (mortality, emersion and flight activity) to the combination of acute heat and osmotic stress. These responses are compared between lotic and lentic congeneric species of saline water beetles across different genera to test for differences in tolerance to acute stress associated with habitat specialisation.

Chapter 3. Examine inter- and intraspecific variation in several desiccation resistance traits (water content, cuticle content and survival and water loss rates under desiccation), in congeneric species of water beetles with marked habitat specificity (lentic *vs*. lotic systems and different salinity optima from fresh- to hypersaline waters).

Chapter 4. Identify cross-tolerance responses to desiccation and salinity stress in two saline beetle species from different families. In particular, the effects of i) exposure to stressful salinity on desiccation resistance and ii) exposure to desiccation stress on salinity tolerance are evaluated by examining patterns in survival and water and ionic balance.

Chapter 5. Explore the evolution of hyporegulation ability, desiccation resistance, and habitat transitions across the saline gradient in a water beetle lineage. Experimental, ecological and molecular data are combined to assess whether desiccation resistance and salinity tolerance are correlated and to infer the temporal sequence of development for these adaptations.

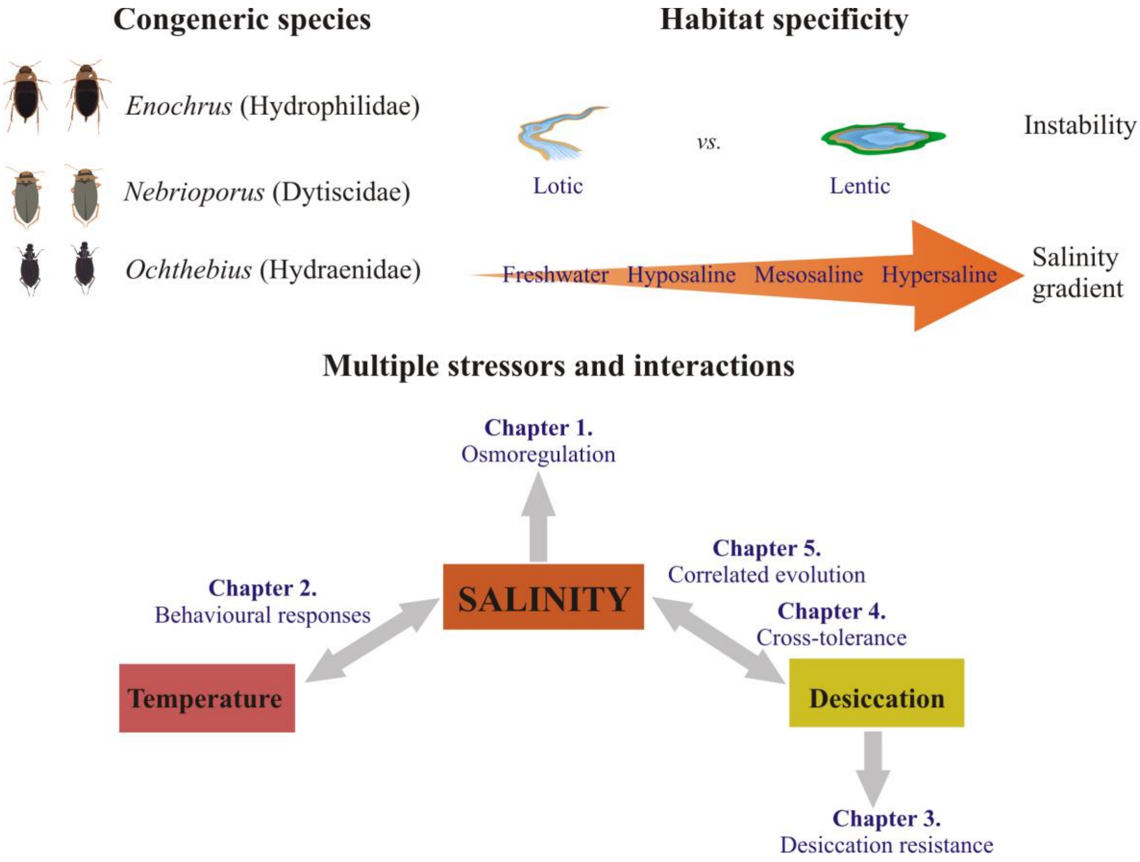


Figure 4. Scheme of the methodological approach and thesis structure.

Chapters 1 to 4 are published in international peer-reviewed journals indexed in SCI, while Chapter 5 is under review for publication at the moment of completion of this thesis. The five articles on which this thesis is based are:

- **Chapter 1**: Pallarés, S., Arribas, P., Bilton, D.T., Millán, A. & Velasco, J. (2015) The comparative osmoregulatory ability of two water beetle genera whose species span the fresh-hypersaline gradient in inland waters (Coleoptera: Dytiscidae, Hydrophilidae). *PLoS ONE*, **10**, e0124299.

- **Chapter 2**: Pallarés, S., Arribas, P., Céspedes, V., Millán, A. & Velasco, J. (2012) Lethal and sublethal behavioural responses of saline water beetles to acute heat and osmotic stress. *Ecological Entomology*, **37**, 508–520.

- **Chapter 3**: Pallarés, S., Velasco, J., Millán, A., Bilton, D.T. & Arribas, P. (2016) Aquatic insects dealing with dehydration: do desiccation resistance traits differ in species with contrasting habitat preferences? *PeerJ*, **4**, e2382.

- **Chapter 4**: Pallarés, S., Botella-Cruz, M., Arribas, P., Millán, A. & Velasco, J. (2017) Aquatic insects in a multistress environment: cross-tolerance to salinity and desiccation. *Journal of Experimental Biology*. In press.

- **Chapter 5**: Pallarés, S., Arribas, P., Bilton, D.T., Millán, A., Velasco, J. & Ribera, I. Adaptation to desiccation and salinity tolerance in a lineage of water beetles. Under review.

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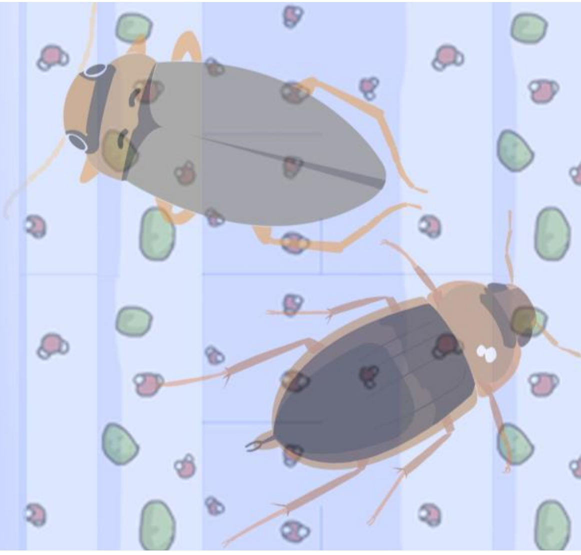
CHAPTERS

Rambla Salada, Murcia (Spain). Author: Josefa Velasco

CHAPTER 1

The comparative osmoregulatory ability of two water beetle genera whose species span the fresh-hypersaline gradient in inland waters (Coleoptera: Dytiscidae, Hydrophilidae)



INTRODUCTION

Of all the physiological challenges organisms face in the aquatic environment, an ability to maintain the osmotic concentration of body fluids in the face of fluctuations in the external environment is essential (Barton-Browne, 1964; Evans, 2008). For primarily freshwater organisms such as aquatic insects, salinity is a natural stressor that can disrupt metabolism and water balance (Kefford *et al.*, 2002), and therefore species inhabiting saline waters have developed a range of physiological mechanisms to deal with ionic fluctuations, which fall into two main strategies: osmoconformity and osmoregulation. Osmoconformers concentrate organic osmolytes intracellularly and/or extracellularly in response to increasing salinity, thus avoiding the toxicity associated with salt accumulation. In osmoregulators, the internal fluid compartment is instead strictly regulated regardless of the external osmotic fluctuation mainly by the active transport of ions via specialized organs (Bradley, 2008, 2009; Evans, 2008).

Osmoregulation has been widely studied in marine organisms (e.g. Deaton, 1981; Evans, 1981; Sang & Fotedar, 2004; Serrano *et al*., 2011; Torres *et al*., 2011), where the vast majority of taxa are osmoconformers (Willmer *et al*., 2005; Bradley, 2009). In contrast, our knowledge of the osmotic mechanisms of organisms inhabiting inland waters is severely limited, despite the fact that information on the salinity tolerance of such taxa is essential for understanding their ecology and evolutionary history (e.g. Muñoz *et al.*, 2008; Pinceel *et al.*, 2013; Arribas *et al.*, 2014). Inland aquatic systems encompass a wide variety of habitats across a large salinity range, from freshwaters to hypersaline water bodies (up to six times the salinity of the sea, i.e. around 200 g L^{-1}), which also differ in ionic composition (Bayly, 1972; Millán *et al*., 2011). In addition, organisms inhabiting inland saline systems frequently experience large osmotic and ionic fluctuations, far exceeding those seen in most marine systems, as a result of freshwater input from rainfall, or evaporation during dry periods (Velasco *et al.*, 2006; Millán *et al.*, 2011). As a consequence, tolerance of osmotic stress is one of the main constraints to colonisation and survival in such ecosystems. Despite these challenges, specialisation in saline waters has occurred in many primarily freshwater lineages (Bradley *et al.*, 2009; Albers & Bradley, 2011; Arribas *et al.*, 2014), which offer an ideal comparative framework within which to study the physiological traits of related species adapted to different salinity optima.

In aquatic insects, osmotic patterns have been well documented in a range of larval Diptera (reviewed in Bradley, 1987). However, in other orders such as Odonata, Hemiptera and Coleoptera, osmotic and ionic regulation patterns are much less well-known; most studies to date focusing on single, unrelated species or only on larval stages (e.g. Treherne, 1954; Nemenz, 1969; Tones & Hammer, 1975; Tones, 1977; Nicholls, 1983; Frisbie & Dunson, 1988a,b). The osmotic responses of aquatic organisms generally appear to correlate well with the salinity range occupied in nature. Strictly freshwater forms can hyperregulate in dilute waters, but die when the external osmotic concentration reaches or exceeds that of their haemolymph (e.g. Wigglesworth, 1938; Patrick & Bradley, 2000), whilst in salinity-tolerant taxa two patterns have been found. Some species osmoregulate at concentrations below the isosmotic point and osmoconform at higher concentrations (e.g. Nicholls, 1983; Garret & Bradley, 1984, 1987; Patrick & Bradley, 2000; Havird *et al.*, 2014). In nature these species are generally limited to external ion concentrations no greater than those found in sea water (about 1000 mOsmol kg^{-1}). In contrast, it is thought that all species that show tolerance to osmotic concentrations above 1000 mOsmol kg^{-1} are efficient osmoregulators (e.g. Nayar & Sauerman, 1974; Bradley & Phillips, 1975; Neuman, 1976; Kokkinn, 1986; Herbst *et al.*, 1988; Herbst, 1999; Albers & Bradley, 2011). Osmoregulation therefore seems to be the most recurrent adaptation in aquatic insects inhabiting highly saline media, where the additional energetic costs required by osmoregulatory mechanisms may be compensated by the competitive release afforded by these habitats (Herbst, 2001). However, to date, this apparent association between osmotic capacity and species salinity ranges has never been explored from a comparative perspective within clades of closely related species whose members occupy different parts of the salinity gradient.

Coleoptera is one of the most specious insect orders in inland waters, including saline habitats, having colonized water at least 20 times from separate terrestrial ancestors (Jäch & Balke, 2008). Recent molecular phylogenetic analyses suggest that the aquatic Adephaga (which includes the familiar diving beetles and whirligigs) have entered the aquatic environment only once (Shull *et al.*, 2001; Hunt *et al.*, 2007; Jäch & Balke, 2008), whilst in some polyphagan families, as hydrophilids, multiple transitions from the terrestrial to the aquatic environment and back again have occurred (Bernhard *et al.*, 2006; Short & Fikáček, 2006; Hunt *et al.*, 2007). As well as these shifts between media, the evolutionary history of beetles includes multiple independent transitions from freshwater to saline habitats (e.g. Arribas *et al*., 2014) in which the evolution of specialized mechanisms to deal with salinity must have been crucially important. A number of 'true water beetle' genera (sensu Jäch & Balke, 2008; i.e. with both larvae and adults strictly aquatic) have occupied the full salinity gradient, including closely related species with contrasted habitat preferences, i.e. from strictly freshwater species to hypersaline specialists that are able to survive at salinity levels too toxic for any aquatic vertebrate (Millán *et al.*, 2011). Such taxa are therefore ideal models with which to explore the evolution and physiological diversity of osmotic stress mechanisms and their relation to habitat occupation. However, within Coleoptera, a number of studies have explored osmoregulation in terrestrial species in relation to desiccation (e.g. Coutchie & Crowe, 1979; Nicolson, 1980; Riddle, 1986; Naidu, 2001, 2006), but information on the osmotic mechanisms of aquatic beetles is almost entirely lacking, with only a few aquatic species having been studied, e.g. the freshwater *Dytiscus verticalis* (Frisbie & Dunson, 1988a,b), larvae of freshwater *Elodes* (Treherne, 1954) and a handful of saline water species such as *Berosus spinosus* (Nemenz, 1969) and *Hygrotus salinarius* (Tones, 1977). A better understanding of the osmotic strategies of different lineages of water beetles should provide insights into the evolutionary processes of physiological adaptation to saline waters. In addition, information on species responses to osmotic stress may assist in the assessment of the potential for communities to deal with environmental change (Hofmann & Todgham, 2010; Somero, 2011). In particular, studies on osmoregulation are important in the context of increasing aridity and salinization of inland waters, which may result in severe biodiversity losses (Kefford *et al.*, 2004; Cañedo-Argüelles *et al.*, 2013), especially in regions which already experience dry and Mediterranean climates (Sala *et al.*, 2000).

Here we explore osmotic responses and survival to acute salinity exposure in adults of 8 water beetle species belonging to the genera *Nebrioporus* (Adephaga: Dytiscidae) and *Enochrus* (Polyphaga: Hydrophilidae). Within each lineage, we study species inhabiting the different parts of the fresh – hypersaline gradient (see Table 1). Our aims were to: 1) identify and describe species osmoregulatory strategies (i.e. osmoconformity or osmoregulation) to determine whether the same mechanisms of dealing with salinity have evolved in these two genera which have independently colonized inland saline waters from freshwater ancestors and 2) compare species osmoregulatory strategies and osmotic capacities (i.e. the osmotic gradient between the animal’s internal medium and the external medium), checking for correlation with species salinity preferences in nature and for differences between co-habiting species of the two lineages.

MATERIAL AND METHODS

Studied species

The Mediterranean basin hosts a wide variety of inland aquatic habitats, covering the full salinity range, from freshwater to hypersaline water bodies (Montes & Martino, 1987; Arribas *et al.*, 2009; Millán *et al.*, 2011). *Enochrus* (Polyphaga: Hydrophilidae) and *Nebrioporus* (Adephaga: Dytiscidae) are amongst the most common and representative genera found in water bodies across the Mediterranean region (Millán *et al.*, 2006, 2011), including species occupying different parts of the salinity gradient, with both larval and adult stages being strictly aquatic.

Within each of these genera, we selected four species with different salinity occupancy ranges in the field (see Table 1), including species that commonly are found in freshwater (*N. bucheti cazorlensis* (Lagar, Fresneda and Hernando, 1987)), subsaline (*E. salomonis* (Sahlberg, 1900)), hyposaline (*N. clarkii* (Wollaston 1862) and *E. politus* (Küster, 1849)), mesosaline (*N. baeticus* (Schaum 1864) and *E. bicolor* (Fabricius, 1792)) and hypersaline waters (*N. ceresyi* (Aube 1838) and *E. jesusarribasi* Arribas and Millán, 2013).

Animal collection, maintenance and experimental design

Adults of each species were collected in different areas in Spain (Table 1), most of them located in public land not covered by any special legal protection. For those localities placed in protected areas, the collections were made with the corresponding permissions from the local authorities. None of the studied species is included in national or international lists of protected or endangered species. Specimens were maintained for one week in 7 L aquaria placed in an environmental chamber (SANYO MLR-351, Sanyo Electric Co., Ltd., Moriguchi City, Osaka, Japan) at 20ºC and 12:12 L:D cycle. Each species was maintained at their optimum salinity (see mean conductivity of habitat in Table 1), using water from collection sites. Food was provided daily (chironomid larvae for *Nebrioporus* species and macrophytes for *Enochrus*).

Table 1. Data the common habitats occupied by the studied species (from Biodiversity database of the Aquatic Ecology Research Group at the University of Murcia) and location of the sites where specimens were collected for the experiments.

	Occupied habitats			**Collection sites**		
Species	**Conductivity range** (mS cm^{-1})	**Mean conductivity** (mS cm^{-1})	**Habitat preference***	**Locality**	**Latitude**	**Longitude**
N. b. cazorlensis	0.15 - 0.61	0.40	Freshwater	Río Tus, Albacete	38.3707	-2.4459
N. clarkii	0.11 - 9.00	1.26	Subsaline-Hyposaline	Río Corneros, Murcia	37.7173	-1.9053
N. baeticus	4.10 - 160.00	36.65	Mesosaline	Río Chícamo, Murcia	38.2175	-1.0511
N. ceresyi	4.50 - 129.00	53.68	Mesosaline-Hypersaline	Laguna Cotorrillo, Murcia	37.8251	-0.7619
E. salomonis	0.70 - 2.16	1.23	Subsaline	Arroyos en Laguna de Pétrola, Albacete	38.8471	-1.5589
E. politus	1.50 - 133.40	19.32	Hyposaline	Río Chícamo, Murcia	38.2175	-1.0511
E. bicolor	2.10 - 86.00	34.96	Mesosaline	Laguna Mojón Blanco, Albacete	38.8002	-1.4301
E. jesusarribasi	14.90 - 160.00	62.14	Hypersaline	Rambla Salada, Murcia	38.1263	-1.1182

* Ranges of conductivity of each category (mS cm^{-1}): Freshwater: < 1, Subsaline: 1 - 10, Hyposaline: 10 - 30, Mesosaline: 30 - 60, Hypersaline: > 60 (Montes & Martino, 1987)

Groups of 15-25 animals were exposed for 48 h to different salt concentrations that include the range that each of the species commonly occupies, and lower and upper extremes, as follow: 1, 3, 5, 10, 20, 40 and 50 mS cm^{-1} for *N. b. cazorlensis* and *E. salomonis*; 1, 5, 20, 50, 75 and 100 mS cm^{-1} for *N. clarkii* and *E. politus*; 1, 20, 50, 100, 140 and 180 mS cm^{-1} for *N. baeticus, N. ceresyi, E. bicolor* and *E. jesusarribasi* (see equivalent osmolalities in Table S1). Pilot trials showed that haemolymph osmolality stabilized by 2 days after transfer, as has been previously shown in other studies (e.g. Havird *et al.*, 2014). Waters of different conductivity were prepared by dissolving an appropriate quantity of marine salt (Ocean Fish, Prodac, Cittadella, Padua, Italy) in distilled water. Experimental aquaria (1 L capacity) were filled with 400 mL of water at the test salinity and held in the environmental chamber at constant temperature (20ºC) and 12:12 L:D cycle. Food was not supplied during this period in order to avoid variation in dietary ion intake between the species. Each treatment was replicated three times for each species. Mortality was recorded after 48 h exposure and surviving animals used for haemolymph sampling.

Measurements of haemolymph osmolality

Haemolymph samples were obtained in those treatments with ≤ 50% mortality. Specimens were rinsed in distilled water, dried on blotting paper and placed between two parafilm layers under the binocular microscope. A puncture was made in the pronotum and the resulting haemolymph droplet immediately collected with a 2 µl micro-syringe (Hamilton Company, Reno, Nevada, USA), transferred to cooled eppendorf tubes filled with type B immersion oil (Cargille Laboratories, Cedar Grove, New Jersey, USA) to avoid sample evaporation and melanisation, and stored at -80ºC until osmolality measurements. Haemolymph samples from beetles of each treatment (i.e. 15-25 individuals) were pooled to produce the minimum volume of 2 µl required for osmolality measurements. The osmolality of the haemolymph was measured in a Wescor 5520 vapour pressure osmometer (Wescor Logan, Utah, USA) previously calibrated using Wescor standard solutions of 90, 290 and 1000 mOsmol kg^{-1}. A special sample holder disc was used following manufacturer instructions for small sample volumes (2 µl). Haemolymph was previously separated from the immersion oil by centrifugation in a Sprout mini-centrifugue (Heathrow Scientific LLC, Vernon Hills, Illinois, USA). Samples of 10 µl of the experimental solutions were also measured with the standard sample disc to obtain external media osmolalities. A calibration curve was made to extrapolate the osmolalities of the two highest conductivities (140 and 180 mS cm^{-1}), which exceeded the range of the osmometer. No permits or ethical approval were required for the experimental procedures.

Data analysis

The osmotic concentration of haemolymph was plotted against external medium osmolality and compared with the isosmotic line (slope = 1) to determine if each species was an osmoconformer or osmoregulator. We also used generalized linear models (GLM) to define the relationship between haemolymph and external media osmotic concentration, assuming a gaussian error distribution and an identity link function (Quinn & Keough, 2002). Osmolality would scale linearly with proportional salinity in the absence of osmoregulation, and deviation from this theoretical linearity reflects the degree of osmoregulation. Therefore, linear and quadratic relationships were tested and the model that best fitted our data was selected based on lower AIC and higher deviance.

Osmotic capacity (OC) is defined as the difference between the osmotic concentration of the body fluids and that of the external medium (Charmantier *et al.*, 1984). OC represents an integrated measure of an organism's physiological ability to compensate for the osmotic gradient that may occur between the internal and external environments (Lignot *et al.*, 2000; Calosi *et al.*, 2005) in both hyposmotic (hyper-OC, positive values) and hyperosmotic (hypo-OC, negative values) conditions. The magnitude of this osmotic gradient across the conductivity range tested, i.e. the absolute value of osmotic capacity, was compared between *Nebrioporus* and *Enochrus* species pairs with similar salinity preferences (Table 1); i.e., *N. b. cazorlensis − E. salomonis, N. clarkii − E. politus, N. baeticus − E. bicolor* and *N. ceresyi − E. jesusarribasi*. For this, we employed two-way ANOVA with OC as the dependent variable and external medium osmolality, species and the interaction of both as factors. When the interaction of species x medium osmolality was significant, Tukey HSD post-hoc tests were used to identify the specific treatments in which OC differed amongst species. All analyses were performed with R v. 3.0.1 (R Development Core team, 2011).

Salinity tolerance limits of the species for 48 h exposure were estimated as the LC_{50} (the osmotic concentration which resulted in the death of 50% of individuals), using Trimmed Spearman–Karber analysis (USEPA TSK Programme Version 1.5).

RESULTS

Pattern of osmotic regulation

All studied species showed a capacity to hyperegulate in hyposmotic media (from 30 to 340 mOsmol kg^{-1}), maintaining haemolymph osmotic concentration within a range of 280 - 440 mOsmol kg^{-1} (Fig. 1). The primarily freshwater *Nebrioporus b. cazorlensis*, *N. clarkii* and *E. salomonis* were unable to hyporegulate in media that reach or exceed their haemolymph osmotic concentration (i.e. over 340 mOsmol kg^{-1}), whilst the remaining saline water species (*N. baeticus*, *N. ceresyi*, *E. politus*, *E. bicolor* and *E. jesusarribasi*) were effective hyporegulators in hyperosmotic media. In these species, haemolymph concentration values ranged from 250 to 670 mOsmol kg^{-1}, across a range of external osmolalities close to lethal levels (Table 2), i.e. until 1580 mOsmol kg^{-1} in *E. politus,* 2470 mOsmol kg^{-1} in *N. baeticus* and *E. bicolor,* 3550 mOsmol kg^{-1} in *N. ceresyi* and 4280 mOsmol kg^{-1} in *E. jesusarribasi* (Fig. 1).

Positive and/or negative deviations of haemolymph osmotic concentration from the isosmotic line representing the theoretical osmolalitity of a strict osmoconformer (slope=1), reflect the degree of hyper- and hyporegulation of the different species (Fig. 1). *Nebrioporus b. cazorlensis* and *N. clarkii* had the lowest hyperegulation capacity, showing a gradual linear increase of haemolymph osmotic concentration as external medium concentration increased. In these species, the isosmotic point between haemolymph and the external medium was not reached at any of the salt concentrations tested. *Enochrus salomonis* maintained almost constant haemolymph concentration below the isosmotic point and reached this at around 300 mOsmol kg^{-1}, being unable to hyporegulate above this concentration. *Enochrus politus* showed a slight linear increase of haemolymph osmolality across the experimental conductivity range, but it remained both hyper and hyposmotic to the external media. Mesosaline and hypersaline species in both genera showed the strongest deviation (mainly downside) from the isosmotic line, and the relationship between haemolymph and external osmolality was non-linear (see the fitted models in Fig. 1) reflecting their high osmoregulatory potential. In *N.*

baeticus, haemolymph osmolality increased nonlinearly across the conductivity gradient (Fig. 1), whilst in *E. bicolor* it was maintained within a narrow range (255 - 336 mOsmol kg^{-1}). In the hypersaline *N. ceresyi* and *E. jesusarribasi*, haemolymph osmolality increased more markedly at the highest salinities, but in any case, hyporegulation capacity was detected until the most extreme salt concentrations tested (3550 and 4280 mOsmol kg^{-1}, respectively).

Osmotic capacity

There were significant differences in osmotic capacity (OC) between the four species pairs compared, except in the case of hyposaline species (Table 3). There was also a significant species x external medium osmolality interaction, showing that species differed in their specific response patterns of OC across the range of osmotic concentrations tested.

Species occupying fresh-subsaline waters (*N. b. cazorlensis* and *E. salomonis*), showed similar salinity tolerances (see LC_{50} values in Table 2) and similar mean hyper-OCs in media up to 90 mOsmol kg^{-1}. This was followed by a significant reduction in OC as haemolymph osmolality was closer to the isosmotic point with the external medium, at 180 and 340 mOsmol kg^{-1}. OC was significantly lower in *E. salomonis* at these osmotic concentrations ($P < 0.01$) (Fig. 2a).

In the hyposaline species (*N. clarkii* and *E. politus*), hyper-OC showed the same decreasing tendency across an osmolality range of 30-340 mOsmol kg^{-1}, which was within the tolerance limits of both species (Table 2). The main differences between these species were due to the fact that *E. politus* was also able to osmoregulate in hyperosmotic conditions, i.e. at 1000 and 1580 mOsmol kg^{-1}, which were over the lethal limit of *N. clarkii* (Fig. 2b, Table 2).

Mesosaline species displayed similar mean values of hyper-OC at 30 and 340 mOsmol kg^{-1}. From this concentration, hypo-OC increased with increasing osmotic stress in both species, and was significantly higher in *E. bicolor* than *N. ceresyi* at 2470 mOsmol kg^{-1} (Fig. 2c).

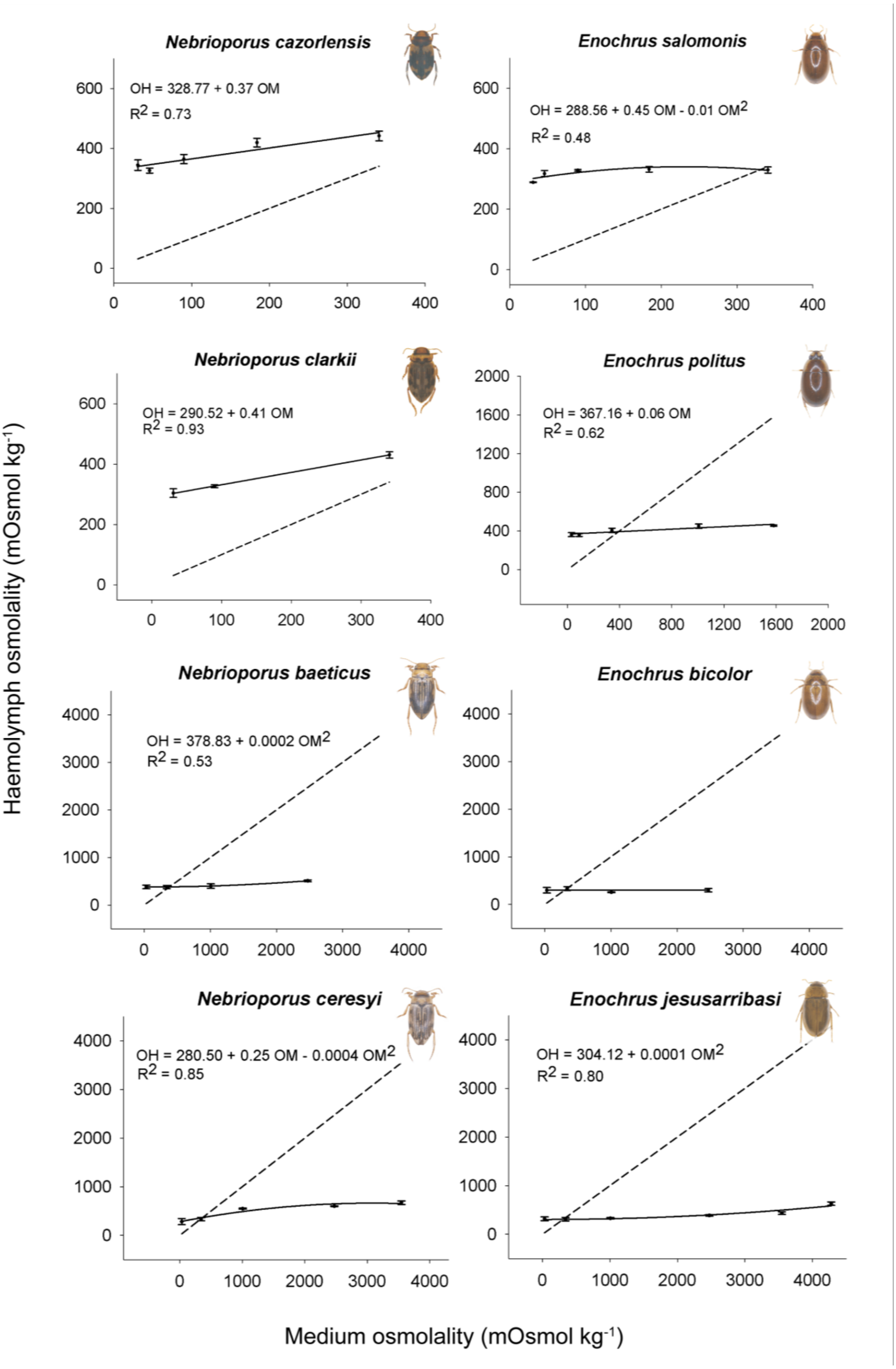


Figure 1. Relationship between osmotic concentration of the haemolymph and the external medium. Data are expressed as mean ± s.e. (n=3). The isosmotic line is represented by the discontinuous line. OM: osmolality of external medium, OH: osmolality of haemolymph.

Table 2. LC_{50} values (mosmol kg^{-1}) and 95% confidence intervals estimated by the Trimmed Spearman–Karber analysis.

Species	**LC50 (95%CI)**
N. b. cazorlensis	682.68 (610.83 − 762.02)
N. clarkii	557.12 (479.47 − 644.94)
N. baeticus	2738.20 (2643.12 − 2836.65)
N. ceresyi	4190.87 (3884.61 − 4521.32)
E. salomonis	841.37*
E. politus	2249.05 (2109.71− 2400.25)
E. bicolor	3076.87 (2711.59 − 3489.81)
E. jesusarribasi	> 4280 **

* 95% confidence interval was not reliable
** mortality was lower than 50% in all tested conductivities

Hypersaline species showed an identical pattern of hyper-OC to mesosaline taxa in media below the isosmotic point. Above this osmolality, hypo-OC progressively increased, being significantly higher in *E. jesusarribasi* than in *N. ceresyi* across all hyperosmotic treatments (Fig. 2d). In addition, this species could still osmoregulate at the highest experimental concentration (4280 mOsmol kg^{-1}), which was above the lethal limit for *N. ceresyi* (Table 2).

DISCUSSION

We have, for the first time, characterised the osmoregulatory strategies of adult aquatic beetles with different salinity tolerances in nature. Our study shows that species from two genera that have independently invaded saline waters are able to osmoregulate in chloride-rich waters, with no osmoconformity being observed in any of the species studied.

In media below 340 mOsmol kg^{-1} (i.e. 20 mS cm^{-1}), all species showed a similar pattern of hyperegulation, maintaining haemolymph concentration within a narrow

Table 3. Effect of osmolality of external medium (OM), species (Sp) and their interaction on osmotic capacity (OC).

Source	df	SS	F-value	P - value
Subsaline species (*N. b. cazorlensis*, *E. salomonis*)				
OM	4	215826	131434	< 0.001
Sp	1	22792	55519	< 0.001
Sp*OM	4	7080	4312	0.011
Residual	20	8210		
Hyposaline species (*N. clarkii*, *E. politus*)				
OM	2	166533	106368	< 0.001
Sp	1	2225	2842	0.118
Sp*OM	2	5272	3368	0.069
Residual	12	9394		
Mesosaline species (*N. baeticus*, *E. bicolor*)				
OM	3	14582203	1290.717	< 0.001
Sp	1	29470	7.825	0.013
Sp*OM	3	78381	6.938	< 0.001
Residual	16	60255		
Hypersaline species (*N. ceresyi*, *E. jesusarribasi*)				
OM	4	38580493	3262.603	< 0.001
Sp	1	149390	50.533	< 0.001
Sp*OM	4	79415	6.716	0.001
Residual	20	59125		

range close to the typical osmolality of insect haemolymph (300 mOsmol kg^{-1}) (Chown & Nicolson, 2004). Hyperegulation is a universal adaptation for life in freshwater (Bradley *et al.*, 2009), and involves the production of dilute urine to compensate for water that enters the body osmotically coupled with the replacement of lost salts by dietary intake (Frisbie & Dunson, 1988c) and active ion uptake (Bradley 1987, 2009). In most insects, Malphigian tubules and the rectum are responsible for urine formation (Pannabecker, 1995; O'Donnell, 1997), and some species possess anal papillae for active ion uptake from the external environment (e.g. Treherne, 1954; Wichard & Komnick, 1974; Donini *et al.*, 2005; Patrick *et al.*, 2006; Larsen *et al.*, 2014).

In terms of the ecological implications of the osmotic patterns found here in hyposmotic media, the high survival and osmoregulatory capacity of the studied saline species in these conditions demonstrate that, at least during the adult stage, they can potentially survive in freshwaters, despite rarely being found in fresh or low conductivity habitats in nature. This is in agreement with another recent study on saline

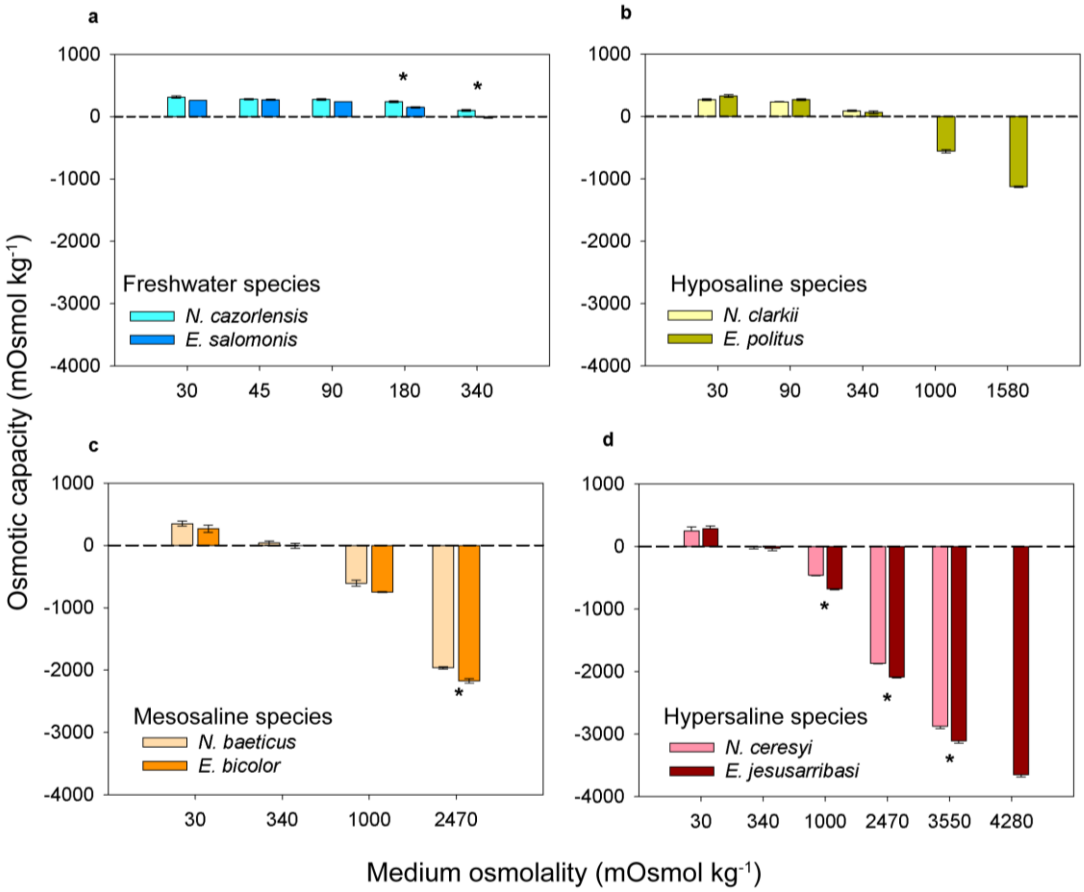


Figure 2. Osmotic capacities of *Nebrioporus* and *Enochrus* species pairs. Data are expressed as mean ± s.e. (n=3). Asterisks indicate statistically significant differences between species ($P \leq 0.01$) at each conductivity treatment. OC: osmotic capacity.

beetle species that tolerate a wide range of salinities (including freshwater) under experimental conditions, but are restricted to waters with salinities close to their upper tolerance limits in nature (Céspedes *et al.*, 2013), something also observed in saline water corixids (e.g. Tones & Hammer, 1975; Carbonell *et al.*, 2012). Therefore, restriction to saline habitats may be driven by other factors such as interspecific competition and/or larval requirements (see below).

In media above the isosmotic point, only the hyposaline *E. politus* and the meso and hypersaline species studied were able to regulate their haemolymph osmotic concentrations. Hyporegulation capacity has previously been reported in other water beetles, as adults of the dytiscid *Hygrotus salinarius* (Tones, 1977), and larvae of the hydrophilid *Berosus spinosus* (Nemenz, 1969). However, the very wide osmotic gradients that the hypersaline species here studied were able to maintain have never been demonstrated in any beetle species before. For example, the Iberian endemic species *E. jesusarribasi* was able to maintain its haemolymph at approximately 3500 mOsmol kg^{-1} below that of the media, displaying a hyporegulation capacity comparable to those reported for some of the most effective insect osmoregulators known to date, such as the larvae of *Ephydra* brine flies (Herbst *et al.*, 1988; Herbst, 1999), the dolichopodid *Hydrophorus plumbeus* (Herbst & Bradley, 1988), the soldier fly *Odontomyia cincta* (Stratiomyidae) (Gainey, 1984) or larvae and adults of some species of corixid bugs as *Trichocorixa verticalis interiores* (Tones & Hammer, 1975) or *Sigara stagnalis* (Scudder, 1976).

A diversity of mechanisms could be behind the extraordinary hyporegulation capacity showed by the species studied here. In general, insect adaptations to live in saline waters are designed to a) limit the entry of ions into the body and the loss of water to the external medium by osmosis, and b) actively excrete excess ions and retain water via specialized organs and tissues. The cuticle of insects represents a relatively impermeable boundary with their environment, with epicuticular lipids, and especially hydrocarbons, serving as a barrier to water loss and a waterproofing layer. However, aquatic insects seem to be in general more permeable to water than their terrestrial counterparts (Holdgate, 1956; Beament, 1961; Armold *et al.*, 1969). Data on epicuticular hydrocarbons are available for a few freshwater beetle species (Jacob & Hansen, 1986; Alaire *et al.*, 1998), but nothing is known to date regarding these in saline aquatic taxa. Despite the adaptations to minimize fluxes of water through the body wall, ion entry by drinking the external medium and feeding on food rich in salts (aquatic macrophytes and biofilms in *Enochrus* species and macroinvertebrates in *Nebrioporus*) is likely to represent an important salt input for the saline species studied here. Since access to freshwater or to food with low osmotic concentration is not available in inland saline habitats, regulating drinking rates could be a complementary behavioural adjustment to minimize ionic input (Bradley & Phillips, 1977a; Patrick & Bradley, 2000). Such adaptations, coupled with mechanisms for active ion excretion and water conservation in specialized excretory organs likely account for the osmoregulatory capacities observed here. Insect excretory adaptations typically involve the Malpighian tubules for primary urine formation and the hindgut (the rectum in particular) as the major site of water conservation (Bradley, 2002; Bradley *et al.*, 2009; Albers & Bradley, 2011). Osmotic and water homeostasis are often under complex hormonal control (e.g. oxytocin- and vasopressin-like peptides have been related to osmoregulatory functions in several invertebrate species (Aikins *et al.*, 2008; Stafflinger *et al.*, 2008; Gruber, 2014) and the specific mechanisms at morphological, biochemical and cellular levels are widely diverse between different insect groups (see Larsen *et al.* (2014) for an extensively review) or even between related taxa with different ecological requirements. For example, some saline-tolerant dipteran larvae possess a two-part rectum, with the posterior segment serving as a salt gland (Bradley & Phillips, 1977b; Bradley, 2002), whilst closely related freshwater species do not show such morphological differentiation (Albers & Bradley, 2011). The osmotic regulation patterns reported here provide an ideal basis for further comparative studies on the specific osmoregulatory mechanisms in saline and freshwater beetles, which are so far unknown for this group of aquatic insects.

We found that species in both genera show parallel osmotic strategies in relation to the salinity ranges they occupy in nature, i.e. the species living in fresh-subsaline waters possess hyperegulation but not hyporegulation capacity, whilst species found in more highly saline waters are euryhaline osmoregulators. Osmotic capacities were similar between species of the two lineages occupying habitat with similar salinity, differing only significantly at the most elevated osmotic stress levels. Likewise, individual species were able to osmoregulate within a specific range of osmotic concentrations, which correlate with the upper salinity levels they commonly occupy in nature. This is clear, for example, in *N. clarkii* and *E. politus*. Although both species can be found in hyposaline waters, *N. clarkii* occupies a narrower range of salinities (Table 1) and accordingly its osmotic response and LC_{50} were similar to that of the freshwater-subsaline species, whilst *E. politus*, which lives within a broader salinity range, showed a similar hyper- and hyporegulation pattern to the mesosaline species *E. bicolor* and *N. baeticus*. These results together sustain the idea that within each genus, the differing osmotic capacities of the species may mediate their differential tolerances to salinity and consequently their habitat segregation across the salinity gradient (Arribas *et al.*, 2009; Céspedes *et al.*, 2013; Gutiérrez-Cánovas *et al.*, 2013).

On the other hand, despite the general concordance between field salinity and osmotic response ranges observed, our experimental data show that the saline-tolerant species studied could osmoregulate and survive at salinities that exceed both the upper and lower limits they commonly occupy in nature. The balance between the metabolic costs of osmoregulation and interspecific competition may play an important role in constraining habitat occupancy in saline waters. The osmotic stress posed by inland saline environments limits the number of species that are able to colonize them, resulting in a significant reduction in interspecific interactions, such as competition or predation in such habitats (Scudder, 1983; Herbst, 2001). The high energy demands required for homeostatic adjustment in the face of osmotic stress may result in trade-offs with other biological traits, resulting in a negative correlation between tolerance to salt and competitive ability (Bradley, 1994; Herbst, 2001). This may at least partly explain the absence of euryhaline hyper- and hyporegulator species (e.g. *N. baeticus*, *N. ceresyi*, *E. bicolor* and *E. jesusarribasi*) in physiologically suitable habitats with higher species richness, such as freshwaters. In addition, however, our experiments may overestimate the true osmotic capacity of these species at salinities exceeding their natural ranges, since their regulatory mechanisms might not be maintained in the long term at these conditions (Chown & Nicolson, 2004). Also, although larval and adult stages are truly aquatic (Jäch & Balke, 2008) and apparently coexist during their entire life at similar salinities in nature, experimental data on salinity tolerance of larvae are lacking and it is unknown if their osmotic capacities differ to that found in adults (e.g. Tones, 1977). This could also be behind the absence of saline water species in freshwater habitats. Further studies on the osmoregulatory mechanisms and capacities of larvae would be welcome, as they may aid our understanding of the ecological and evolutionary implications of salinity tolerance in water beetles. Unfortunately, such studies are hampered by a number of factors, including short larval lifespan in most species, and the taxonomic intractability of the majority of relevant larval stages.

Our understanding of the evolutionary history of colonisation of saline waters by beetles is limited, but it is clear that salinity tolerance has arisen independently in a number of different aquatic lineages; for example, independent and direct transitions from freshwater to saline habitats have been reported in *Enochrus* species of the subgenus *Lumetus* (Arribas *et al.*, 2014). Our results and previous work on osmoregulation in beetles (Treherne, 1954; Nemenz, 1969; Tones, 1977) suggest that hyporegulation capacity has arisen in independent lineages to deal with salinity. Salt tolerance has also apparently arisen independently in larvae of many genera of mosquitoes (Bradley, 1987, 1994, 2008), but in this case instead a diversity of osmotic strategies has evolved: from strictly freshwater hyperegulators, to osmoconformers and true euryhaline osmoregulators (Herbst & Bradley, 1988). To date, the osmoconformist strategy seems to be absent amongst aquatic beetles, and generally in those lineages of insects that have successfully colonized highly saline waters (Albers & Bradley, 2011). Therefore, despite the fact that osmoconformity is less energetically costly (Edwards, 1982; Bradley, 2009) and the most common osmotic strategy amongst marine invertebrates, osmoregulation appears as the most effective and successful adaptation to osmotic stress in insects in inland waters.

In a recent study on the evolution of salinity tolerance in *Enochrus*, Arribas *et al*., (2014) found evidence of multiple direct transitions to saline waters, apparently associated with periods of global aridification, as well as strong concordance between the position of species on habitat salinity and aridity gradients. The authors therefore hypothesised that the mechanisms behind salinity and desiccation tolerance might have co-evolved in this lineage. Our discovery of a generalised osmoregulation strategy in saline water beetles is consistent with the idea of correlated evolution of such tolerances, since the physiological basis of osmoregulation has multiple commonalities with mechanisms underlying desiccation resistance (Beadle, 1943; Cloudsley-Thompson, 1975; Gibbs *et al.*, 1997; Gómez-Mestre & Tejedo, 2005). In fact, examples of the correlation between good osmoregulatory ability and tolerance to arid conditions are abundant amongst a variety of terrestrial xeric beetles, e.g. desert tenebrionids (Broza *et al.*, 1976; Riddle *et al.*, 1976; Nicolson, 1980; Riddle, 1986) or the meloid *Cysteodemus armatus* (Cohen *et al.*, 1986). In the case of aquatic Coleoptera, the development of drought tolerance in lineages subjected to strong seasonal aridity may, therefore, have provided the genetic and physiological basis behind hyporegulation capacity, making colonisation and diversification in saline waters possible.

In conclusion, our findings suggest that osmoregulation could be a generalized strategy to deal with osmotic stress among adult aquatic beetles, and reveal that the evolution of enhanced hyporegulation capacities might have played a key role in the colonisation of saline waters by some lineages.

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SUPPORTING INFORMATION

Table S1. Equivalent osmolalities of the experimental conductivities.

Conductivity (mS cm^{-1})	Osmolality (mOsmol kg^{-1})
1	30
3	45
5	90
10	180
20	340
40	780
50	1000
75	1580
100	2470
140	3550
180	4280

CHAPTER 2

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

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 the 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 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 in order 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 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, since 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 aquatic species (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 acute 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 in adult beetles that 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, 2013), 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. In particular, we aimed to address the following questions: i) Does the combination of high temperature and salinity have a negative synergistic effect on species performance (i.e. survival and behavioural avoidance)? ii) Are flight and emersion responses good indicators of sublethal stress limits? iii) Do lotic species have lower physiological amplitude than lentic ones (i.e. lower stress thresholds for survival and avoidance activity).

MATERIALS 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. jesusarribasi*[1] Arribas & Millán, 2013; family Hydrophilidae) and *Ochthebius* (*O. notabilis* Rosenhauer, 1856 and *O. glaber* Montes

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[1] Referred to as *E. falcarius* Hebauer, 1991 in the publication derived from this chapter (Pallarés *et al.* Ecol. Entomol. 37, 508–520 (2012))

& 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 south eastern Spain, and is found in lotic meso and 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. jesusarribasi,* has a narrower distribution and occupies saline streams in the southern of Iberian Peninsula (Arribas *et al.*, 2013; Millán *et al.*, 2014). *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). Approximately 400 individuals of each *Enochrus* and *Nebrioporus* species and 600 of *Ochthebius* were collected from different areas (one locality per species) in south eastern Spain (see Table 1 for collection locations). Specimens were maintained under laboratory conditions for one 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 (20ºC), 12:12 light:dark cycle and light intensity of 15 μmol.m-2.s-1.

Finally, ten 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, 35ºC) to extreme temperatures (40, 45ºC) that are close to the sublethal and upper lethal limits (UTLs) 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 (SE Spain), the observed daily water temperature amplitude could commonly reach 10-12ºC and up to 18ºC, and water temperatures of 35ºC and high rates of heating are frequent during the summer (J. Velasco, unpublished data).

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. ±1ºC). 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 two minutes in order to determine the frequency of emersion response. Specimens that flew or were dead at each interval of two minutes were counted and removed. However, due to the small size of individuals from the *Ochthebius* species, it was impossible to determine the exact time of the specimens' death, 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 minutes 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 that were present in the aquaria at the moment of recording (i.e. each two minutes). For *Ochthebius* species, since 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.

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

Species	Habitat type	Conductivity range (mS cm^{-1})	Sample location	Latitude	Longitude	Locality mean conductivity (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

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 GLM 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 amongst treatments (Underwood, 1997; Rutherford, 2001). All statistical analyses were conducted using SPSS for Windows, Rel. 15.0.1. 2006. Chicago: SPSS Inc.

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, 4 and 5). The interaction of temperature x 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 x Species interaction in Table 3). *Enochrus bicolor* and *E. jesusarribasi* displayed high survival at all temperature levels tested, except at 45 ºC, where most individuals died after 30 minutes of exposure (Fig. 1a).

Similarly, for both *Nebrioporus* species survival decreased with increasing temperature, and most individuals died at 45ºC (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 40ºC ($P < 0.001$ in post hoc test for species difference at 40 °C; Fig. 1b).

Ochthebius species displayed varying tolerances to both stressors (Temperature x Conductivity x 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 the higher temperatures (40-45ºC), 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 40ºC; only 45ºC 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).

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

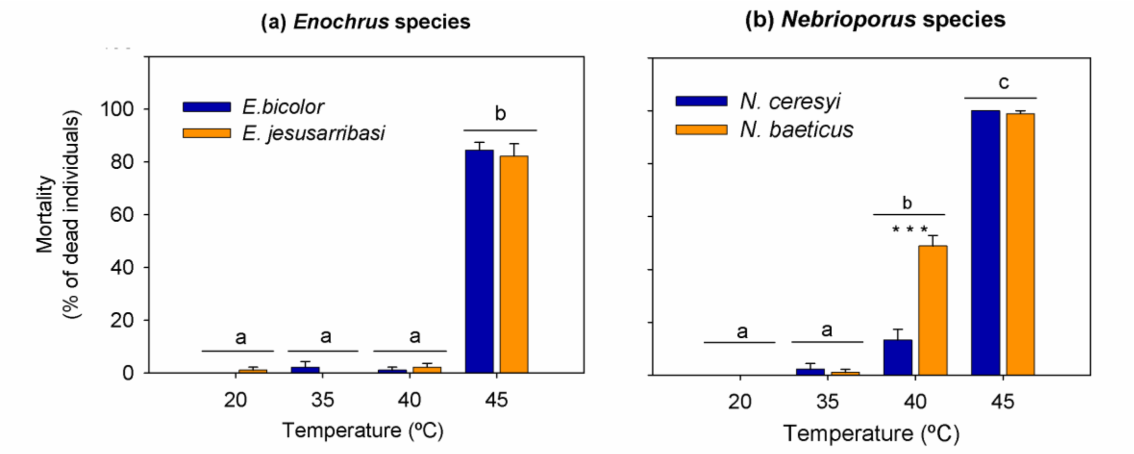
Effect	**Pillai's trace**	**F**	**d.f**	**P - value**
Enochrus				
Temperature	1.597	18.223	9	< 0.001
Conductivity	0.065	0.523	6	0.789
Species	0.361	8.654	3	< 0.001
Temperature x Conductivity	0.312	0.929	18	0.545
Temperature x Species	0.377	2.302	9	0.019
Conductivity x Species	0.182	1.572	6	0.164
Temperature x Conductivity x 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 x Conductivity	0.199	0.568	18	0.918
Temperature x Species	0.622	4.185	9	< 0.001
Conductivity x Species	0.008	0.060	6	0.999
Temperature x Conductivity x 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 x Conductivity	0.895	2.835	36	< 0.001
Temperature x Species	0.604	6.718	9	< 0.001
Conductivity x Species	0.527	4.259	12	< 0.001
Temperature x Conductivity x Species	0.796	2.408	36	< 0.001

d.f., degrees of freedom

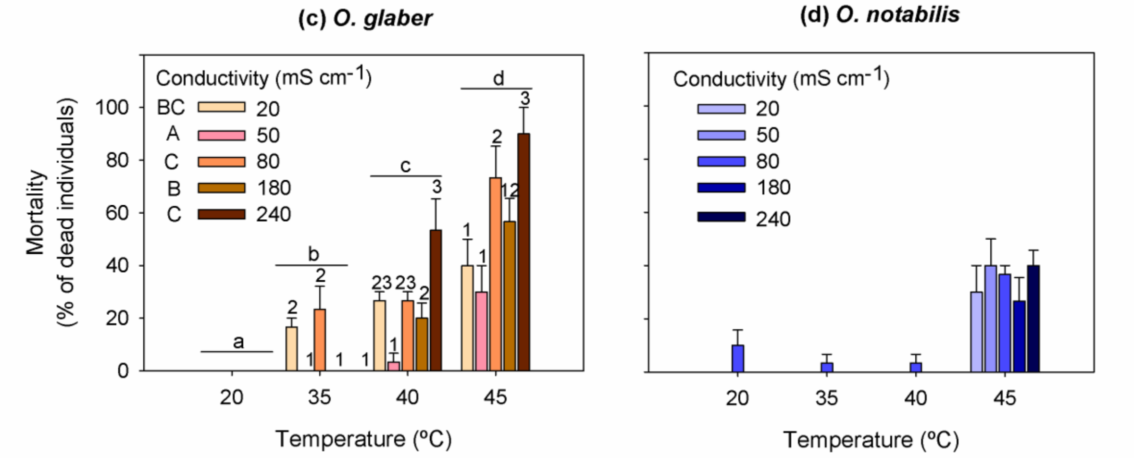
Table 3. Effect of temperature and conductivity on mortality for *Enochrus*, *Nebrioporus* and *Ochthebius* species.

Effect	SS	d.f.	F	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 x Conductivity	0.183	6	1.539	0.186
Temperature x Species	0.023	3	0.389	0.761
Conductivity x Species	0.014	2	0.350	0.706
Temperature x Conductivity x Species	0.046	6	0.387	0.884
Error	0.949	48		
Nebrioporus				
Full model	32189.498	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 x Conductivity	0.013	6	0.122	0.993
Temperature x Species	0.807	3	15.577	< 0.001
Conductivity x Species	0.000	2	0.007	0.993
Temperature x Conductivity x 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 x Conductivity	0.874	12	4.432	< 0.001
Temperature x Species	1.084	3	21.982	< 0.001
Conductivity x Species	0.466	4	7.082	< 0.001
Temperature x Conductivity x Species	0.858	12	4.351	< 0.001
Error	1.315	80		

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



(a) ***Enochrus*** **species** (b) ***Nebrioporus*** **species**



(c) ***O. glaber*** (d) ***O. notabilis***

Figure 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. Blue colors for lentic species, orange colors for lotic ones.

Sublethal behavioural responses

Emersion. The lentic species *E. bicolor* emerged more frequently than the lotic *E. jesusarribasi* (see Species in Table 4). However, *Enochrus* species showed no significant differences in emersion activity pattern across temperature treatments (Temperature x Species in Table 4), i.e. emersion increased from 20 to 40ºC, when the maximum emersion activity was displayed 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 x Species in Table 4). Thus, 40ºC 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* species (Species in Table 4) and a similar response pattern across temperature and conductivity treatments was displayed by both species (Temperature x Conductivity x Species in Table 4). The number of emersions increased with increasing temperature, reaching the maximum response at 45ºC (Fig. 2c,d), and decreased significantly at high conductivities. The combination of the highest temperatures (40-45ºC) and conductivity (240 mS cm^{-1}) caused a significant reduction in emersion response (Conductivity x Temperature in Table 4, Fig. 2c,d).

Flight. Between *Enochru*s species, the lentic *E. bicolor* showed a more intense 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 x 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 35º- 40ºC and minimum response was shown at 20 and 45ºC (Temperature and Species x 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 x Conductivity x Species in Table 5). Flight activity increased with increasing heat stress in both species but *O. glaber* reached the maximum response at 45ºC and *O. notabilis* at 40ºC (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 x conductivity interaction differed between species (Table 5). At the most extreme temperatures (40-45ºC), the lotic species *O. glaber* showed a significant decrease in flight response at 240 ms cm^{-1} (Fig. 3c). In contrast, at the highest temperatures (40 and

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

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

Effect	**SS**	**d.f.**	**F**	**P - value**
Enochrus				
Full model	37775.999	23	4.504	< 0.001
Intercept	104761.476	1	287.253	< 0.001
Temperature	30534.568	3	27.908	< 0.001
Conductivity	313.644	2	0.430	0.653
Species	2702.195	1	7.409	0.009
Temperature x Conductivity	810.362	6	0.370	0.894
Temperature x Species	2429.023	3	2.220	0.098
Conductivity x Species	809.732	2	1.110	0.338
Temperature x Conductivity x Species	176.76	6	0.081	0.998
Error	17505.627	48		
Nebrioporus				
Full model	32189.498	23	15.393	< 0.001
Intercept	21606.420	1	237.638	< 0.001
Temperature	31030.047	3	113.761	< 0.001
Conductivity	63.235	2	0.348	0.708
Species	108.586	1	1.194	0.280
Temperature x Conductivity	456.951	6	0.838	0.547
Temperature x Species	497.775	3	1.825	0.155
Conductivity x Species	2.028	2	0.011	0.989
Temperature x Conductivity x Species	30.876	6	0.057	0.999
Error	4364.243	48		
Ochthebius				
Full model	51983.679	39	6.748	< 0.001
Intercept	67635.235	1	342.389	< 0.001
Temperature	32140.818	3	54.235	< 0.001
Conductivity	6787.594	4	8.590	< 0.001
Species	1306.765	1	6.615	0.012
Temperature x Conductivity	7640.230	12	3.223	0.001
Temperature x Species	348.966	3	0.589	0.624
Conductivity x Species	1171.376	4	1.482	0.215
Temperature x Conductivity x Species	2587.929	12	1.092	0.379
Error	15803.137	80		

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

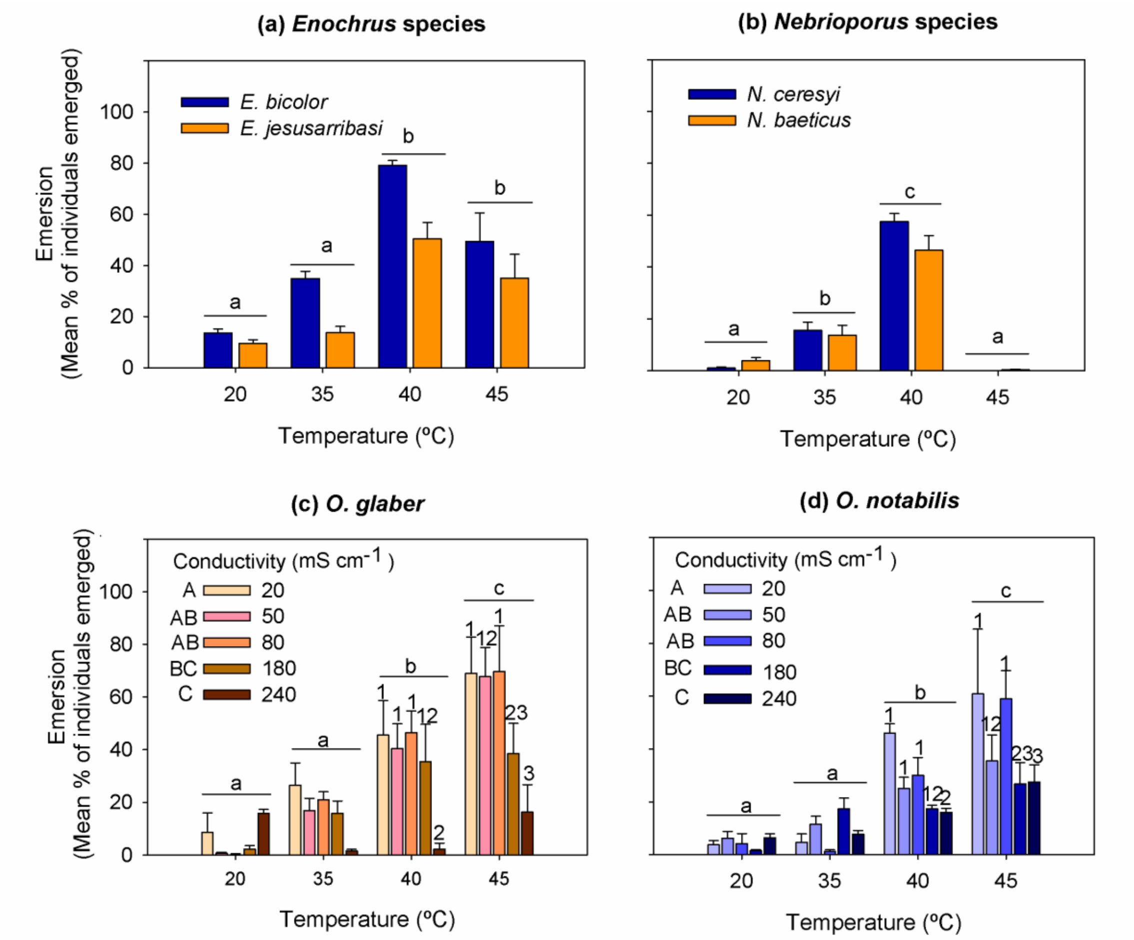


Figure 2. Mean ± SE emersion of each species. Significant differences determined by post-hoc analysis employing Bonferroni correction are indicated as follows: by capital letters in the legend for conductivity levels, by lower case above the bars for temperature levels, by numbers above the bars for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

DISCUSION

Does the combination of high temperature and salinity has a negative synergistic effect on species performance?

In our acute stress experiments, salinity and its interaction with temperature had different effects across the three genera studied. For the *Ochthebius* species, the combination of high conductivities and extreme temperatures had a synergistic effect, reducing emersion response of both species, and also 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.

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

Effect	SS	d.f.	F	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 x Conductivity	8.524	6	0.419	0.862
Temperature x Species	23.795	3	2.342	0.085
Conductivity x Species	7.509	2	1.109	0.338
Temperature x Conductivity x 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 x Conductivity	22.756	6	1.027	0.420
Temperature x Species	29.103	3	2.626	0.061
Conductivity x Species	1.257	2	0.170	0.844
Temperature x Conductivity x 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 x Conductivity	35.477	12	2.169	0.021
Temperature x Species	19.307	3	4.721	0.004
Conductivity x Species	30.157	4	5.531	0.001
Temperature x Conductivity x Species	45.236	12	2.766	0.003
Error	711.040	80		

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

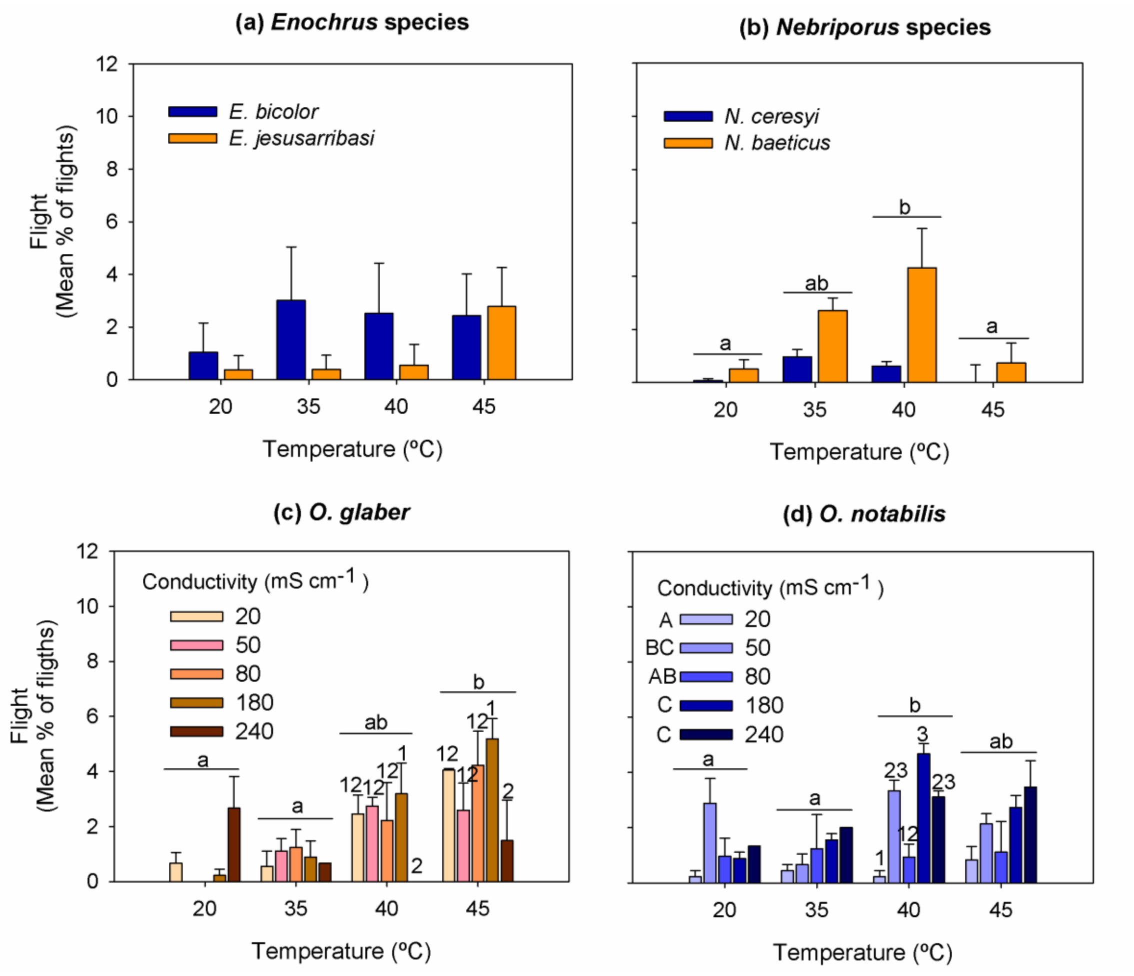


Figure 3. Mean ± SE total flight of each species. Significant differences determined by post-hoc analysis employing Bonferroni correction are indicated as follows: by capital letters in the legend for conductivity levels, by lower case above the bars for temperature levels, by numbers above the bars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

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.

Ochthebius species were the most heat tolerant, with a relatively high survival at 45ºC in some salinity treatments if compared with the other species pairs. 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). Some of the mechanisms used to deal with extreme osmotic stress might be also involved in protection for heat stress (e.g. synthesis of heat shock proteins or reduction of metabolic rate), which could provide these species a cross-tolerance to

salinity and heat (e.g. Chen & Stillman, 2012). 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, to date there have been no studies on the mechanism of such cross-tolerance in saline beetle species.

In contrast, 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. jesusarribasi* (Arribas *et al*., 2012a); the upper thermal limits of these species are higher in individuals acclimated to relatively high salinities and temperatures. Therefore, the effect of salinity and interactive effects with temperature are highly mediated by exposure time, in agreement with many studies that have found that lethal and sublethal strongly differ depending on the duration of exposure to a determined stressor (e.g. Reynaldi & Liess, 2005; Terblanche *et al*., 2008; Nel *et al*., 2011).

Are flight and emersion responses good indicators of sublethal stress limits?

As a general pattern in all of the studied species, avoidance responses increased in magnitude as stress levels intensified and decreased near tolerance limits. In *Enochrus* and *Nebrioporus* species, low mortality and increasing emersion and flight activity were recorded between 20 and 40ºC. At 45ºC only a few individuals survived, and among these, behavioural responses were significantly reduced indicating a likely irreversible physiological damage caused by the extreme heat stress. *Ochthebius* species, the most heat tolerant, displayed more intense behavioural activity and this was maintained at higher stress level than *Enochrus* and *Nebrioporus*.

Within each genus, differences in behavioural responses between species pairs were consistent with survival patterns (see below). Therefore, flight and emersion responses were useful measures to estimate and compare sublethal limits between the studied species. 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.

Do lotic species would have lower physiological amplitude than lentic ones?

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 limits 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 (35-40ºC), although *N. baeticus*, the less tolerant species, showed higher flight activity at such temperature than *N. ceresyi*, indicating a higher sensitivity to the stress conditions. Parallel differences in stress responses to that observed in *Nebrioporus* 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* at lower salinities, while at the most extreme conductivity (240 mS cm^{-1}) such pattern was reversed.

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. jesusarribasi*. 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. jesusarribasi*).

Differences in the environmental stability of lentic and lotic habitats could promote the evolution of different stress response strategies among closely related species in each kind of habitat (Ribera, 2008). Our results suggest habitat stability may have an important role in mediating tolerance to environmental stress in water beetles, but thermal tolerance seems to be phylogenetically conserved in some lineages (e.g. *Enochrus*). Thus, species adapted to less stable lentic habitats would have developed

higher colonisation capabilities that would be mediated by either improved physiological tolerances (e.g. *Nebrioporus* and *Octhebius* species) or dispersal abilities (e.g. *Enochrus* species) compared to their lotic counterparts.

Despite the wide tolerance of the saline species to environmental stress if compared with those from freshwaters (Millán *et al*., 2011), on the basis of the responses studied here, the saline lotic species *N. baeticus* and *O. glaber* could be more vulnerable than their respective lentic congeners *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. jesusarribasi* (Arribas *et al*., 2012a) also points to a higher vulnerability to environmental changes than for the lentic *E. bicolor*.

Despite our study provides valuable information about potential species' sensitivity to environmental change, the static acute approach employed did not consider possible acclimation responses through short-term physiological plasticity (Stillman, 2003). However, in unpredictable and highly variable environments such as saline water bodies, acclimation effects can be expected to be reduced because species live close to their lethal limits, with small margin for acclimation capacity (Chown & Terblanche, 2007; Tomanek, 2010). 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 environments' biodiversity (Millán *et al*., 2011). We therefore propose that biomonitoring and extra conservation efforts be focussed on these singular habitats.

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CHAPTER 3

Aquatic insects dealing with dehydration: do desiccation resistance traits differ in species with contrasting habitat preferences?

INTRODUCTION

Maintaining water balance is fundamental for organismal survival, small animals such as insects being especially vulnerable to dehydration (Addo-Bediako *et al.*, 2001). Desiccation resistance therefore shapes the distribution of insect species at multiple spatial scales, both ecologically and biogeographically (Kellermann *et al.*, 2009), and will determine the way insect taxa respond to increased temperatures and greater seasonal fluctuations in water availability in the face of climate change (Chown *et al.*, 2011).

The role of desiccation resistance for insect vulnerability could be particularly important in arid and semiarid regions such as the Mediterranean Basin. In these areas, many lowland inland waters show spatial and temporal flow intermittency, because they are subjected to intense summer droughts (Millán *et al.*, 2011; Hershkovitz & Gasith, 2013). During the dry period, some small and shallow lentic waterbodies can remain completely dry for months. In intermittent streams, flow connectivity is disrupted; some reaches dry out while others can retain water in receding pools. The predicted increase of the duration and frequency of droughts in Mediterranean-climate regions in the context of global change threatens the persistence of many of their endemic aquatic organisms (Lawrence *et al.*, 2010; Filipe *et al.*, 2013; IPCC, 2013).

However, a large knowledge gap still exists in the way aquatic macroinvertebrates respond to droughts (Robson *et al.*, 2011). In the case of aquatic insects, most studies are focused on desiccation-resistant eggs or dormant larvae stages (e.g. Woods & Singer, 2001; Juliano *et al.*, 2002; Benoit, 2010), but little is known on the effects of dryness on species which lack these resistant stages like most aquatic beetles (Strachan *et al.*, 2015). Although some species of water beetles resist the dry phase of temporary waters in microrefuges *in situ*, as adult or larvae stages (e.g. Davy-Bowker, 2002; Stubbington *et al.*, 2016), winged adults of many species show a more resilient strategy, dispersing by flying from drying sites to more favourable wet habitats (Bilton, 2014; Strachan *et al.*, 2015). The duration of exposure to drying stress during dispersal depends on specific biological traits (e.g. flight ability) and landscape configuration, i.e. the availability of suitable habitats that may serve as wet refuges and the distance and connectivity between them (Larned *et al.*, 2010; Datry *et al.*, 2016). But even short exposures to drying stress are challenging for flying aquatic insects, because flight activity is associated to a strong dehydration (Dudley, 2000).

Studies of geographical variation in responses to desiccation stress in terrestrial insects have typically demonstrated that species and populations from xeric environments show a greater ability to deal with dehydration than those from mesic areas (e.g. Schultz *et al*., 1992; Chown, 1993; Le Lagadec *et al*., 1998; Gibbs & Matzkin, 2001). Different responses to desiccation have also been related to patterns of habitat and microhabitat choice in arthropods (e.g. Chown, 1993; Gereben, 1995; De Vito *et al*., 2004; Lapinski & Tschapka, 2014), including aquatic species (e.g. Wissinger *et al*., 2003; Yoder *et al*., 2015). In general, these studies show that some physiological mechanisms linked to drying stress such as the control of water loss rate have an important plastic and adaptive component, whilst others, such as the tolerance of water loss, are less variable across species (Chown *et al*., 1999; Hoffmann & Harshman, 1999). In inland water ecosystems, even congeneric macroinvertebrate species show remarkable differences in the strategies and particular traits to deal with desiccation, and such variability is strongly associated with the frequency and duration of droughts in their habitats (Strachan *et al*., 2015).

In Mediterranean inland waters, a number of genera of water beetles belonging to different families contain species which are adapted to different parts of the fresh – hypersaline gradient (Millán *et al*., 2011). Organisms dealing with osmotic stress in saline waters face similar physiological challenges to those imposed by desiccation; i.e. maintaining water balance and compensating for the increase in the osmotic concentration of internal fluids (Bradley, 2009). In light of this, interspecific differences in desiccation resistance may correlate with salinity tolerance, so that species inhabiting saline waters are expected to be potentially more resistant to desiccation than those in lower salinity ranges (Arribas *et al*., 2014). In addition, species living in relatively short-lived small lentic water bodies, which are more unstable systems over evolutionary and ecological time-scales than lotic systems (see Ribera, 2008 for details), may also be expected to have higher desiccation resistance compared to related lotic taxa. These predictable differences have already been found between lotic and lentic congeneric beetle species in other traits such as dispersal capacity (Arribas *et al*., 2012), behavioural responses to acute thermal stress (Pallarés *et al*., 2012) and salinity tolerance (Céspedes *et al*., 2013).

Together with behavioural responses (e.g. use of microrefuges, burrowing) or aestivation, invertebrates have evolved a variety of physiological adaptations to cope with desiccation stress (Hershkovitz & Gasith, 2013; Strachan *et al*., 2015). These fall under two main strategies (Edney, 1997; Chown & Nicolson, 2004): 1) avoiding desiccation through the reduction of water loss and increases in body water content (*desiccation resistance*, e.g. Gray & Bradley, 2005) and 2) withstanding the loss of a significant proportion of body water (*desiccation tolerance*, e.g. Suemoto *et al*., 2004; Benoit *et al*., 2007). In insects, mechanisms regulating cuticle permeability are the major component of desiccation resistance because the cuticle represents their main avenue for water loss (Hadley, 1994; Chown & Nicolson, 2004; Benoit *et al*., 2010). Cuticle permeability is related with the cuticle thickness (Crowson, 1981; Harrison *et al*., 2012; Reidenbach *et al.*, 2014), but can be actively regulated through changes in the amount and composition of surface lipids (Gibbs & Rajpurohit, 2010; Stinziano *et al.*, 2015). Water loss has shown to be non-linear following exposure to desiccation in a range of taxa (e.g. Arlian & Staiger, 1979; Benoit *et al*., 2007). Greater water loss rates occur during initial hours of exposure and decrease as body water content approaches lethal levels, suggesting that water loss is actively regulated by dynamic mechanisms. As a consequence, the water content of an individual at a particular moment could influence its water loss dynamics and ultimately its survival under drying stress. On the other hand, body size can affect desiccation resistance in arthropods in a number of ways. In general, larger body mass allows a higher proportion of water and lipid content (Lighton *et al*., 1994; Prange & Pinshow, 1994), and additionally smaller insects may show higher mass-specific water loss rates due to higher surface area-volume ratios (Schmidt-Nielsen, 1984; Chown *et al*., 1998; Williams & Bradley, 1998). Such effects of size on water loss rates have been seen both inter- (e.g. Le Lagadec *et al*., 1998; Chown & Klok, 2003) and intraspecifically (e.g. Renault & Coray, 2004).

Here we compared responses to desiccation stress in flying adults of four aquatic beetle species of the genus *Enochrus*. These species are specialists of either lentic or lotic waters of differing salinity, providing an ideal model to explore the potential relationship between specific desiccation resistance traits of aquatic insects and the main inland waters habitat types. We measured their survival and recovery ability following controlled exposure to drying stress and explored key traits related to desiccation resistance to: i) determine whether congeneric species with different habitat preferences differ in desiccation resistance and ii) explore the extent to which inter-individual differences in water loss rates are shaped by body size, cuticle thickness and/or water content in these insects. We predicted that species from most saline habitats would show higher desiccation resistance than less saline tolerant ones. Additionally, species living in lentic waters could have evolved a higher resistance to desiccation than lotic ones.

MATERIAL AND METHODS

Study species

The genus *Enochrus* (Coleoptera: Hydrophilidae) has representatives living across a wide variety of inland waters of differing salinities (from 0.5 to >80 g L^{-1} in the study area). The four species used in this study show different salinity ranges and optima both in laboratory (Pallarés *et al*., 2015) and nature (Arribas *et al*., 2014): *E. halophilus* (Bedel, 1878) (fresh-subsaline waters), *E. politus* (Kuster, 1849) (hyposaline), *E. bicolor* (Fabricius, 1792) (mesosaline) and *E. jesusarribasi* Arribas & Millán, 2013 (hypersaline). All species live in shallow water close to the margins of occupied waterbodies, but differ in their habitat preferences across the lentic-lotic divide, being found in lentic (*E. halophilus* and *E. bicolor*) and lotic waters (*E. politus* and *E. jesusarribasi*) (see Table 1 for more detailed habitat information). These species do not show any resistant form to face desiccation *in situ* at any stage of their life cycle. Therefore, their main strategy to deal with droughts in temporal and/or intermittent systems relies on the dispersal ability of adults, which move from drying to wet sites. These movements may occur between different or within the same waterbodies, depending on the landscape configuration and habitat availability (Larned *et al*., 2010; Datry *et al*., 2016).

Experimental procedures

Adult specimens (approx. 50 per species) were collected from different localities all in southeastern Spain and representing the optima salinity conditions of each species (see Table 1). For logistic reasons, we used specimens from one single location per species. Such locations were selected minimizing distances between each other and so have a comparable climatic regime. All species were collected during the spring 2014, therefore the specimens used for the experiments were all mature adults from the winter generation and presumably had not been previously exposed to desiccation stress in natural conditions.

Specimens were maintained for 4-7 days in the laboratory at 20±1°C in aerated tanks with water from collection sites (i.e. at the same salinity of their habitat) and fed with macrophytes also collected in the source localities. For comparative purposes, insects were kept 48 h before desiccation experiments in a dilute medium (ca. 0.1 mS cm^{-1}) at 20±1°C and 12:12 light:day cycle in a climatic chamber (SANYO MLR-351, Sanyo Electric Co., Ltd., Moriguchi City, Osaka, Japan), without access to food. The medium was prepared by dissolving the appropriate amount of marine salt (Ocean Fish, Prodac, Cittadella, Italy) in distilled water.

The experimental protocol and variables recorded in controlled desiccation experiments are showed in Fig. 1. For each specimen studied we obtained the initial fresh mass (M_0) as a surrogate of size, initial water content (WC_0; % wet mass to initial fresh mass), cuticle content as a surrogate of cuticle thickness (CC; % of cuticle mass to initial fresh mass), water loss rates (WLR; % of water lost to initial fresh mass per hour) and total water loss after the corresponding treatment (WL, % of water loss to total water content). For this, groups of 20-25 individuals of each species were dried on blotting paper, weighed on a balance accurate to 0.01 mg and placed individually into clean 15 mL open glass vials. These were kept for 6 h in a glass desiccator containing silica gel (Sigma-Aldrich, Madrid, Spain) at 20±1°C. Relative humidity, monitored with a hygrometer (OM-EL-USB-2-LCD, Omega Engineering, Seville, Spain), dropped from approx. 40% (laboratory humidity) to 20±5% within the first 2 h and remained stable within this range until the end of the trial. The experimental conditions were optimized trough pilot trials in order to detect differences among species, within their tolerance limits and in a reasonable experimental time (to avoid additional stress such as starvation). The remaining specimens (N=10-20 individuals per species) were used as a control under no desiccation stress. They were kept in glass vials placed in a closed tank with deionized water in the base, producing a relative humidity $\geq$ 90%. After 6 h, surviving specimens from control and test groups were re-weighed for estimation of water loss rates and allowed to recover for 24 h in 100 mL containers with 40 mL of the dilute solution. Some studies have shown that rehydration may result in an excessive increase in specimens' water content (overhydration stress, e.g. Lopez-Martinez *et al.*, 2009; Yoder *et al*., 2015). However, we checked in pilot trials (data not shown) that the species here studied recovered their initial water content after rehydration, with no significant water gains. Mortality was monitored after desiccation exposure and after the recovery period. Specimens were killed by freezing at -80ºC, dried at 50ºC for 48 h and re-weighed for estimation of the initial water content. A subgroup of 20 individuals per species from the test group were also immersed in 4 mL of 2M NaOH (aq.) for 48 h at room temperature to allow tissue digestion, rinsed in distilled water, dried and weighed again for estimation of cuticle content (Harrison *et al*., 2012). Specimens were sexed after the experiment by examining genitalia under a Leica M165C stereomicroscope.

Data analyses

Interspecific comparison of desiccation traits

Fresh mass, water loss rate, water content and cuticle content were compared among species using generalized linear models (GLMs) with species as factor, followed by Bonferroni post-hoc tests. Gaussian error distribution and identity link function were used for fresh mass, water content and cuticle content models; and gamma distribution for water loss rate which showed a positively skewed distribution. To account for the potential effects of sex and body size in desiccation resistance, sex and the interaction of sex and species were included as predictors, as well as fresh mass in comparisons of water loss rate, water content and cuticle content (e.g. Terblanche *et al*., 2005; Addo-Bediako *et al*., 2001). Model residuals were checked for normality and homoscedasticity assumptions.

Relationships between desiccation resistance traits within species

To determine the possible effects of initial water content, cuticle content and size (fresh mass) on inter-individual variation in water loss rate, the relationship between water loss rate and each variable was explored for each species separately using GLMs. Gaussian error distribution and identity link function were used when data met a normal distribution. When this assumption was not met, different link functions (log) or different error distributions (Gamma) were implemented, and the model with the lowest AIC was selected.

All the statistical analyses were carried out using R v. 3.0.1 (R Core Team, 2015).

Table 1. Habitat parameters of *Enochrus* species, together with collection sites.

	Habitat preferences			**Collection sites**		
Species	**Conductivity range** [a] (mS cm^{-1})	**Conductivity optimum** [b] (mS cm^{-1})	**Habitat type**	**Locality**	**Latitude**	**Longitude**
E. halophilus	0.47- 23.00	6.25 (subsaline)	Temporary-Lentic	Pétrolapond, Albacete	38.8471	-1.5589
E. politus	1.50 - 133.40	19.32 (hyposaline)	Intermittent-Lotic	Chícamostream, Murcia	38.2175	-1.0511
E. bicolor	2.10 - 86.00	34.96 (mesosaline)	Temporary-Lentic	Mojón Blanco pond, Albacete	38.8002	-1.4301
E. jesusarribasi	14.90 - 160.00	62.14 (hypersaline)	Intermittent-Lotic	Rambla Salada stream, Murcia	38.1263	-1.1182

[a] Field conductivity data were obtained from Biodiversity database of the Aquatic Ecology Research Group, University of Murcia.

[b] Ranges of conductivity of each category (mS cm^{-1}): Freshwater: < 1, Subsaline: 1–10, Hyposaline: 10–30, Mesosaline: 30–60, Hypersaline: > 60 (Montes & Martino, 1987).

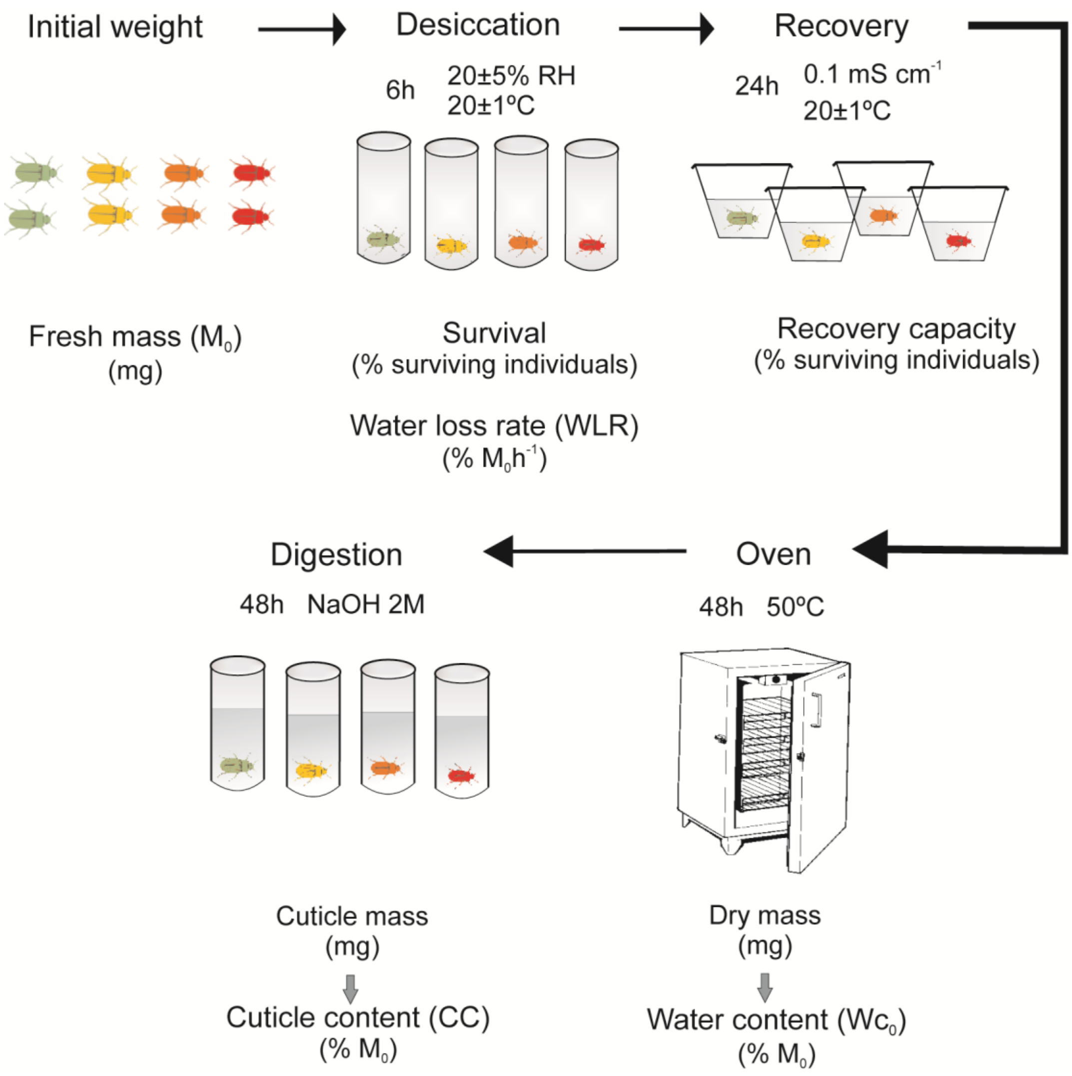

Figure 1. Experimental procedure and variables measured in desiccation experiments.

RESULTS

Interspecific comparison of desiccation traits

The water and cuticle contents of the four studied species ranged from 60 to 68 % and 12 − 23 % M_0, respectively. Mean water loss rates of specimens exposed to desiccation ranged from 2.22 to 3.57 % M_0 h^{-1}, with a total water loss after 6 h of desiccation exposure of 19.3 – 39.1 % WC_0. Specimens in the control group showed very little water loss (approx. 0.5 % M_0 $h^{-1}$and a maximum water loss of 6 % WC_0) (see Table S1 for species comparative data).

All desiccation resistance traits differed significantly between species (Table 2). Females showed higher fresh mass and water content than males in all species (see sex and sex x species effects in Table 2). Despite significant interspecific differences in mean fresh mass (Fig. 2a), the effect of initial body mass on the other trait comparisons was not significant (Table 2).

The species living in fresh-subsaline waters (*E. halophilus*) showed a significantly higher water loss rate, but this did not differ significantly amongst the other three species (Fig. 2b). Initial water content was higher in the meso and hypersaline species (*E. bicolor* and *E. jesusarribasi*) than in the subsaline and hyposaline ones (*E. halophilus* and *E. politus*) (Fig. 2c). The species showed similar cuticle contents, except for *E. halophilus* which had the highest value (Fig. 2d). No consistent patterns between lotic and lentic species were observed for any of the measured traits.

No mortality occurred during exposure to desiccation (except for one specimen of *E. halophilus*). *Enochrus halophilus* showed a limited capacity to recover after desiccation (44% of the tested specimens died during the recovery period *vs* only one specimen in each of the other species). The observed mortality can be mainly attributed to desiccation stress because 100% survival occurred in the control group in all species.

Relationships between desiccation resistance traits within species

In general, the desiccation resistance traits showed high inter-individual variability in all species studied (see Figs. 2 and 3). A significant positive relationship was found between individual water loss rates and water content in all species except for *E. halophilus* (Fig. 3a). In contrast, cuticle content was not related to water loss rate in any species (Fig. 3b), and these were also independent of initial body mass (Fig. 3c).

DISCUSSION

On the basis of our investigations, desiccation resistance in the *Enochrus* water beetle species studied appears to be associated with habitat salinity, but does not differ between species occupying lotic and lentic water bodies. The most saline-tolerant species studied (*E. bicolor, E. jesusarribasi* and *E. politus*) showed lower water loss rates than the freshwater-subsaline species (*E. halophilus*). Furthermore, within these three saline species, the meso and hypersaline ones (*E. bicolor* and *E. jesusarribasi*) had significantly higher initial water content than the hyposaline *E. politus.* Indeed, these interespecific differences in water control efficiency showed to be relevant in terms of

Table 2. GLM results on interspecific differences in fresh mass (M_0), water loss rate (WLR), water content (WC_0) and cuticle content (CC) across *Enochrus* species (N=20 per species).

Trait	**Predictors**	**df**	**F-value/ χ^2 [a] (Explained deviance) [b]**	**P-value**
M_0 (mg)	Sp	3	37.627	< 0.001
	Sex	1	14.206	< 0.001
	Sp x Sex	3	0.607	0.613
			(0.651)	
WLR (%M_0 h-1)	Sp	3	2.718	< 0.001
	M_0	1	0.126	0.161
	Sex	1	0.004	0.799
	Sp x Sex	3	0.007	0.990
			(0.397)	
WC_0 (% M_0)	Sp	3	22.086	< 0.001
	M_0	1	1.387	0.243
	Sex	1	4.736	0.033
	Sp x Sex		0.335	0.800
			(0.519)	
CC (% M_0)	Sp	3	27.019	< 0.001
	M_0	1	3.067	0.085
	Sex	1	0.027	0.870
	Sp x Sex	3	1.629	0.192
			(0.593)	

[a] F-value for GLMs with gaussian distribution (M_0, WC and CC); χ^2 for GLMs with gamma distribution (WLR)

[b] (null deviance − residual deviance/null deviance)

survival under drying stress, as *E. halophilus* was also the most sensitive species to the conditions tested here. In consequence, assuming that the species may tolerate similar levels of water loss (Chown *et al.*, 1999; Hoffmann & Harshman, 1999), the studied saline tolerant species showed a clear physiological advantage over freshwater ones under desiccation conditions.

Arribas *et al.* (2014) suggested that salinity tolerance in water beetles could be based on a co-opted mechanism originally developed for desiccation resistance, relying on the temporal correlation of global aridification events and the phylogenetic ages of saline lineages. The pattern found here of stronger desiccation resistance in aquatic species living in saline waters is clearly consistent with this hypothesis and emphasises the important role that traits associated with coping with osmotic and desiccation stress

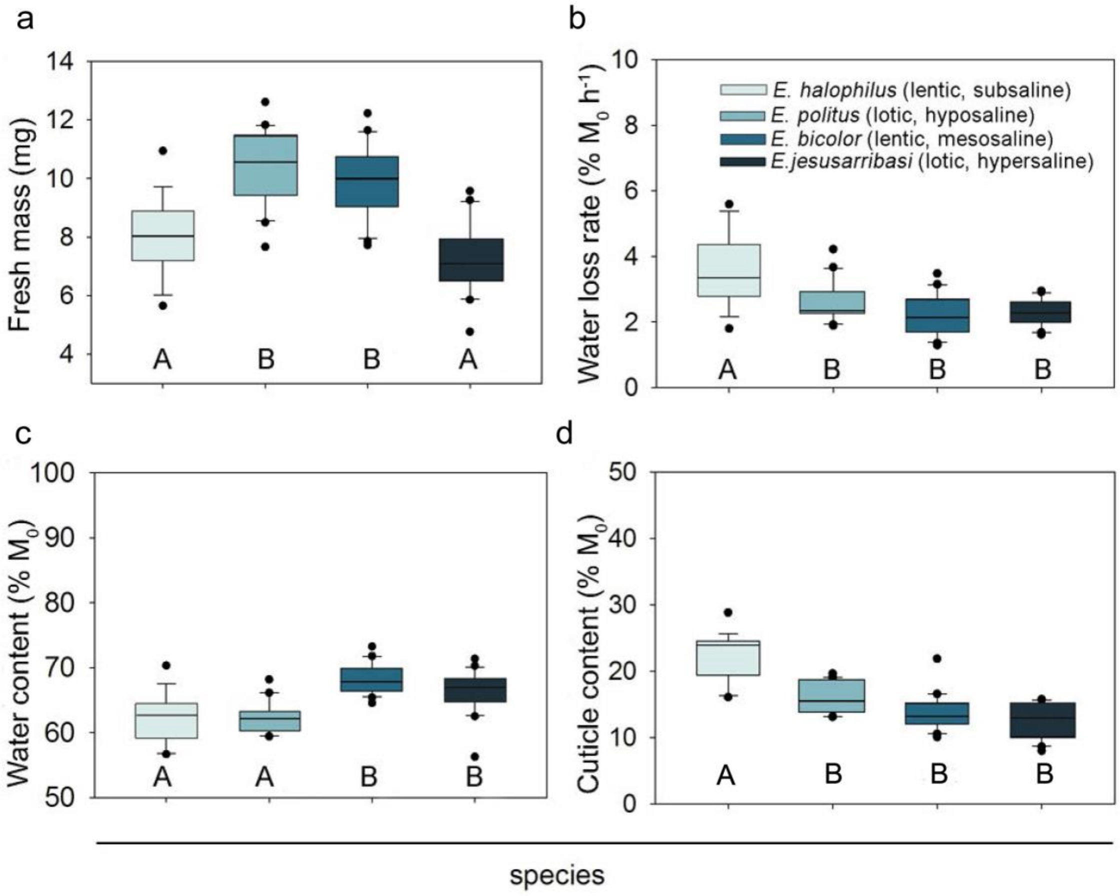

Figure 2. Interspecific comparison of desiccation resistance traits in *Enochrus* species. Letters below the boxes indicate significant differences between species (Bonferroni post-hoc tests, $P < 0.05$). Boxplots represent Q25, median and Q75, whiskers are Q10 and Q90 and dots are outliers.

could have in shaping the ecological diversification of *Enochrus*. Also in line with the relationship between desiccation and salinity tolerance seen across the beetles studied here, intraspecific studies of corixid populations found similar responses to the two stressors (e.g. Cannings, 1981), and salinity acclimation was showed to confer desiccation resistance in an Antarctic midge (Elnitsky *et al*., 2009). Salinity imposes similar stress on aquatic organisms as that resulting from desiccation during air exposure at the cellular level (i.e. water loss and increase of the osmotic pressure) (Evans, 2008; Bradley, 2009). In consequence, shared genetic and physiological mechanisms might underlie resistance to these two factors, as found with other related stressors such as desiccation and cold (e.g. Holmstrup *et al*., 2002; Levis *et al*., 2012; Everatt *et al*., 2014).

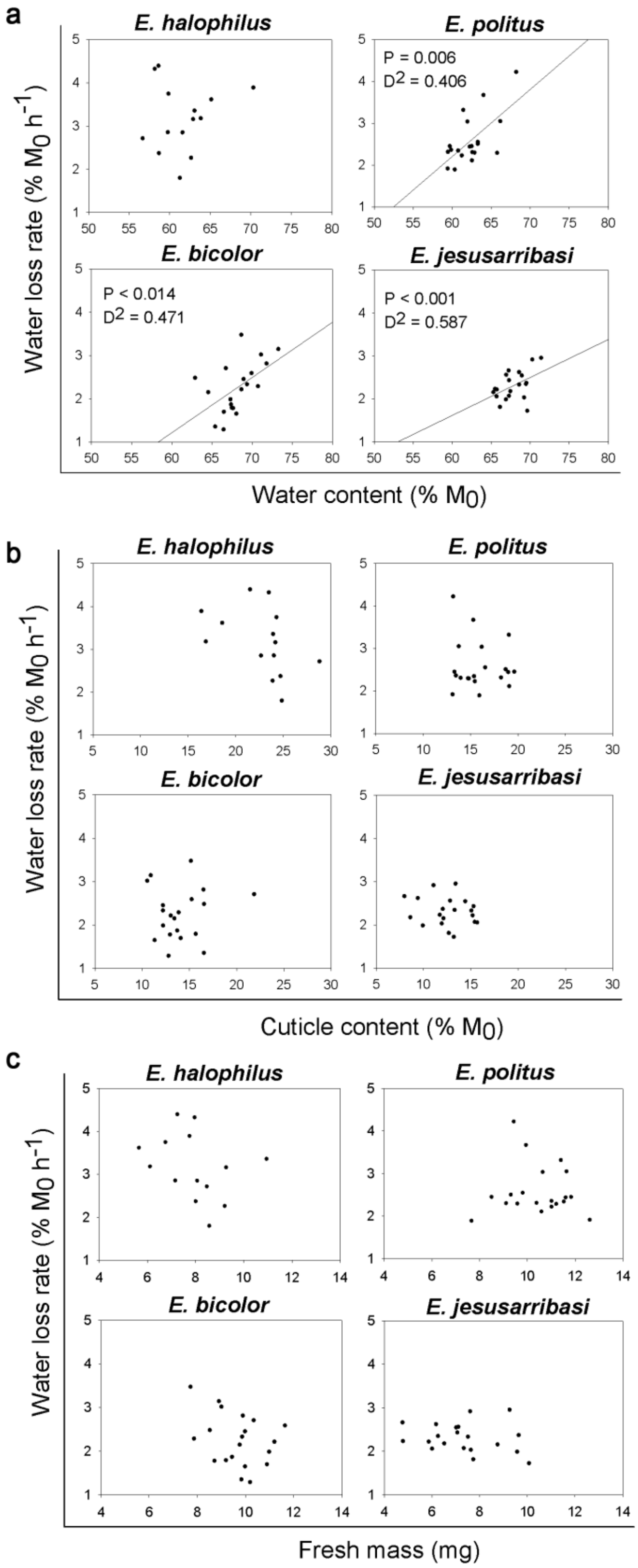

Figure 3. Relationships between individual water loss rates and a) initial water content, b) cuticle content and c) fresh mass for *Enochrus* species. Regression line, P-values and deviance (D^2) are showed for the statistically significant relationships ($P<0.05$).

Our study found no direct association between desiccation resistance and the lotic/lentic habitat divide. Previous studies on water beetles have shown that lentic taxa have a higher colonisation ability (i.e. the ability of a species to disperse and establish new populations) than lotic related species, resulting in larger geographical ranges and lower population genetic structure (Ribera, 2008; Abellán *et al*., 2009; Hof *et al*., 2012). Dispersal capacity and thermal tolerance seem to be the main traits driving this lotic/lentic pattern in water beetles (e.g. Hjalmarsson *et al*., 2015) and particularly in two of the species here studied, *E. jesusarribasi* and *E. bicolor* (Arribas *et al*., 2012; Pallarés *et al*., 2012). The two lotic species studied here are restricted to the Iberian Peninsula and Morocco whilst the lentic ones are distributed across larger areas, including northern Europe (Millán *et al*., 2014), but no clear differences in desiccation resistance traits were found accordingly. Therefore, desiccation resistance could play a secondary role to differences in dispersal capacity in shaping the colonisation ability of water beetles. In this point it should be noted that *Enochrus* species' occurrence across different habitat types will be also constrained by the limited desiccation resistance of eggs and larvae, being the latter likely the most desiccation-sensitive stage because of their thinner cuticles. In addition, desiccation resistance might show inter-population variability (e.g. Hoffmann & Harshman, 1999) as a result of physiological plasticity or local adaptations. Despite our study on adults from populations on similar climatic regimes but different habitats allows for a robust comparison across species, further studies on multiple stages and populations are needed to deeply understand the relationship between habitat occupation and resistance to desiccation in this group.

Beetles are one of the groups of arthropods best adapted to desiccation, with species from desert or semi-desert areas typically representing the extremes in tolerance to dehydration. For example, the terrestrial spider beetle *Mezium affine* shows daily water losses as little as 0.3% per day and the ability to survive up to 3 months with no food or water (Benoit *et al*., 2005). Surprisingly, the highest tolerance to water loss (89% of the body water content) has been reported for a fully aquatic beetle, the haliplid *Peltodytes muticus* (Arlian & Staiger, 1979). Since they occupy the shallow margins of waterbodies, *Enochrus* species may be intuitively expected to be intermediate in desiccation resistance between strictly terrestrial beetles and those occupying deeper water such as many diving beetles (Dytiscidae) (Wigglesworth, 1945; Holdgate, 1956; Beament, 1961). However, it is difficult to establish a comparative framework because of the few existent data on desiccation resistance traits in adult aquatic insects and the multiple and contrasting approaches and/or experimental conditions used to measure them. The water contents of the four *Enochrus* species (60-68% of fresh mass) were consistent with the typical 62% of most beetles (Hadley, 1994). Water loss rates, ranging from 2.2 to 3.6% of initial mass at 20% RH, appear to be comparable to those reported for the extraordinarily desiccation resistant *P. muticus*, which lost ca. 5.4% of initial mass per hour under more severe conditions (0% RH) (Arlian & Staiger, 1979). Nevertheless, the total water losses that the studied species reached after the desiccation treatment (Table S1) were close to the limit of dehydration tolerance of most insects (20-30 % of water content) (Hadley, 1994). Although such water loss was measured under an unrealistic humidity in natural conditions (20% RH), a combination of high temperatures (>30ºC) and low humidity (40-50% RH) is frequent in the natural habitats of these species. Prolonged exposures to such conditions in nature may result in extremely stressful conditions and high mortalities of local populations of the studied species, but further research is needed to identify desiccation level and duration thresholds under natural conditions for each particular species.

The analysis of traits at the individual level is essential for further exploration of the mechanisms regulating water loss rate. In *Enochrus* species, water loss rates were positively related to the specimens' initial water content. These relationships were relatively weak ($r^2<0.5$) due to high inter-individual variation in both traits, which might be associated to age, sex or the physiological state of the individuals (e.g. Chown *et al*., 1999; Matzkin *et al*., 2007; Lyons *et al*., 2014). Despite this variability, resistance to water loss seems to be partly a function of individual water content, as beetles with a higher initial proportion of water lost it faster than those with lower values. This suggests that a critical level of water loss may induce active mechanisms for water conservation (e.g. changes in cuticular permeability), which might be "relaxed" when organismal water content rises above this threshold. Such regulation is concordant with the nonlinearity of water loss following exposure to desiccation found in many fully terrestrial insects (e.g. Arlian & Staiger, 1979; Benoit *et al*., 2007).

Although we used cuticle content as a potential surrogate of cuticle permeability, since increased cuticle thickness is associated with desiccation resistance in insects adapted to arid conditions (Crowson, 1981; Elias, 2010), this trait showed no relationship with water loss rates in any *Enochrus* species. In addition, in interspecific comparisons, the species with the highest mean water loss rate had the highest cuticle content. A recent study also showed that cuticle thickness in adult mosquitoes appeared not to affect desiccation resistance (Reidenbach *et al*., 2014). Therefore, the validity of cuticle content as proxy for cuticular permeability could be very different across taxa and may perhaps have low resolution for intra-generic comparisons. In some terrestrial insects, changes in the composition and quantity of cuticular hydrocarbons appear to be the main mechanism through which they can modulate cuticular permeability (e.g. Hadley, 1978; Toolson, 1982; Nelson & Lee, 2004; Stinziano *et al*., 2015). In aquatic insects, similar mechanisms may shape responses to dehydration occurring both in exposure to air or hyperosmotic aquatic medium but to date even basic cuticular properties in such taxa have received little study (but see Alarie *et al.*, 1998 for an example).

Despite the fact that many previous studies suggest that body size affects water loss rate in arthropods (e.g. Lighton *et al.*, 1994; Prange & Pinshow, 1994; Chown *et al*., 1998) our results suggest that both interspecific and inter-individual size differences do not significantly affect desiccation resistance in these water beetles. Although large size (lower area-to-volume ratio) might be expected to be beneficial for survival under desiccating conditions (Schmidt-Nielsen, 1984; Chown *et al*., 1998), important trade-offs could arise as a result of increases in body size (Chown & Klok, 2003; Chown & Gaston, 2010). This could be particularly true in the case of aquatic insects living in fluctuating or temporary waters, such as the beetles studied here, where rapid larvae development and small body size are common, alongside other r-selected traits (Williams, 1985; Millán *et al*., 2011).

This study is the first to explore both interspecific and inter-individual variation in desiccation resistance traits within a group of closely related aquatic insects. Our results suggest that control of both water loss rate and water content may be key mechanisms for dealing with desiccation stress in adult water beetles and suggest an association between salinity tolerance and desiccation resistance. Further studies are required to evaluate the ecological and evolutionary consequences of interspecific variation in key desiccation resistance traits, but our results point to habitat-mediated differences (saline *vs*. freshwater) in the vulnerability of water beetle species to a higher frequency and intensity of droughts expected in semi-arid regions.

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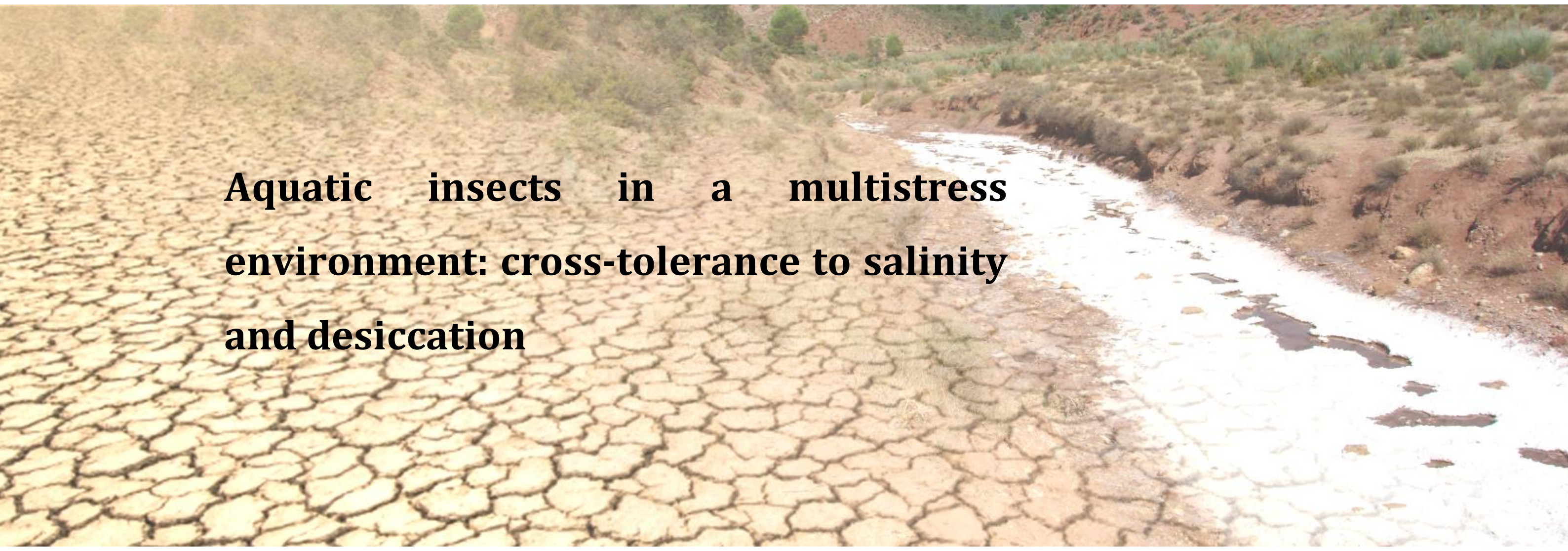
Table S1. Summary of variation in desiccation resistance traits in control and treatment groups of *Enochrus* species.

	N	**Initial fresh mass** (mg)	**Initial water content** (% of fresh mass)	**Cuticle content** (% of fresh mass)	**Total water loss** (% of water content)	**Water loss rate** (% of fresh mass per hour)
Control group						
E. halophilus	10	8.703 ± 1.528	60.6 ± 2.7		5.9 ± 1.1	0.51 ± 0.32
E. politus	20	10.239 ± 1.258	62.5 ± 3.0		6.1 ± 0.5	0.67 ± 0.25
E. bicolor	15	10.175 ± 1.274	66.4 ± 3.3		4.3 ± 0.5	0.48 ± 0.25
E. jesusarribasi	20	7.211 ± 1.174	65.3 ± 2.4		4.3 ± 0.3	0.48 ± 0.15
Treatmentgroup						
E. halophilus	17	8.035 ± 1.288	62.3 ± 3.5	22.3 ± 3.5	39.1 ± 2.9	3.57 ± 1.12
E. politus	20	10.439 ± 1.252	62.4 ± 2.3	15.9 ± 2.3	24.8 ± 1.1	2.59 ± 0.59
E. bicolor	20	9.784 ± 1.024	68.3 ± 2.2	14.0 ± 2.6	19.3 ± 1.1	2.22 ± 0.60
E. jesusarribasi	20	7.048 ± 1.304	66.3 ± 3.2	12.6 ± 2.3	20.0 ± 0.6	2.28 ± 0.40

Data are reported as mean ± SD. N, sample size.

CHAPTER 4



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

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. Holmstrup *et al.*, 2002; Elnitsky *et al.*, 2009) or, conversely, can cause the organisms to be more susceptible to the second stress (cross-susceptibility) (Sinclair *et al.*, 2013; Todgham & 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 & Kelly, 2016; Gunderson *et al.*, 2016) as well as to increase our understanding of responses to global change in the 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. Todgham *et al.*, 2005; Pansch *et al.*, 2012; Huth & Place, 2016)

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 & 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 & 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 & 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 (Evans, 2008; Bradley, 2009; Cohen, 2012). 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) (Dow & Davies, 2006; Bradley, 2009; Gibbs & 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).

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. Todgham *et al*., 2005; Kumlu *et al*., 2010; Sánchez-Fernández *et al*., 2010; Chen & Stillman, 2012). 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 the 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 tolerances in this group, potentially as an exaptation process due to linked physiological mechanisms to deal with both 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 tolerances 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 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 colonised 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 could allow us to infer the importance of the adaptation to both stressors in inland saline waters and provide new insights into the processes of colonisation and diversification in these systems. This information could be also highly relevant to understanding how aquatic insects may respond to the ongoing aridification in Mediterranean inland aquatic ecosystems, where more extreme and prolonged droughts and increased salinity levels are predicted (Sala *et al*., 2000; Bonada & Resh, 2013; Filipe *et al*., 2013).

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 (Sánchez-Fernández *et al*., 2008; Arribas *et al*., 2013; Millán *et al*., 2014).

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 20ºC 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) the exposure to stressful salinity on the subsequent resistance to desiccation and ii) the 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 (Sánchez-Fernández *et al*., 2010; Arribas *et al*., 2012b; Céspedes *et al*., 2013; Pallarés *et al*., 2015, 2016). Pilot trials were also conducted to determine the number of replicates (i.e. specimens) needed to ensure adequate power to detect the stressors effect.

The experiments were conducted at a constant temperature (20ºC) 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 50ºC for 48 h) with an electronic high-precision balance (± 0.00001 g) and beetles were sexed by examination of genitalia.

1 Effect of salinity on desiccation resistance

Groups of 30-40 specimens of each species were randomly assigned to the following pre-treatments, for one week: i) optimum salinity (OS), i.e. the most frequent salinity levels of each species' habitat or to ii) a higher sublethal salinity (SLS) (Fig. 1).

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% RH in an environmental chamber. Survival was checked and specimens were re-weighed every two hours. Using such fresh mass measures and the dry mass (M_{dry}) of each specimen (see above), we estimated their water content after salinity exposure (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 initial water content (WC_s) and water loss rates (WLR) under the subsequent desiccation. Because the change in water loss rates was not linear over desiccation exposure, this variable was estimated for each individual as i) the mean of the rates measured every 2 hours, ii) the maximum and iii) the final water loss rate (i.e. the rate measured at the interval previous 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 (Le Lagadec *et al*., 1998; Chown & Nicolson, 2004), initial fresh mass and sex were included as covariates, plus initial water content in the analysis of water loss rates. In this case, as initial fresh mass and initial water content are highly correlated, their effects were evaluated separately to avoid statistical problems of collinearity between predictors.

2 Effect of desiccation on salinity tolerance

Groups of 50 specimens of each species were exposed to i) a non-desiccation control treatment (CD; at RH > 90%), ii) a rapid desiccation (RD; at 10% RH) or iii) a slow desiccation treatment (SD; at 40% RH) (the specific exposure times for each treatment and species are shown in Fig. 1). Allocation of individuals to each pre-treatment was made randomly. For the slow desiccation pre-treatment, specimens were placed in individual open glass tubes in the environmental chamber set at 40±5% RH. For the rapid desiccation, 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 (approx. 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 it was not provided throughout the experiments. All specimens were initially weighed (M_0) and after desiccation, survival and ability to move were checked, discarding individuals which showed movement difficulties. A subgroup of 20-30 specimens were re-weighed (M_d) and used for estimation of the initial and final water contents (WC_0 and WC_d, before and after the desiccation pre-treatment, respectively) as well as water loss rates (WLR). These same specimens were subsequently used for the survival assay under stressful high salinity. For this purpose, specimens were placed in individual plastic containers with 40 mL of the specific sublethal saline 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 previous experiment. In this case, interval censored data were specified in Kaplan-Meier curves for those deaths registered between 12 h intervals. Water loss rates (WLR) and final water contents (WC_F) 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 following salinity exposure. Initial fresh mass (M_0), sex and initial water content (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 nanoliter osmometer (Otago Osmometers, Dunedin, New Zealand) (see details of the same measurement procedure in Williams *et al*., 2004). Some haemolymph samples were discarded for measurement because the volume was insufficient or they showed a dark color 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 (both as percentages and absolute units in the case of water content and water loss rate), are supplied in Tables S1-S3.

1 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: $\chi^2 = 2.2$, P = 0.135), although survivorship curves showed a tendency of higher survival in individuals from the sublethal salinity pre-treatment than those from the optimum one (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 optimum one (4.92 ± 0.14 mg) (Table 1). Water loss rates tended to decrease along exposure to desiccation (Fig. S1).

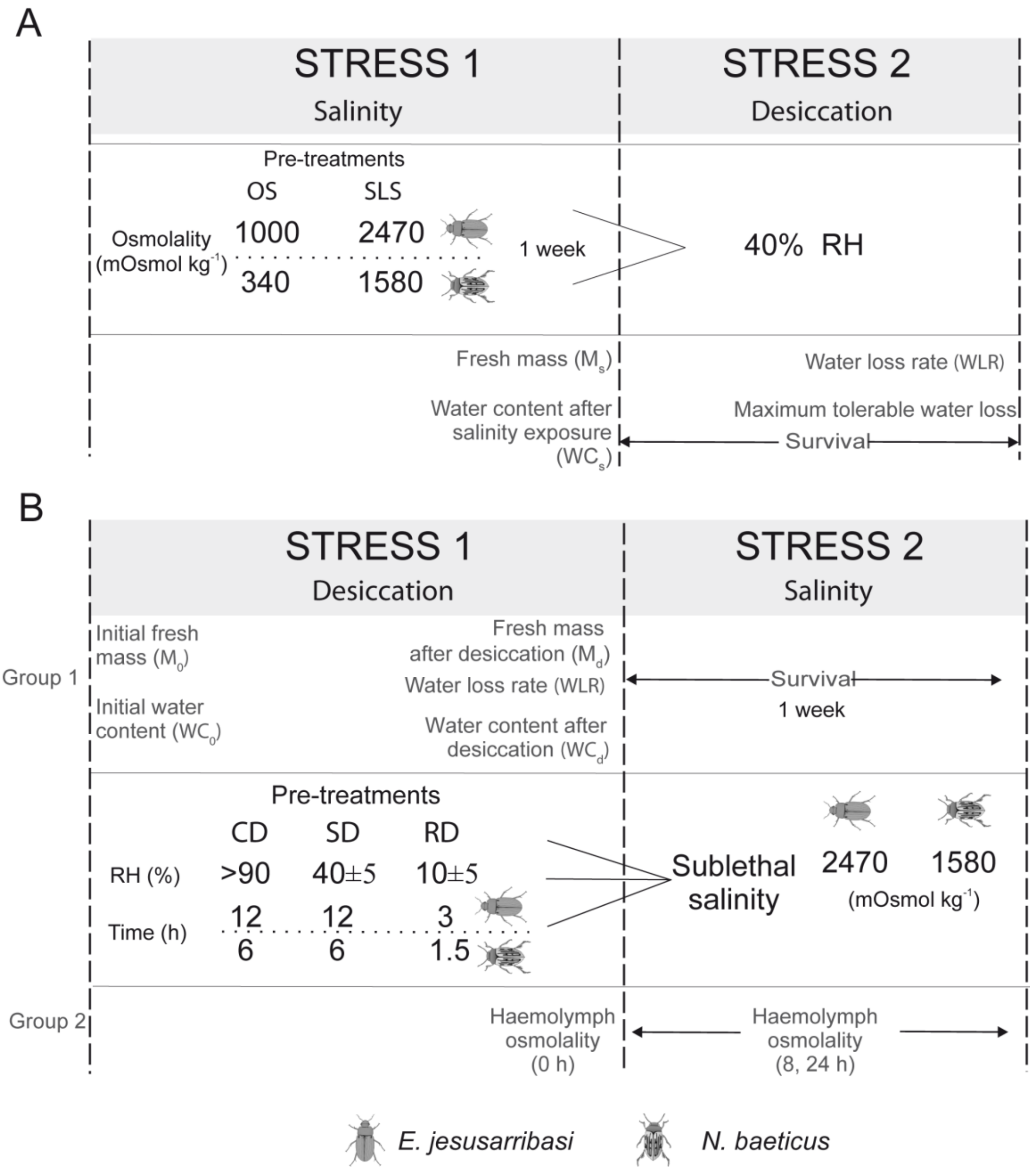


Figure 1. Experimental conditions (relative humidity, RH; osmolality and exposure time) and variables obtained at each experimental step. (A) Experiment testing the effect of salinity on desiccation resistance; (B) Experiment testing the effect of desiccation on salinity tolerance. Experimental conditions 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.

Mean water loss rates did not differ between salinity pre-treatments (0.113 ± 0.004 and 0.117 ± 0.006 mg h^{-1} in the sublethal and optimum salinity, respectively). Maximum and final water loss rates did not differ either (Tables 2 and S4). The mean water content at death (WC_f) was 2.58 ± 0.07 mg (i.e. approx. 52 % of WC_s).

A clear cross-tolerance response was observed in *N. baeticus*; individuals exposed to sublethal salinity showed higher survival than those exposed to optimum salinity ($\chi^2 = 6.5$, $P = 0.011$; Fig. 2). Similarly to *E. jesusarribasi*, water content after the sublethal salinity exposure (5.17 ± 0.10 mg) was higher than in the optimum one (4.83 ± 0.08 mg) (Table 1). The change in water loss rate with exposure time was not linear, reaching a maximum at 4 h in both pre-treatments (Fig. S1). In agreement with survival patterns, mean and final water loss rates during desiccation were slightly but significantly lower in individuals from the sublethal salinity pre-treatment (mean water loss rate: 0.226 ± 0.012 mg h^{-1}) if compared with those from the optimum one (mean water loss rate: 0.262 ± 0.069 mg h^{-1}). However, maximum water loss rates did not differ between treatments (Tables 2 and S4). The mean water content at death (WC_f) was 2.53 ± 0.06 mg (i.e. approx 59 % of WC_s).

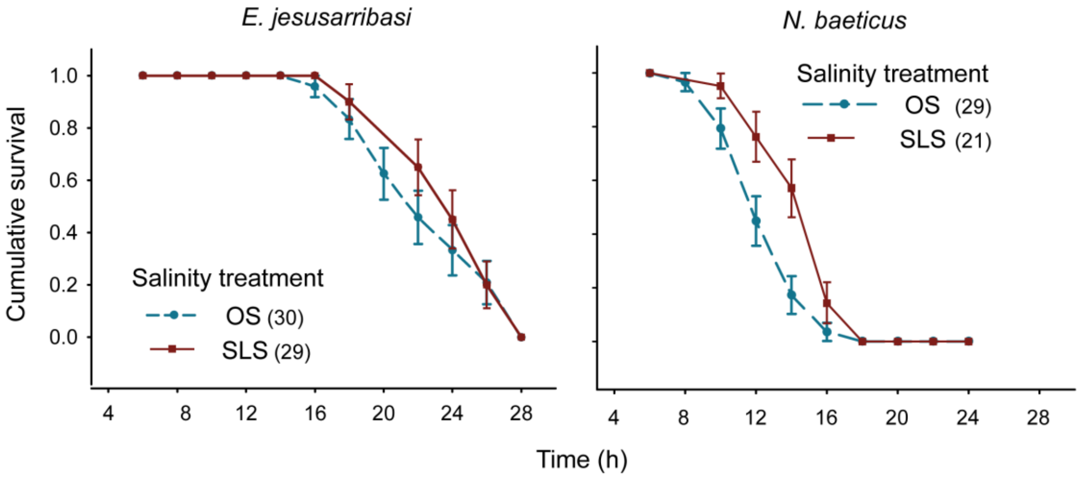


Figure 2. Kaplan-Meir survivorship curves along exposure to desiccation for each salinity pre-treatment. Each data point represents survival probability ± s.e. Numbers in parentheses indicate the number of specimens in each group. OS: optimum salinity, SLS: sublethal salinity.

2 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 slow desiccation treatment.

Table 1. GLM results on the differences in water content between salinity pre-treatments (Trat), and effect of individuals´ body mass (M_s) and sex in the experiment testing the effect of salinity on desiccation resistance.

Species	Predictors	Slope ± SE	df	F-statistic	Explained deviance (%)
E. jesusarribasi	Intercept	-0.415 ± 0.153**			96.8
	Trat (SLS)	0.185 ± 0.043***	1	327.203***	
	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***			76.6
	Trat (SLS)	0.083 ± 0.073	1	22.271***	
	M_s	0.563 ± 0.050***	1	127.984***	
	Sex (male)	-0.057 ± 0.071	1	0.629	

Significance levels: *P < 0.05; **; P < 0.01; ***P < 0.001.
SLS: sublethal salinity

In *E. jesusarribasi*, survival under stressful salinity conditions after the slow desiccation exposure showed a rapid decline if compared with that of individuals exposed to rapid desiccation or not desiccated (Log rank test CD vs SD: $\chi^2 = 14.4$, $P < 0.001$; Fig. 3). Although differences in mean survival between the rapid desiccation and the control were not significant (Log rank test: $\chi^2 = 1.2$, $P = 0.277$), Kaplan-Meier survivorship curves showed a better performance in individuals previously subjected to rapid desiccation 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 water loss rates were recorded in the rapid desiccation (in accordance with the nature of this treatment), but specimens lost a significantly higher amount of water (lower WC_d) in the longer slow desiccation pre-treatment (Tables 3 and S5, Fig. 4). Haemolymph osmolality differed among treatments ($\chi^2 = 10.1$, $P = 0.006$) and exposure time ($\chi^2 = 7.5$, $P = 0.023$). Specimens’ osmotic concentration at time 0 (i.e. after the pre-treatment) in the rapid desiccation treatment was higher than both the control and the

slow desiccation one ($P < 0.05$ in Dunn's post hoc comparisons), but remained stable along salinity exposure. In contrast, in specimens from the slow desiccation pre-treatment and the control, haemolymph osmolality significantly increased along the salinity exposure ($P < 0.05$) (Fig. 5).

In *N. baeticus*, the specimens exposed to the rapid and slow desiccation pre-treatments showed lower survival to salinity than those from the control (Log rank test CD vs SD: $\chi^2 = 4.1$, $P = 0.043$; CD vs RD: $\chi^2 = 6.5$, $P = 0.011$). Such a decline in performance occurred in both treatments after 12 h of exposure to salinity (Fig. 3). The rapid desiccation produced a significantly higher water loss rates than the slow desiccation and control treatments, but the final water content was similar between both desiccation pre-treatments (Tables 3 and S5, Fig. 4). Haemolymph osmolality followed a similar temporal variation pattern in all treatments, remaining stable along salinity exposure ($\chi^2 = 4.5$, $P = 0.106$) but differed in magnitude among treatments ($\chi^2 = 13.4$, $P = 0.001$). Specimens previously exposed to slow desiccation showed a higher osmotic concentration than those from the control and rapid desiccation 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 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 tolerances to these co-occurring stressors in water beetles, which could have played a key role in the colonisation of these systems and may have important implications in the context of climate change.

Table 2. GLM results on the differences in mean (WLR_{mean}), maximum (WLR_{max}) and final (WLR_f) water loss rate under desiccation between salinity pre-treatments (Trat), and effect of individuals´ initial water content (WC_s) and sex in the experiment testing the effect of salinity on desiccation resistance.

Species	Variable	Predictors	Slope ± SE	df	F-statistic	Explained deviance (%)
E. jesusarribasi	WLR_{mean}	Intercept	0.033 ± 0.026			19.2
		Trat (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
		Trat (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
		Trat (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
		Trat (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
		Trat (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
		Trat (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	

Significance levels: *P < 0.05;**; P < 0.01; ***P < 0.001
SLS: sublethal salinity

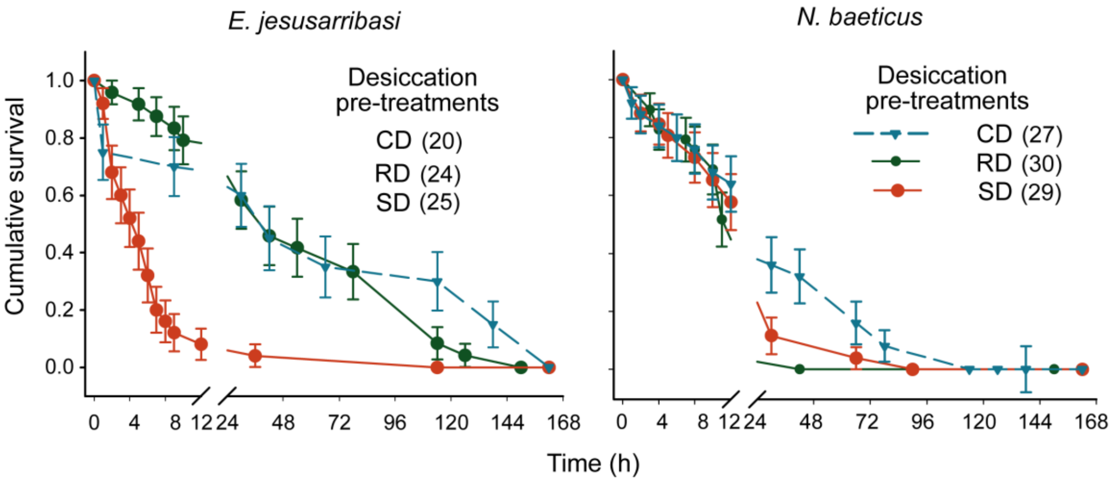


Figure 3. Kaplan-Meir survivorship curves along exposure to salinity for each desiccation pre-treatment. Each data point represents survival probability ± s.e. Numbers in parentheses indicate the number of specimens in each group. CD: control desiccation, SD: slow desiccation, RD: rapid desiccation.

Table 3. GLM results on variation in water loss rate (WLR) and final water content (WC_d) between desiccation pre-treatments (Trat) and effect of individuals´ initial water content (WC_0) and sex in the experiment testing the effect of desiccation on salinity tolerance.

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

Significance levels: *P < 0.05; **; P < 0.01; ***P < 0.001
RD: rapid desiccation, SD: slow desiccation

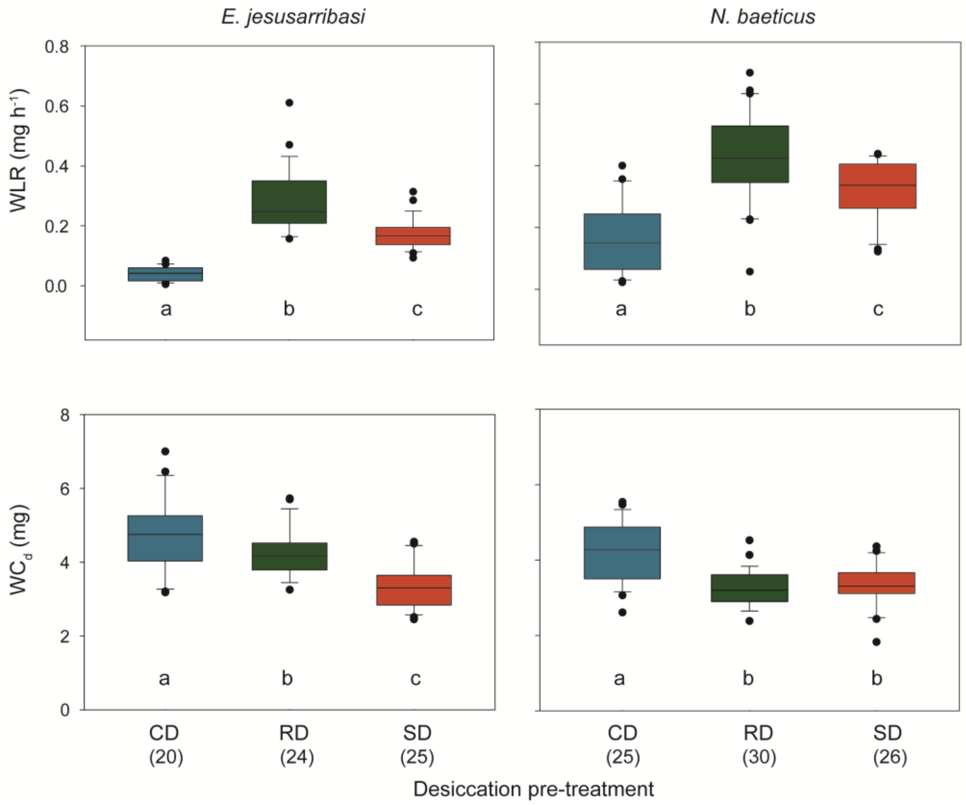


Figure 4. Water loss rate and final water content in each desiccation pre-treatment. Box length represents the interquartile range (IQR) of the data and whiskers are 1.5 times the IQR. Data outside of this range are represented as points. Letters indicate significant differences between treatments (Bonferroni post-hoc comparisons, P<0.05) and numbers in parentheses indicate the number of specimens in each group. CD: control desiccation, SD: slow desiccation, RD: rapid desiccation.

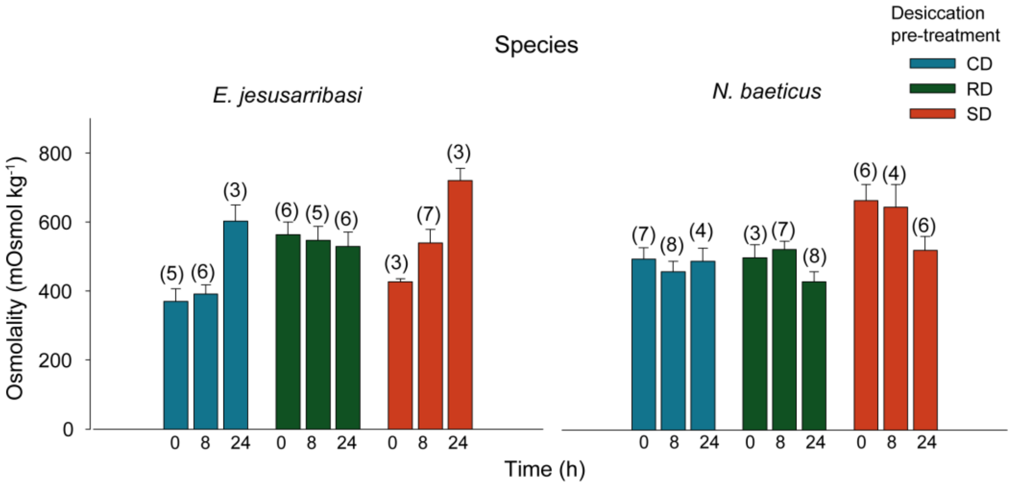


Figure 5. Haemolymph osmolality measured immediately after desiccation (time 0) and after 8 and 24 h of exposure to sublethal salinity (2470 and 1580 mOsmol kg^{-1} for *E. jesusarribasi* and *N. baeticus*, respectively). Bars represent mean ± s.e. and numbers in parentheses indicate the number of samples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

Physiological mechanisms linking tolerances to salinity and desiccation

In our first experiment, specimens of the two species studied 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 extend survival time in *N. baeticus* and had little effect, but still positive, on the performance of *E. jesusarribasi*.

Although the 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 the water loss by osmosis, a common behavior in other saline aquatic insects such as mosquito larvae (e.g. Bradley & Phillips, 1975; Patrick & 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 ingesting 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 & King, 1985; Machin & O’Donnell, 1991; Ramsay, 1994). The pre-activation of these osmoregulatory organs and tissues to maintain water and osmotic balance during salinity exposure likely contributed to minimize water loss along 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. Terblanche *et al.*, 2010; Stinziano *et al.*, 2015), although its role in aquatic insects has been less explored (e.g. Jacob & Hanssen, 1986; Alarie *et al.*, 1998). 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 the aerial medium and therefore would have a relatively 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 *vs* 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 rate and total water loss produced. 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 & Hatting, 1988; Naidu, 2001) and *Drosophila* (Albers & Bradley, 2004). Nevertheless, osmolalities 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 specimens were subjected to rapid dehydration, this species 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 detrimentral under the subsequent salinity stress in the long-term for *E. jesusarribasi*. 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 following 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. Todgham *et al*., 2005; Kleynhans *et al*., 2014).

Ecological and evolutionary implications of linked salinity and desiccation tolerances

To our knowledge, only one case of cross-tolerance between salinity and desiccation has been previously reported in insects, specifically in larvae of the Antarctic midge *Belgica antarctica* Jacobs, 1900 (Elnitsky *et al*., 2009). However, there is evidences of cross-tolerance between salinity and thermal tolerance in diverse saline-tolerant taxa in inland waters, as for the branchiopod *Daphnia pulex* (Linnaeus, 1758) (Chen & Stillman, 2012), the water boatmen *Tricocorixa verticalis* (Fieber, 1851) and *Sigara lateralis* (Leach, 1817) (Coccia *et al*., 2013) as well as several water beetle species, including those studied here (Sánchez-Fernández *et al*., 2010; Arribas *et al*., 2012b; Botella-Cruz *et al*., 2016). The finding of these patterns in ecologically similar species from different lineages highlights the ecological relevance of the cross-tolerance phenomenom (Hochacka & 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 waters, drying is often preceded by an increase in salinity levels. Acclimatization to such increasing salinities might allow insect populations showing crossed-tolerances 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 *vs* autumn-winter) could show different desiccation and thermal resistance, in relation to the seasonal salinity levels of their habitat (e.g. Kalra & Parkash, 2016).

Considering the predicted intensification of droughts and water salinization across the Mediterranean region (Sala *et al*., 2000; Bonada & Resh, 2013; Filipe *et al*., 2013), 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 (Sánchez-Fernández *et al*., 2010; Arribas *et al*., 2012b), compromising their persistence in their current localities.

Salinity and desiccation play essential roles on the distribution and diversification of aquatic lineages. Tolerances 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 both tolerances 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 colonisation 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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Table S1. Data on the experiment testing the effect of salinity on desiccation resistance.

Species	treat	cond	ID	M_s	M_f	M_{dry}	sex	WC_s	$\%WC_s$	surv. time	status	WLR_{mean}	$\%WLR_{mean}$	WLR_{max}	$\%WLR_{max}$	WLR_f	WLR_f	WC_f	$\% WC_f$
E. jesusarribasi	OP	50	JO18	5.62	4.08	2.02	F	3.6	64.06	20	1	0.084	1.49	0.084	1.49	0.086	1.52	2.06	50.49
E. jesusarribasi	OP	50	JO26	5.69	NA	1.77	M	3.92	68.89	28	0	0.136	2.40	NA	NA	NA	NA	NA	NA
E. jesusarribasi	OP	50	JO17	5.8	4.39	1.88	F	3.92	67.59	22	1	0.099	1.70	0.103	1.77	0.097	1.67	2.51	50.34
E. jesusarribasi	OP	50	JO19	6.06	4.51	2.18	M	3.88	64.03	22	1	0.084	1.39	0.086	1.42	0.084	1.38	2.33	51.22
E. jesusarribasi	OP	50	JO5	6.08	5.38	2.22	M	3.86	63.49	28	1	0.077	1.26	0.092	1.51	0.096	1.58	3.16	46.84
E. jesusarribasi	OP	50	JO6	6.08	3.97	2.09	M	3.99	65.63	24	1	0.092	1.51	0.080	1.32	0.082	1.35	1.88	47.36
E. jesusarribasi	OP	50	JO4	6.36	4.83	2.2	M	4.16	65.41	26	1	0.081	1.27	0.084	1.33	0.080	1.26	2.63	46.17
E. jesusarribasi	OP	50	JO14	6.42	4.27	2.06	M	4.36	67.91	24	1	0.096	1.49	0.098	1.52	0.098	1.52	2.21	51.76
E. jesusarribasi	OP	50	JO12	6.55	3.86	2.2	M	4.35	66.41	22	1	0.095	1.45	0.097	1.48	0.102	1.56	1.66	51.30
E. jesusarribasi	OP	50	JO25	6.57	NA	2.34	M	4.23	64.38	28	0	0.171	2.60	NA	NA	NA	NA	NA	NA
E. jesusarribasi	OP	50	JO11	6.63	4.23	2.12	M	4.51	68.02	18	1	0.143	2.15	0.143	2.15	0.150	2.26	2.11	49.88
E. jesusarribasi	OP	50	JO9	6.64	4.49	2.26	F	4.38	65.96	18	1	0.124	1.87	0.124	1.87	0.134	2.02	2.23	49.67
E. jesusarribasi	OP	50	JO21	6.71	4.95	2.08	M	4.63	69.00	20	1	0.132	1.96	0.133	1.97	0.129	1.93	2.87	50.10
E. jesusarribasi	OP	50	JO22	6.9	6.22	2.39	M	4.51	65.36	28	1	0.084	1.22	0.091	1.32	0.088	1.28	3.83	53.05
E. jesusarribasi	OP	50	JO20	7.03	5.2	2.6	F	4.43	63.02	26	1	0.085	1.21	0.089	1.27	0.092	1.30	2.6	50.00
E. jesusarribasi	OP	50	JO33	7.03	NA	2.42	F	4.61	65.58	28	0	0.090	1.28	NA	NA	NA	NA	NA	NA
E. jesusarribasi	OP	50	JO34	7.44	NA	2.46	M	4.98	66.94	28	0	0.088	1.19	NA	NA	NA	NA	NA	NA
E. jesusarribasi	OP	50	JO16	7.65	4.43	2.6	M	5.05	66.01	26	1	0.099	1.30	0.102	1.33	0.102	1.33	1.83	50.34
E. jesusarribasi	OP	50	JO8	7.74	5.41	2.47	M	5.27	68.09	20	1	0.153	1.98	0.157	2.03	0.155	2.00	2.94	49.35
E. jesusarribasi	OP	50	JO7	7.9	4.61	2.86	F	5.04	63.80	28	1	0.097	1.23	0.099	1.25	0.097	1.23	1.75	48.16
E. jesusarribasi	OP	50	JO30	7.92	NA	2.71	M	5.21	65.78	28	0	0.101	1.27	NA	NA	NA	NA	NA	NA
E. jesusarribasi	OP	50	JO1	7.95	3.94	2.87	M	5.08	63.90	28	1	0.104	1.31	0.106	1.33	0.101	1.27	1.07	43.65
E. jesusarribasi	OP	50	JO10	7.99	5.01	2.52	F	5.47	68.46	24	1	0.137	1.72	0.141	1.76	0.135	1.70	2.49	49.70
E. jesusarribasi	OP	50	JO27	8.08	NA	3.09	F	4.99	61.76	28	0	0.079	0.98	NA	NA	NA	NA	NA	NA
E. jesusarribasi	OP	50	JO2	8.11	5.31	2.99	F	5.12	63.13	20	1	0.167	2.06	0.169	2.08	0.156	1.92	2.32	43.69
E. jesusarribasi	OP	50	JO15	8.16	4.38	2.74	F	5.42	66.42	20	1	0.152	1.87	0.153	1.87	0.153	1.87	1.64	52.51
E. jesusarribasi	OP	50	JO23	8.24	5.16	2.42	F	5.82	70.63	16	1	0.220	2.67	0.220	2.67	0.220	2.67	2.74	53.10
E. jesusarribasi	OP	50	JO28	8.39	NA	2.88	F	5.51	65.67	28	0	0.087	1.04	NA	NA	NA	NA	NA	NA
E. jesusarribasi	OP	50	JO29	8.69	NA	2.87	F	5.82	66.97	28	0	0.098	1.13	NA	NA	NA	NA	NA	NA
E. jesusarribasi	OP	50	JO31	8.69	NA	2.87	F	5.82	66.97	28	0	0.102	1.18	NA	NA	NA	NA	NA	NA
E. jesusarribasi	OP	50	JO13	8.91	5.71	2.77	F	6.14	68.91	18	1	0.202	2.27	0.202	2.27	0.200	2.24	2.94	51.49

E. jesusarribasi	OP	50	JO32	8.94	NA	3.03	F	5.91	66.11	28	0	0.101	1.13	NA	NA	NA	NA	NA	NA
E. jesusarribasi	OP	50	JO3	9.13	5.32	2.92	M	6.21	68.02	28	1	0.111	1.21	0.115	1.26	0.112	1.23	2.4	46.05
E. jesusarribasi	OP	50	JO24	10.1	6.16	2.84	F	7.3	71.99	22	1	0.196	1.94	0.201	1.98	0.199	1.96	3.32	53.90
E. jesusarribasi	SLS	100	JS19	6.76	4.9	2.09	F	4.67	69.08	18	1	0.109	1.62	0.109	1.62	0.107	1.58	2.81	49.18
E. jesusarribasi	SLS	100	JS1	6.8	5.33	2.11	F	4.69	68.97	22	1	0.117	1.72	0.118	1.74	0.119	1.74	3.22	53.28
E. jesusarribasi	SLS	100	JS6	6.92	4.99	2.05	M	4.87	70.38	24	1	0.118	1.71	0.123	1.78	0.124	1.79	2.94	55.91
E. jesusarribasi	SLS	100	JS14	6.97	NA	2.29	M	4.68	67.14	28	0	0.077	1.10	NA	NA	NA	NA	NA	NA
E. jesusarribasi	SLS	100	JS20	7	5.09	2.18	M	4.82	68.86	22	1	0.141	2.02	0.144	2.06	0.141	2.01	2.91	49.90
E. jesusarribasi	SLS	100	JS10	7.36	5.56	2.18	F	5.18	70.38	28	1	0.102	1.39	0.107	1.46	0.102	1.38	3.38	57.01
E. jesusarribasi	SLS	100	JS22	7.51	4.2	2.49	M	5.02	66.84	28	1	0.100	1.33	0.102	1.36	0.100	1.34	1.71	51.19
E. jesusarribasi	SLS	100	JS11	7.59	6.41	2.36	F	5.23	68.91	26	1	0.100	1.32	0.104	1.37	0.109	1.44	4.05	57.41
E. jesusarribasi	SLS	100	JS21	7.6	5.35	2.49	F	5.11	67.24	24	1	0.111	1.46	0.116	1.53	0.103	1.36	2.86	50.09
E. jesusarribasi	SLS	100	JS25	7.66	4.97	2.31	F	5.35	69.84	26	1	0.099	1.29	0.111	1.45	0.098	1.29	2.66	52.52
E. jesusarribasi	SLS	100	JS27	7.66	5.29	2.28	M	5.38	70.23	28	1	0.098	1.28	0.102	1.33	0.099	1.29	3.01	56.33
E. jesusarribasi	SLS	100	JS28	7.68	7.02	2.2	F	5.48	71.35	24	1	0.120	1.56	0.126	1.65	0.122	1.59	4.82	56.98
E. jesusarribasi	SLS	100	JS9	7.76	NA	2.19	F	5.57	71.78	28	0	0.085	1.10	NA	NA	NA	NA	NA	NA
E. jesusarribasi	SLS	100	JS24	7.78	4.43	2.36	F	5.42	69.67	24	1	0.116	1.49	0.119	1.53	0.116	1.49	2.07	52.37
E. jesusarribasi	SLS	100	JS23	7.81	5.74	2.55	F	5.26	67.35	26	1	0.117	1.49	0.124	1.59	0.113	1.45	3.19	51.57
E. jesusarribasi	SLS	100	JS29	7.81	NA	2.6	M	5.21	66.71	28	0	0.091	1.16	NA	NA	NA	NA	NA	NA
E. jesusarribasi	SLS	100	JS3	8.02	5.97	2.67	M	5.35	66.71	26	1	0.109	1.36	0.111	1.39	0.111	1.39	3.3	53.94
E. jesusarribasi	SLS	100	JS4	8.13	5.23	2.39	F	5.74	70.60	18	1	0.166	2.05	0.166	2.05	0.161	1.98	2.84	54.88
E. jesusarribasi	SLS	100	JS12	8.46	5.05	2.78	M	5.68	67.14	28	1	0.095	1.12	0.107	1.27	0.105	1.24	2.27	58.61
E. jesusarribasi	SLS	100	JS15	8.72	NA	2.8	M	5.92	67.89	28	0	0.085	0.97	NA	NA	NA	NA	NA	NA
E. jesusarribasi	SLS	100	JS30	8.73	NA	2.84	F	5.89	67.47	28	0	0.077	0.88	NA	NA	NA	NA	NA	NA
E. jesusarribasi	SLS	100	JS7	8.75	NA	2.5	M	6.25	71.43	28	0	0.112	1.28	NA	NA	NA	NA	NA	NA
E. jesusarribasi	SLS	100	JS17	9.18	5.6	2.75	M	6.43	70.04	22	1	0.168	1.83	0.170	1.85	0.161	1.75	2.85	47.14
E. jesusarribasi	SLS	100	JS5	9.22	5.1	2.96	F	6.26	67.90	26	1	0.147	1.59	0.151	1.64	0.151	1.64	2.14	55.29
E. jesusarribasi	SLS	100	JS16	9.37	NA	2.79	F	6.58	70.22	28	0	0.097	1.04	NA	NA	NA	NA	NA	NA
E. jesusarribasi	SLS	100	JS18	9.67	4.18	2.73	F	6.94	71.77	22	1	0.166	1.71	0.169	1.74	0.163	1.69	1.45	47.85
E. jesusarribasi	SLS	100	JS2	9.82	4.72	3.02	M	6.8	69.25	22	1	0.153	1.56	0.159	1.62	0.140	1.43	1.7	53.81
E. jesusarribasi	SLS	100	JS8	9.97	NA	3.14	M	6.83	68.51	28	0	0.105	1.06	NA	NA	NA	NA	NA	NA
E. jesusarribasi	SLS	100	JS13	10.3	NA	2.86	F	7.45	72.26	28	0	0.105	1.02	NA	NA	NA	NA	NA	NA
N. baeticus	OP	20	BO16	4.79	3.02	1.09	F	3.7	77.24	8	1	0.294	6.14	0.318	1.59	0.318	1.59	1.93	63.91
N. baeticus	OP	20	BO29	5.46	3.7	1.14	F	4.32	79.12	10	1	0.244	4.48	0.305	1.53	0.215	1.08	2.56	69.19
N. baeticus	OP	20	BO8	5.78	3.99	1.66	F	4.12	71.28	10	1	0.242	4.19	0.258	1.29	0.233	1.17	2.33	58.40

N. baeticus	OP	20	BO27	5.84	3.18	1.52	F	4.32	73.97	10	1	0.366	6.27	0.440	2.20	0.377	1.88	1.66	52.20
N. baeticus	OP	20	BO24	5.99	3.17	1.22	F	4.77	79.63	12	1	0.293	4.89	0.330	1.65	0.306	1.53	1.95	61.51
N. baeticus	OP	20	BO28	6.03	3.78	1.51	F	4.52	74.96	12	1	0.269	4.46	0.330	1.65	0.244	1.22	2.27	60.05
N. baeticus	OP	20	BO22	6.06	4.16	1.96	M	4.1	67.66	14	1	0.188	3.11	0.235	1.18	0.159	0.80	2.2	52.88
N. baeticus	OP	20	BO18	6.16	3.81	1.5	M	4.66	75.65	12	1	0.268	4.36	0.330	1.65	0.255	1.28	2.31	60.63
N. baeticus	OP	20	BO26	6.16	3.92	1.66	M	4.5	73.05	14	1	0.234	3.79	0.325	1.63	0.200	1.00	2.26	57.65
N. baeticus	OP	20	BO15	6.17	3.67	1.63	M	4.54	73.58	14	1	0.243	3.93	0.265	1.33	0.218	1.09	2.04	55.59
N. baeticus	OP	20	BO5	6.22	4.12	1.68	F	4.54	72.99	12	1	0.266	4.28	0.355	1.78	0.235	1.18	2.44	59.22
N. baeticus	OP	20	BO20	6.29	3.88	1.72	F	4.57	72.66	14	1	0.248	3.94	0.310	1.55	0.215	1.08	2.16	55.67
N. baeticus	OP	20	BO1	6.53	4.25	1.63	M	4.9	75.04	16	1	0.216	3.31	0.300	1.50	0.167	0.83	2.62	61.65
N. baeticus	OP	20	BO19	6.54	4.43	1.65	F	4.89	74.77	16	1	0.182	2.78	0.358	1.79	0.324	1.62	2.78	62.75
N. baeticus	OP	20	BO7	6.54	3.67	1.3	F	5.24	80.12	12	1	0.297	4.55	0.235	1.18	0.163	0.82	2.37	64.58
N. baeticus	OP	20	BO11	6.71	3.72	1.5	M	5.21	77.65	12	1	0.296	4.41	0.480	2.40	0.324	1.62	2.22	59.68
N. baeticus	OP	20	BO3	6.71	3.65	1.65	F	5.06	75.41	12	1	0.367	5.47	0.340	1.70	0.319	1.59	2	54.79
N. baeticus	OP	20	BO6	6.72	4.39	1.67	F	5.05	75.15	14	1	0.215	3.19	0.245	1.23	0.186	0.93	2.72	61.96
N. baeticus	OP	20	BO2	6.73	4.58	1.37	M	5.36	79.64	14	1	0.218	3.24	0.295	1.48	0.172	0.86	3.21	70.09
N. baeticus	OP	20	BO12	6.82	3.83	1.64	M	5.18	75.95	14	1	0.279	4.09	0.318	1.59	0.282	1.41	2.19	57.18
N. baeticus	OP	20	BO10	7.03	4.46	1.94	M	5.09	72.40	16	1	0.195	2.77	0.219	1.09	0.197	0.98	2.52	56.50
N. baeticus	OP	20	BO13	7.05	4.31	1.92	F	5.13	72.77	16	1	0.256	3.64	0.378	1.89	0.200	1.00	2.39	55.45
N. baeticus	OP	20	BO9	7.14	5.05	1.81	F	5.33	74.65	10	1	0.316	4.43	0.358	1.79	0.322	1.61	3.24	64.16
N. baeticus	OP	20	BO14	7.23	4.42	1.85	M	5.38	74.41	18	1	0.225	3.11	0.313	1.56	0.184	0.92	2.57	58.14
N. baeticus	OP	20	BO25	7.42	5.31	1.95	F	5.47	73.72	12	1	0.233	3.14	0.255	1.28	0.224	1.12	3.36	63.28
N. baeticus	OP	20	BO17	7.49	5.23	2.63	M	4.86	64.89	14	1	0.216	2.89	0.273	1.36	0.191	0.96	2.6	49.71
N. baeticus	OP	20	BO30	7.53	5.34	2.66	M	4.87	64.67	12	1	0.262	3.48	0.320	1.60	0.234	1.17	2.68	50.19
N. baeticus	OP	20	BO21	7.63	5.2	2.77	M	4.86	63.70	10	1	0.373	4.89	0.445	2.23	0.345	1.73	2.43	46.73
N. baeticus	OP	20	BO4	7.78	5.4	2.14	M	5.64	72.49	12	1	0.312	4.01	0.445	2.23	0.264	1.32	3.26	60.37
N. baeticus	SLS	70	BS35	5.16	5.38	1.19	F	3.97	76.94	12	1	0.174	3.38	0.190	0.27	0.169	0.24	4.19	65.99
N. baeticus	SLS	70	BS42	5.51	3.5	1.4	M	4.11	74.59	12	1	0.236	4.28	0.245	0.35	0.244	0.35	2.1	66.00
N. baeticus	SLS	70	BS50	6.51	3.71	1.76	M	4.75	72.96	16	1	0.172	2.65	0.205	0.29	0.163	0.23	1.95	66.58
N. baeticus	SLS	70	BS60	6.53	5.56	1.67	F	4.86	74.43	14	1	0.173	2.65	0.255	0.36	0.188	0.27	3.89	53.96
N. baeticus	SLS	70	BS44	6.55	4.24	1.24	M	5.31	81.07	17	1	0.213	3.25	0.303	0.43	0.179	0.26	3	60.61
N. baeticus	SLS	70	BS41	6.65	3.92	1.87	F	4.78	71.88	14	1	0.304	4.58	0.575	0.82	0.234	0.33	2.05	58.67
N. baeticus	SLS	70	BS53	6.67	3.9	1.54	M	5.13	76.91	16	1	0.192	2.88	0.237	0.34	0.208	0.30	2.36	60.51
N. baeticus	SLS	70	BS39	6.7	4.98	1.62	F	5.08	75.82	10	1	0.375	5.59	0.505	0.72	0.372	0.53	3.36	55.02
N. baeticus	SLS	70	BS38	6.75	4.82	1.87	M	4.88	72.30	16	1	0.212	3.14	0.363	0.52	0.161	0.23	2.95	63.69

N. baeticus	SLS	70	BS33	6.97	4.63	1.63	F	5.34	76.61	12	1	0.289	4.14	0.400	0.57	0.250	0.36	3	61.56
N. baeticus	SLS	70	BS55	7.1	4.95	1.65	M	5.45	76.76	16	1	0.248	3.49	0.323	0.46	0.236	0.34	3.3	51.92
N. baeticus	SLS	70	BS34	7.21	5.19	1.78	F	5.43	75.31	16	1	0.178	2.47	0.243	0.35	0.172	0.25	3.41	51.06
N. baeticus	SLS	70	BS32	7.3	5.02	1.83	F	5.47	74.93	16	1	0.142	1.95	0.175	0.25	0.132	0.19	3.19	58.76
N. baeticus	SLS	70	BS56	7.4	4.71	2.41	M	4.99	67.43	12	1	0.264	3.57	0.350	0.50	0.216	0.31	2.3	65.39
N. baeticus	SLS	70	BS36	7.48	3.85	1.86	M	5.62	75.13	17	1	0.167	2.23	0.201	0.29	0.178	0.25	1.99	57.14
N. baeticus	SLS	70	BS45	7.53	4.01	1.75	M	5.78	76.76	16	1	0.211	2.81	0.273	0.39	0.201	0.29	2.26	53.37
N. baeticus	SLS	70	BS31	7.56	4.25	2.24	M	5.32	70.37	14	1	0.225	2.97	0.267	0.38	0.221	0.32	2.01	56.00
N. baeticus	SLS	70	BS40	7.65	3.24	2.07	F	5.58	72.94	16	1	0.236	3.08	0.463	0.66	0.191	0.27	1.17	56.79
N. baeticus	SLS	70	BS54	7.79	4.16	2.38	F	5.41	69.45	16	1	0.229	2.93	0.315	0.45	0.197	0.28	1.78	57.69
N. baeticus	SLS	70	BS37	8.16	4.61	2.56	M	5.6	68.63	14	1	0.299	3.67	0.553	0.79	0.227	0.32	2.05	59.65
N. baeticus	SLS	70	BS43	8.16	5.27	2.54	F	5.62	68.87	17	1	0.211	2.59	0.260	0.37	0.184	0.26	2.73	54.27

treat: salinity pre-treatment (OP: optimum, SLS: sublethal); **cond**: conductivity of pre-treatment (ms cm^{-1}); **ID**: specimen identity number; **M_s**: initial fresh mass (before desiccation), mg; **M_f**: final fresh mass (at death), mg; **M_{dry}**: dry mass, mg; **WC_s**: initial water content (before desiccation), mg; **% WC_s**: percentage water content, % of M_s; **surv. time**: survival time, h; **status**: specimen status (0 = alive, 1 = dead); **WLR_{mean}, WLR_{max}** and **WLR_f**: mean, maximum and final water loss rate, mg h^{-1}; **%WLR_{mean}, % WLR_{max}, %WLR_f**: percentage mean, maximum and final water loss rate, % of M_s h^{-1}, **WC_f**: final water content (at death), mg; **%WC_f**: percentage final water content (at death), % of M_f

Table S2. Data on the experiment testing the effect of desiccation on salinity tolerance (survival).

species	treat	RH	exp. time	ID	M_0	M_d	M_{dry}	status 1	sex	WC_0	$\%WC_0$	WC_d	$\%WC_d$	WLR	%WLR	t_1	t_2	status 2
Enochrus jesusarribasi	CD	90	12	JC1	8.44	7.91	2.44	0	M	6	71.09	5.47	69.15	0.044	0.523	156	168	3
Enochrus jesusarribasi	CD	90	12	JC10	8.77	8.07	2.79	0	F	5.98	68.19	5.28	65.43	0.058	0.665	1	1	1
Enochrus jesusarribasi	CD	90	12	JC11	6.34	5.98	2.13	0	NA	4.21	66.40	3.85	64.38	0.023	0.355	36	48	3
Enochrus jesusarribasi	CD	90	12	JC12	6.59	5.97	1.92	0	F	4.67	70.86	4.05	67.84	0.052	0.784	132	144	3
Enochrus jesusarribasi	CD	90	12	JC13	10.25	9.84	2.84	0	F	7.41	72.29	7	71.14	0.034	0.333	156	168	3
Enochrus jesusarribasi	CD	90	12	JC14	6.55	6.07	1.74	0	M	4.81	73.44	4.33	71.33	0.040	0.611	60	72	3
Enochrus jesusarribasi	CD	90	12	JC15	6.83	6.1	2.07	0	F	4.76	69.69	4.03	66.07	0.061	0.891	1	1	1
Enochrus jesusarribasi	CD	90	12	JC16	6.32	6.07	2.18	0	M	4.14	65.51	3.89	64.09	0.016	0.247	36	48	3
Enochrus jesusarribasi	CD	90	12	JC17	7.61	7.4	2.82	0	F	4.79	62.94	4.58	61.89	0.013	0.172	132	144	3
Enochrus jesusarribasi	CD	90	12	JC18	7.83	7.62	2.69	0	M	5.14	65.64	4.93	64.70	0.013	0.168	24	36	3
Enochrus jesusarribasi	CD	90	12	JC19	10.5	9.63	3.18	0	M	7.32	69.71	6.45	66.98	0.073	0.690	24	36	3
Enochrus jesusarribasi	CD	90	12	JC2	7.03	6.88	2.21	0	M	4.82	68.56	4.67	67.88	0.009	0.133	36	48	3
Enochrus jesusarribasi	CD	90	12	JC20	4.74	4.66	1.48	0	M	3.26	68.78	3.18	68.24	0.005	0.105	108	120	3
Enochrus jesusarribasi	CD	90	12	JC3	7.4	6.85	1.75	0	F	5.65	76.35	5.1	74.45	0.046	0.619	156	168	3
Enochrus jesusarribasi	CD	90	12	JC4	7.45	7.08	2.82	0	M	4.63	62.15	4.26	60.17	0.031	0.414	1	1	1
Enochrus jesusarribasi	CD	90	12	JC5	5.56	5.1	1.9	0	M	3.66	65.83	3.2	62.75	0.038	0.689	9	9	1
Enochrus jesusarribasi	CD	90	12	JC6	8.05	7.31	2.12	0	M	5.93	73.66	5.19	71.00	0.062	0.766	132	144	3
Enochrus jesusarribasi	CD	90	12	JC7	8.95	8.34	2.96	0	F	5.99	66.93	5.38	64.51	0.051	0.568	1	1	1
Enochrus jesusarribasi	CD	90	12	JC8	8.24	7.41	2.45	0	M	5.79	70.27	4.96	66.94	0.069	0.839	60	72	3
Enochrus jesusarribasi	CD	90	12	JC9	8.46	7.45	2.61	0	M	5.85	69.15	4.84	64.97	0.084	0.995	1	1	1
Enochrus jesusarribasi	RD	10	3	JR1	8.03	6.2	2.95	0	M	5.08	63.26	3.25	52.42	0.610	7.597	2	2	1
Enochrus jesusarribasi	RD	10	3	JR10	7.19	6.52	2.57	0	F	4.62	64.26	3.95	60.58	0.223	3.106	24	36	3
Enochrus jesusarribasi	RD	10	3	JR11	6.83	6.34	2.16	0	F	4.67	68.37	4.18	65.93	0.163	2.391	24	36	3
Enochrus jesusarribasi	RD	10	3	JR12	7.5	6.32	2.45	0	M	5.05	67.33	3.87	61.23	0.393	5.244	7	7	1
Enochrus jesusarribasi	RD	10	3	JR13	7.93	6.96	2.66	0	M	5.27	66.46	4.3	61.78	0.323	4.077	24	36	3
Enochrus jesusarribasi	RD	10	3	JR14	6.39	5.72	2.07	0	M	4.32	67.61	3.65	63.81	0.223	3.495	72	84	3
Enochrus jesusarribasi	RD	10	3	JR15	7.19	6.51	2	0	M	5.19	72.18	4.51	69.28	0.227	3.153	108	120	3
Enochrus jesusarribasi	RD	10	3	JR16	6.49	6.02	1.88	0	M	4.61	71.03	4.14	68.77	0.157	2.414	108	120	3
Enochrus jesusarribasi	RD	10	3	JR17	6.35	5.86	1.99	0	M	4.36	68.66	3.87	66.04	0.163	2.572	108	120	3
Enochrus jesusarribasi	RD	10	3	JR18	8.81	7.92	2.72	0	F	6.09	69.13	5.2	65.66	0.297	3.367	48	60	3
Enochrus jesusarribasi	RD	10	3	JR19	6.66	5.6	2.15	0	F	4.51	67.72	3.45	61.61	0.353	5.305	5	5	1

Enochrus jesusarribasi	RD	10	3	JR2	7.2	5.79	2.34	0	F	4.86	67.50	3.45	59.59	0.470	6.528	9	9	1
Enochrus jesusarribasi	RD	10	3	JR20	7.25	6.63	2.08	0	F	5.17	71.31	4.55	68.63	0.207	2.851	120	132	3
Enochrus jesusarribasi	RD	10	3	JR21	8.29	7.18	2.59	0	F	5.7	68.76	4.59	63.93	0.370	4.463	36	48	3
Enochrus jesusarribasi	RD	10	3	JR22	7.31	6.67	2.39	0	F	4.92	67.31	4.28	64.17	0.213	2.918	36	48	3
Enochrus jesusarribasi	RD	10	3	JR23	7.09	6.02	2.4	0	M	4.69	66.15	3.62	60.13	0.357	5.031	10	10	1
Enochrus jesusarribasi	RD	10	3	JR24	9.34	8.5	2.8	0	F	6.54	70.02	5.7	67.06	0.280	2.998	108	120	3
Enochrus jesusarribasi	RD	10	3	JR3	7.4	6.62	2.38	0	M	5.02	67.84	4.24	64.05	0.260	3.514	36	48	3
Enochrus jesusarribasi	RD	10	3	JR4	7.09	6.51	2.21	0	M	4.88	68.83	4.3	66.05	0.193	2.727	144	156	3
Enochrus jesusarribasi	RD	10	3	JR5	6.95	6.34	2.3	0	M	4.65	66.91	4.04	63.72	0.203	2.926	72	84	3
Enochrus jesusarribasi	RD	10	3	JR6	9.54	8.56	2.83	0	M	6.71	70.34	5.73	66.94	0.327	3.424	24	36	3
Enochrus jesusarribasi	RD	10	3	JR7	7.08	6.06	2.3	0	M	4.78	67.51	3.76	62.05	0.340	4.802	12	24	3
Enochrus jesusarribasi	RD	10	3	JR8	7.16	6.5	2.36	0	M	4.8	67.04	4.14	63.69	0.220	3.073	108	120	3
Enochrus jesusarribasi	RD	10	3	JR9	7.86	7.16	2.64	0	F	5.22	66.41	4.52	63.13	0.233	2.969	108	120	3
Enochrus jesusarribasi	SD	40	12	JS1	6.83	5.32	2.02	0	M	4.81	70.42	3.3	62.03	0.126	1.842	8	8	1
Enochrus jesusarribasi	SD	40	12	JS10	10.09	7.56	3.01	0	F	7.08	70.17	4.55	60.19	0.211	2.090	6	6	1
Enochrus jesusarribasi	SD	40	12	JS11	7.66	5.42	2.41	0	M	5.25	68.54	3.01	55.54	0.187	2.437	2	2	1
Enochrus jesusarribasi	SD	40	12	JS12	8.26	5.55	2.54	0	F	5.72	69.25	3.01	54.23	0.226	2.734	2	2	1
Enochrus jesusarribasi	SD	40	12	JS13	6.86	5.47	2.1	0	F	4.76	69.39	3.37	61.61	0.116	1.689	6	6	1
Enochrus jesusarribasi	SD	40	12	JS14	6.8	4.56	2.11	0	NA	4.69	68.97	2.45	53.73	0.187	2.745	4	4	1
Enochrus jesusarribasi	SD	40	12	JS15	9.47	7.15	2.73	0	F	6.74	71.17	4.42	61.82	0.193	2.042	4	4	1
Enochrus jesusarribasi	SD	40	12	JS16	7.87	5.96	2.59	0	M	5.28	67.09	3.37	56.54	0.159	2.022	7	7	1
Enochrus jesusarribasi	SD	40	12	JS17	10.37	7.68	3.18	0	F	7.19	69.33	4.5	58.59	0.224	2.162	6	6	1
Enochrus jesusarribasi	SD	40	12	JS18	8.56	6.34	2.54	0	F	6.02	70.33	3.8	59.94	0.185	2.161	2	2	1
Enochrus jesusarribasi	SD	40	12	JS19	8.12	6.03	2.41	0	F	5.71	70.32	3.62	60.03	0.174	2.145	3	3	1
Enochrus jesusarribasi	SD	40	12	JS2	6.32	4.56	1.94	0	M	4.38	69.30	2.62	57.46	0.147	2.321	1	1	1
Enochrus jesusarribasi	SD	40	12	JS20	6.41	4.63	1.78	0	M	4.63	72.23	2.85	61.56	0.148	2.314	9	9	1
Enochrus jesusarribasi	SD	40	12	JS21	6.5	4.66	2.15	0	M	4.35	66.92	2.51	53.86	0.153	2.359	2	2	1
Enochrus jesusarribasi	SD	40	12	JS22	7.86	5.69	2.48	0	F	5.38	68.45	3.21	56.41	0.181	2.301	3	3	1
Enochrus jesusarribasi	SD	40	12	JS23	9.95	6.53	2.83	0	F	7.12	71.56	3.7	56.66	0.285	2.864	1	1	1
Enochrus jesusarribasi	SD	40	12	JS24	7.55	5.7	2.29	0	M	5.26	69.67	3.41	59.82	0.154	2.042	5	5	1
Enochrus jesusarribasi	SD	40	12	JS25	6.34	5.03	2	0	NA	4.34	68.45	3.03	60.24	0.109	1.722	108	120	3
Enochrus jesusarribasi	SD	40	12	JS3	7.64	6.09	2.43	0	M	5.21	68.19	3.66	60.10	0.129	1.691	7	7	1
Enochrus jesusarribasi	SD	40	12	JS4	7.39	5.52	2.36	0	M	5.03	68.06	3.16	57.25	0.156	2.109	7	7	1
Enochrus jesusarribasi	SD	40	12	JS5	8.55	6.18	2.7	0	M	5.85	68.42	3.48	56.31	0.198	2.310	5	5	1
Enochrus jesusarribasi	SD	40	12	JS6	5.65	4.53	1.72	0	NA	3.93	69.56	2.81	62.03	0.093	1.652	24	48	3

Enochrus jesusarribasi	SD	40	12	JS7	7.48	5.96	2.59	0	M	4.89	65.37	3.37	56.54	0.127	1.693	12	12	1
Enochrus jesusarribasi	SD	40	12	JS8	9.03	5.26	2.57	0	NA	6.46	71.54	2.69	51.14	0.314	3.479	2	2	1
Enochrus jesusarribasi	SD	40	12	JS9	7.19	5.19	2.48	0	M	4.71	65.51	2.71	52.22	0.167	2.318	2	2	1
Nebrioporus baeticus	CD	90	6	BC1	7.02	5.69	1.08	0	F	5.94	84.62	4.61	81.02	0.266	3.789	12	24	3
Nebrioporus baeticus	CD	90	6	BC10	6.54	5.43	1.63	0	M	4.91	75.08	3.8	69.98	0.222	3.394	108	120	3
Nebrioporus baeticus	CD	90	6	BC11	7.51	7.01	2.91	0	M	4.6	61.25	4.1	58.49	0.100	1.332	12	24	3
Nebrioporus baeticus	CD	90	6	BC12	6.28	6.15	1.51	0	F	4.77	75.96	4.64	75.45	0.026	0.414	60	72	3
Nebrioporus baeticus	CD	90	6	BC13	6.15	6.04	2.98	0	M	3.17	51.54	3.06	50.66	0.022	0.358	132	144	3
Nebrioporus baeticus	CD	90	6	BC14	8.22	6.96	2.57	0	M	5.65	68.73	4.39	63.07	0.252	3.066	12	24	3
Nebrioporus baeticus	CD	90	6	BC15	6.34	6.18	1.91	0	M	4.43	69.87	4.27	69.09	0.032	0.505	144	156	3
Nebrioporus baeticus	CD	90	6	BC16	7.01	6.81	1.56	0	M	5.45	77.75	5.25	77.09	0.040	0.571	144	156	3
Nebrioporus baeticus	CD	90	6	BC17	6.26	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Nebrioporus baeticus	CD	90	6	BC18	6.94	5.57	1.7	0	M	5.24	75.50	3.87	69.48	0.274	3.948	1	1	1
Nebrioporus baeticus	CD	90	6	BC19	4.64	4.32	1.11	0	F	3.53	76.08	3.21	74.31	0.064	1.379	12	24	3
Nebrioporus baeticus	CD	90	6	BC2	6.35	5.59	1.22	0	F	5.13	80.79	4.37	78.18	0.152	2.394	12	24	3
Nebrioporus baeticus	CD	90	6	BC20	6.74	6.31	1.15	0	M	5.59	82.94	5.16	81.77	0.086	1.276	12	12	1
Nebrioporus baeticus	CD	90	6	BC21	6.17	4.17	1.56	0	M	4.61	74.72	2.61	62.59	0.400	6.483	6	6	1
Nebrioporus baeticus	CD	90	6	BC22	5.02	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Nebrioporus baeticus	CD	90	6	BC23	5.54	4.92	1.13	0	F	4.41	79.60	3.79	77.03	0.124	2.238	4	4	1
Nebrioporus baeticus	CD	90	6	BC24	7.62	6.97	1.5	0	F	6.12	80.31	5.47	78.48	0.130	1.706	24	36	3
Nebrioporus baeticus	CD	90	6	BC25	5.72	4.97	1.64	0	F	4.08	71.33	3.33	67.00	0.150	2.622	2	2	1
Nebrioporus baeticus	CD	90	6	BC26	6.76	6.39	2.93	0	F	3.83	56.66	3.46	54.15	0.074	1.095	12	24	3
Nebrioporus baeticus	CD	90	6	BC27	6.94	6.62	1.56	0	F	5.38	77.52	5.06	76.44	0.064	0.922	132	144	3
Nebrioporus baeticus	CD	90	6	BC3	6.82	5.89	0.89	0	F	5.93	86.95	5	84.89	0.186	2.727	8	8	1
Nebrioporus baeticus	CD	90	6	BC4	6.45	6.19	1.46	0	F	4.99	77.36	4.73	76.41	0.052	0.806	108	120	3
Nebrioporus baeticus	CD	90	6	BC5	6.11	4.37	1.05	0	F	5.06	82.82	3.32	75.97	0.348	5.696	11	11	1
Nebrioporus baeticus	CD	90	6	BC6	7.87	6.91	1.37	0	M	6.5	82.59	5.54	80.17	0.192	2.440	108	120	3
Nebrioporus baeticus	CD	90	6	BC7	5.85	5.09	0.91	0	F	4.94	84.44	4.18	82.12	0.152	2.598	108	120	3
Nebrioporus baeticus	CD	90	6	BC8	6.71	4.93	1.39	0	M	5.32	79.28	3.54	71.81	0.356	5.306	1	1	1
Nebrioporus baeticus	CD	90	6	BC9	6.96	5.78	1.02	0	F	5.94	85.34	4.76	82.35	0.236	3.391	11	11	1
Nebrioporus baeticus	RD	10	1.5	BR1	5.27	4.24	1.22	0	F	4.05	76.85	3.02	71.23	0.343	6.515	2	2	1
Nebrioporus baeticus	RD	10	1.5	BR10	4.59	3.91	0.88	0	M	3.71	80.83	3.03	77.49	0.227	4.938	12	24	3
Nebrioporus baeticus	RD	10	1.5	BR11	7.29	5.85	2.25	0	F	5.04	69.14	3.6	61.54	0.480	6.584	12	24	3
Nebrioporus baeticus	RD	10	1.5	BR12	6.1	5.31	1.78	0	M	4.32	70.82	3.53	66.48	0.263	4.317	36	48	3
Nebrioporus baeticus	RD	10	1.5	BR13	5.84	3.91	1.02	0	F	4.82	82.53	2.89	73.91	0.643	11.016	8	8	1

Nebrioporus baeticus	RD	10	1.5	BR14	4.76	3.85	1.21	0	M	3.55	74.58	2.64	68.57	0.303	6.373	12	24	3
Nebrioporus baeticus	RD	10	1.5	BR15	6.56	4.84	1.58	0	F	4.98	75.91	3.26	67.36	0.573	8.740	10	10	1
Nebrioporus baeticus	RD	10	1.5	BR16	7.62	6.4	2.66	0	M	4.96	65.09	3.74	58.44	0.407	5.337	12	24	3
Nebrioporus baeticus	RD	10	1.5	BR17	6.04	4.15	1.04	0	M	5	82.78	3.11	74.94	0.630	10.430	3	3	1
Nebrioporus baeticus	RD	10	1.5	BR18	5.52	4.45	1.48	0	F	4.04	73.19	2.97	66.74	0.357	6.461	11	11	1
Nebrioporus baeticus	RD	10	1.5	BR19	3.6	3.43	1.05	0	M	2.55	70.83	2.38	69.39	0.057	1.574	11	11	1
Nebrioporus baeticus	RD	10	1.5	BR2	5.83	4.41	1.21	0	M	4.62	79.25	3.2	72.56	0.473	8.119	12	24	3
Nebrioporus baeticus	RD	10	1.5	BR20	6.94	5.37	1.24	0	F	5.7	82.13	4.13	76.91	0.523	7.541	12	24	3
Nebrioporus baeticus	RD	10	1.5	BR21	5.6	NA	NA	0	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Nebrioporus baeticus	RD	10	1.5	BR22	5.67	3.57	0.92	0	M	4.75	83.77	2.65	74.23	0.700	12.346	3	3	1
Nebrioporus baeticus	RD	10	1.5	BR23	5.85	4.69	2.05	0	M	3.8	64.96	2.64	56.29	0.387	6.610	10	10	1
Nebrioporus baeticus	RD	10	1.5	BR24	6.56	5.46	1.62	0	M	4.94	75.30	3.84	70.33	0.367	5.589	12	24	3
Nebrioporus baeticus	RD	10	1.5	BR25	6.32	5.65	2.03	0	M	4.29	67.88	3.62	64.07	0.223	3.534	12	24	3
Nebrioporus baeticus	RD	10	1.5	BR26	5.51	4.47	1.15	0	F	4.36	79.13	3.32	74.27	0.347	6.292	11	11	1
Nebrioporus baeticus	RD	10	1.5	BR27	6.02	4.17	1.28	0	F	4.74	78.74	2.89	69.30	0.617	10.244	4	4	1
Nebrioporus baeticus	RD	10	1.5	BR28	7.48	6.29	3.08	0	M	4.4	58.82	3.21	51.03	0.397	5.303	12	24	3
Nebrioporus baeticus	RD	10	1.5	BR29	8.24	6.34	2.71	0	F	5.53	67.11	3.63	57.26	0.633	7.686	12	24	3
Nebrioporus baeticus	RD	10	1.5	BR3	5.65	4.34	1.11	0	M	4.54	80.35	3.23	74.42	0.437	7.729	11	11	1
Nebrioporus baeticus	RD	10	1.5	BR30	6.37	4.77	1.6	0	F	4.77	74.88	3.17	66.46	0.533	8.373	7	7	1
Nebrioporus baeticus	RD	10	1.5	BR4	6.79	5.73	1.21	0	F	5.58	82.18	4.52	78.88	0.353	5.204	12	24	3
Nebrioporus baeticus	RD	10	1.5	BR5	5.72	4.35	1.15	0	M	4.57	79.90	3.2	73.56	0.457	7.984	11	11	1
Nebrioporus baeticus	RD	10	1.5	BR6	5.41	3.86	0.77	0	M	4.64	85.77	3.09	80.05	0.517	9.550	12	24	3
Nebrioporus baeticus	RD	10	1.5	BR7	5.59	4.01	1.31	0	F	4.28	76.57	2.7	67.33	0.527	9.422	4	4	1
Nebrioporus baeticus	RD	10	1.5	BR8	6.56	5.12	2.44	0	M	4.12	62.80	2.68	52.34	0.480	7.317	12	24	3
Nebrioporus baeticus	RD	10	1.5	BR9	7.2	5.97	2.26	0	F	4.94	68.61	3.71	62.14	0.410	5.694	12	24	3
Nebrioporus baeticus	SD	40	6	BS1	6.56	4.38	1.22	0	M	5.34	81.40	3.16	72.15	0.436	6.646	8	8	1
Nebrioporus baeticus	SD	40	6	BS10	5.84	4.92	1.26	0	F	4.58	78.42	3.66	74.39	0.184	3.151	24	36	3
Nebrioporus baeticus	SD	40	6	BS11	5.67	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Nebrioporus baeticus	SD	40	6	BS12	6.01	3.99	0.92	0	F	5.09	84.69	3.07	76.94	0.404	6.722	10	10	1
Nebrioporus baeticus	SD	40	6	BS13	7.03	4.95	1.65	0	F	5.38	76.53	3.3	66.67	0.416	5.917	12	12	1
Nebrioporus baeticus	SD	40	6	BS14	6.72	5.32	1.23	0	M	5.49	81.70	4.09	76.88	0.280	4.167	24	36	3
Nebrioporus baeticus	SD	40	6	BS15	5.57	3.68	1.19	0	F	4.38	78.64	2.49	67.66	0.378	6.786	8	8	1
Nebrioporus baeticus	SD	40	6	BS16	6.92	5.21	1.27	0	F	5.65	81.65	3.94	75.62	0.342	4.942	24	36	3
Nebrioporus baeticus	SD	40	6	BS17	5.58	3.49	1.67	0	F	3.91	70.07	1.82	52.15	0.418	7.491	2	2	1
Nebrioporus baeticus	SD	40	6	BS18	4.86	4.21	0.89	0	F	3.97	81.69	3.32	78.86	0.130	2.675	60	72	3

Nebrioporus baeticus	SD	40	6	BS19	5.68	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Nebrioporus baeticus	SD	40	6	BS2	6.28	4.66	1.54	0	M	4.74	75.48	3.12	66.95	0.324	5.159	5	5	1
Nebrioporus baeticus	SD	40	6	BS20	6.89	5.14	0.97	0	F	5.92	85.92	4.17	81.13	0.350	5.080	12	24	3
Nebrioporus baeticus	SD	40	6	BS21	6.76	4.78	1.19	0	F	5.57	82.40	3.59	75.10	0.396	5.858	12	24	3
Nebrioporus baeticus	SD	40	6	BS22	5.75	4.1	0.99	0	M	4.76	82.78	3.11	75.85	0.330	5.739	12	12	1
Nebrioporus baeticus	SD	40	6	BS23	6.46	4.27	1.16	0	M	5.3	82.04	3.11	72.83	0.438	6.780	12	24	3
Nebrioporus baeticus	SD	40	6	BS24	5.94	4.41	1.17	0	F	4.77	80.30	3.24	73.47	0.306	5.152	24	36	3
Nebrioporus baeticus	SD	40	6	BS25	6.22	4.48	1.78	0	F	4.44	71.38	2.7	60.27	0.348	5.595	2	2	1
Nebrioporus baeticus	SD	40	6	BS26	6.78	5.18	1.61	0	F	5.17	76.25	3.57	68.92	0.320	4.720	4	4	1
Nebrioporus baeticus	SD	40	6	BS27	6.52	4.49	1.31	0	M	5.21	79.91	3.18	70.82	0.406	6.227	10	10	1
Nebrioporus baeticus	SD	40	6	BS28	7.54	6.78	2.54	0	M	5	66.31	4.24	62.54	0.152	2.016	60	72	3
Nebrioporus baeticus	SD	40	6	BS29	6.22	5.61	1.25	0	F	4.97	79.90	4.36	77.72	0.122	1.961	84	96	3
Nebrioporus baeticus	SD	40	6	BS3	7.06	5.3	1.75	0	M	5.31	75.21	3.55	66.98	0.352	4.986	2	2	1
Nebrioporus baeticus	SD	40	6	BS4	6.69	4.54	1.39	0	M	5.3	79.22	3.15	69.38	0.430	6.428	12	24	3
Nebrioporus baeticus	SD	40	6	BS5	5.75	4.49	1.16	0	F	4.59	79.83	3.33	74.16	0.252	4.383	24	36	3
Nebrioporus baeticus	SD	40	6	BS6	3.82	NA	NA	1	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
Nebrioporus baeticus	SD	40	6	BS7	7.25	5.82	2.39	0	F	4.86	67.03	3.43	58.93	0.286	3.945	12	24	3
Nebrioporus baeticus	SD	40	6	BS8	5.88	4.79	1.13	0	F	4.75	80.78	3.66	76.41	0.218	3.707	24	36	3
Nebrioporus baeticus	SD	40	6	BS9	5.51	4.18	1.75	0	F	3.76	68.24	2.43	58.13	0.266	4.828	12	24	3

treat: desiccation pre-treatment (CD: control, RD: rapid, SD: slow); **RH:** relative humidity (%) of pre-treatment; **exp. time:** exposure time (h) of pre-treatment; **ID**: specimen identity number; **M_0**: initial fresh mass (before desiccation), mg; **M_d**: final fresh mass (after desiccation), mg; **M_{dry}**: dry mass, mg; **status** 1: specimens status after desiccation pre-treatment (0 = alive, 1 = dead); **WC_0**: initial water content (before desiccation), mg; **% WC_0**: percentage water content (% of M_0); **WC_d**: final water content (after desiccation), mg; **%WC_d**: percentage final water content (after desiccation), % of M_d; **WLR:** mean water loss rate, mg h^{-1}; **%WLR**: percentage water loss rate, % of M_0 h^{-1}; **t_1**: left end of the time interval of survival monitoring; **t_2**: right end of the time interval of survival monitoring; **status 2:** specimens status at t_2 (1 = dead at t_2, 3= death between t_1-t_2)

Table S3. Data on the experiment testing the effect of desiccation on salinity tolerance (osmoregulatory capacity).

species	treat	RH	exp. time	ext. time	ID	M_0	M_d	T_m	osm
E. jesusarribasi	SD	40	12	0	JS2	8.2	7.41	-0.8	432.29
E. jesusarribasi	SD	40	12	0	JS5	10.01	8.69	-0.81	438.14
E. jesusarribasi	SD	40	12	0	JS6	8.91	7.77	-0.76	410.86
E. jesusarribasi	SD	40	12	8	JS9	8.22	6.94	-0.95	508.27
E. jesusarribasi	SD	40	12	8	JS10	8.74	7.55	-0.91	487.82
E. jesusarribasi	SD	40	12	8	JS11	6.51	5.41	-0.84	453.72
E. jesusarribasi	SD	40	12	8	JS12	6.78	5.5	-0.95	508.27
E. jesusarribasi	SD	40	12	8	JS13	8.69	7.07	-1.42	761.54
E. jesusarribasi	SD	40	12	8	JS15	6.61	5.6	-1.04	561.16
E. jesusarribasi	SD	40	12	8	JS16	9.37	7.95	-0.92	493.34
E. jesusarribasi	SD	40	12	24	JS19	6.67	5.09	-1.44	773.23
E. jesusarribasi	SD	40	12	24	JS20	7.32	6.05	-1.22	654.21
E. jesusarribasi	SD	40	12	24	JS23	10.19	8.93	-1.36	732.44
E. jesusarribasi	CD	90	12	0	JC1	8.51	8.04	-0.62	332.93
E. jesusarribasi	CD	90	12	0	JC3	7.64	7.37	-0.74	400.15
E. jesusarribasi	CD	90	12	0	JC4	6.79	6.67	-0.71	379.69
E. jesusarribasi	CD	90	12	0	JC5	7.55	6.88	-0.89	479.05
E. jesusarribasi	CD	90	12	0	JC8	8.81	8.39	-0.48	256.95
E. jesusarribasi	CD	90	12	8	JC9	7.34	6.79	-0.59	317.35
E. jesusarribasi	CD	90	12	8	JC11	7.18	6.84	-0.71	384.07
E. jesusarribasi	CD	90	12	8	JC12	4.77	4.31	-0.82	442.03
E. jesusarribasi	CD	90	12	8	JC13	7.19	6.86	-0.74	395.28
E. jesusarribasi	CD	90	12	8	JC14	8.4	7.91	-0.61	327.09
E. jesusarribasi	CD	90	12	8	JC16	9.84	9.3	-0.9	482.95
E. jesusarribasi	CD	90	12	24	JC21	8.11	7.6	-1.25	673.31
E. jesusarribasi	CD	90	12	24	JC23	5.34	5.33	-1.15	618.54
E. jesusarribasi	CD	90	12	24	JC24	7.35	6.95	-0.96	516.81
E. jesusarribasi	RD	10	3	0	JC2	9.08	7.52	-1	535.07
E. jesusarribasi	RD	10	3	0	JC3	6.91	6.42	-0.82	438.56
E. jesusarribasi	RD	10	3	0	JC4	5.68	5.3	-1.07	574.2
E. jesusarribasi	RD	10	3	0	JC5	8.91	8.32	-0.99	532.46
E. jesusarribasi	RD	10	3	0	JC6	8.22	7.41	-1.34	720.26
E. jesusarribasi	RD	10	3	0	JC7	6.78	6.33	-1.07	576.81
E. jesusarribasi	RD	10	3	8	JC10	8.29	7.43	-1.1	593.99
E. jesusarribasi	RD	10	3	8	JC11	7.72	7.26	-1.26	678.53
E. jesusarribasi	RD	10	3	8	JC13	8.65	7.76	-0.98	524.64
E. jesusarribasi	RD	10	3	8	JC14	6.47	5.92	-0.86	462.04
E. jesusarribasi	RD	10	3	8	JC15	9.29	8.36	-0.88	472.47
E. jesusarribasi	RD	10	3	24	JC17	7.56	6.8	-1.02	549.18
E. jesusarribasi	RD	10	3	24	JC18	7.44	6.61	-0.75	404.66
E. jesusarribasi	RD	10	3	24	JC19	6.5	6.1	-1.26	678.53
E. jesusarribasi	RD	10	3	24	JC21	9.04	8.33	-0.97	522.03
E. jesusarribasi	RD	10	3	24	JC23	8.43	7.7	-0.61	426.41
E. jesusarribasi	RD	10	3	24	JC24	10.36	9.26	-1.1	592.46
N. baeticus	SD	40	6	0	BS2	5.58	4.82	-1.4	751.56
N. baeticus	SD	40	6	0	BS4	6.49	5.72	-1.04	558.55
N. baeticus	SD	40	6	0	BS5	6.82	5.62	-1.54	827.21
N. baeticus	SD	40	6	0	BS6	6.84	6.05	-1.02	548.11
N. baeticus	SD	40	6	0	BS7	6.99	6.25	-1.3	696.79
N. baeticus	SD	40	6	0	BS8	6.49	5.82	-1.13	605.5
N. baeticus	SD	40	6	24	BS19	5.27	4.21	-0.68	365.53

N. baeticus	SD	40	6	24	BS20	6.63	5.23	-1.07	576.81
N. baeticus	SD	40	6	24	BS21	7.87	6.83	-1.2	647.23
N. baeticus	SD	40	6	24	BS22	6.23	5.34	-0.89	477.69
N. baeticus	SD	40	6	24	BS23	6.47	5.11	-0.9	485.51
N. baeticus	SD	40	6	24	BS24	7.11	6.19	-1.06	571.59
N. baeticus	SD	40	6	8	BS9	6.29	4.31	-1.56	837.64
N. baeticus	SD	40	6	8	BS12	7.81	6.32	-1.13	608.11
N. baeticus	SD	40	6	8	BS13	6.09	4.33	-1.02	550.72
N. baeticus	SD	40	6	8	BS16	6.13	4.76	-1.09	587.24
N. baeticus	CD	90	6	0	BC1	6.81	6.21	-1.03	553.33
N. baeticus	CD	90	6	0	BC2	6.54	6.25	-1.09	584.63
N. baeticus	CD	90	6	0	BC3	6.69	6.59	-1.04	558.55
N. baeticus	CD	90	6	0	BC4	5.87	5.57	-0.59	318.58
N. baeticus	CD	90	6	0	BC5	5.68	5.44	-0.91	490.73
N. baeticus	CD	90	6	0	BC7	6.2	6.04	-0.86	464.65
N. baeticus	CD	90	6	0	BC8	6.94	6.62	-0.92	493.34
N. baeticus	CD	90	6	24	BC17	5.27	5.03	-0.98	527.25
N. baeticus	CD	90	6	24	BC18	8.54	7.57	-0.76	409.87
N. baeticus	CD	90	6	24	BC20	7.05	6.1	-1.07	576.81
N. baeticus	CD	90	6	24	BC24	6.73	6.29	-0.82	441.17
N. baeticus	CD	90	6	8	BC9	6.72	6.04	-0.65	347.27
N. baeticus	CD	90	6	8	BC10	6.71	6.01	-0.82	441.17
N. baeticus	CD	90	6	8	BC11	6.48	6.18	-0.89	480.3
N. baeticus	CD	90	6	8	BC12	6.33	5.63	-0.6	323.8
N. baeticus	CD	90	6	8	BC13	6.71	5.69	-0.9	485.51
N. baeticus	CD	90	6	8	BC14	6.85	5.65	-0.9	482.91
N. baeticus	CD	90	6	8	BC15	7.67	5.92	-0.99	532.46
N. baeticus	CD	90	6	8	BC16	6.29	5.61	-1.07	576.81
N. baeticus	RD	10	1.5	0	BR3	6.89	5.16	-0.8	428.13
N. baeticus	RD	10	1.5	0	BR5	6.31	5.38	-1.04	558.55
N. baeticus	RD	10	1.5	0	BR6	6.43	5.59	-0.95	508.99
N. baeticus	RD	10	1.5	24	BR17	6.71	5.11	-1.05	563.76
N. baeticus	RD	10	1.5	24	BR18	5.48	4.51	-0.6	323.8
N. baeticus	RD	10	1.5	24	BR19	6.31	4.94	-0.62	334.23
N. baeticus	RD	10	1.5	24	BR20	6.26	4.83	-0.9	485.51
N. baeticus	RD	10	1.5	24	BR21	5.23	4.15	-0.68	365.53
N. baeticus	RD	10	1.5	24	BR22	5.67	4.72	-0.84	449
N. baeticus	RD	10	1.5	24	BR23	5.42	4.35	-0.87	469.86
N. baeticus	RD	10	1.5	24	BR24	6.48	4.38	-0.83	445.52
N. baeticus	RD	10	1.5	8	BR9	5.08	4.1	-1.04	584.63
N. baeticus	RD	10	1.5	8	BR11	6.31	4.35	-0.91	516.81
N. baeticus	RD	10	1.5	8	BR12	6.06	5.21	-0.8	456.82
N. baeticus	RD	10	1.5	8	BR13	6.17	5.59	-0.74	428.13
N. baeticus	RD	10	1.5	8	BR14	6.79	5.15	-0.99	555.94
N. baeticus	RD	10	1.5	8	BR15	5.82	4.75	-1.07	600.28
N. baeticus	RD	10	1.5	8	BR16	6.63	5.58	-0.92	519.42

treat: desiccation pre-treatment (CD: control, RD: rapid, SD: slow); **RH:** relative humidity (%) ofpre-treatment; **exp. time:** exposure time of pre-treatment, h; **ext. time:** time of haemolymph extraction after pre-treatment, h; **ID**: specimen identity number; **M_0**: initial fresh mass (before desiccation), mg; **M_d**: final fresh mass (after desiccation), mg; **T_m**: melting temperature of haemolymph sample, ºC; **osm:** osmolality of haemolymph sample, mOsmol kg^{-1}

Table S4. GLM results on the differences in mean (WLR_{mean}), maximum (WLR_{max}) and final (WLR_f) water loss rate under desiccation between salinity pre-treatments (Trat), and effect of individuals´ initial fresh mass (M_s) and sex in the experiment testing the effect of salinity on desiccation resistance.

Species	Variable	Predictors	Slope ± SE	df	F-statistic	Explained deviance (%)
E. jesusarribasi	WLR_{mean}	Intercept	0.042 ± 0.030			13.4
		Trat (SLS)	-0.011 ± 0.008	1	0.182	
		M_s	0.010 ± 0.004**	1	8.537**	
		Sex (male)	-0.005 ± 0.008	1	0.392	
	WLR_{max}	Intercept	0.026 ± 0.028			20.5
		Trat (SLS)	-0.007 ± 0.008	1	0.051	
		M_s	0.012 ± 0.004**	1	13.844***	
		Sex (male)	-0.007 ± 0.008	1	0.871	
	WLR_f	Intercept	0.053 ± 0.030			11.2
		Trat (SLS)	-0.011 ± 0.008	1	0.289	
		M_s	0.009 ± 0.004*	1	6.794*	
		Sex (male)	-0.005 ± 0.008	1	0.345	
N. baeticus	WLR_{mean}	Intercept	0.216 ± 0.071**			13.8
		Trat (SLS)	-0.039 ± 0.016*	1	5.642*	
		M_s	0.008 ± 0.011	1	0.250	
		Sex (male)	-0.019 ± 0.012	1	1.465	
	WLR_{max}	Intercept	0.130 ± 0.120			7.7
		Trat (SLS)	-0.017 ± 0.027	1	0.029	
		M_s	0.032 ± 0.018	1	2.146	
		Sex (male)	-0.034 ± 0.026	1	1.688	
	WLR_f	Intercept	0.274 ± 0.076***			13.4
		Trat (SLS)	-0.036 ± 0.017*		5.424*	
		M_s	0.003 ± 0.012		0.305	
		Sex (male)	-0.020 ± 0.017		1.398	

Significance levels: *P < 0.05; **; P < 0.01; ***P < 0.001
SLS: sublethal salinity

Table S5. GLM results on variation in water loss rate (WLR) water loss rate and final water content (WC_d) between desiccation pre-treatments (Trat) and effect of individuals´ initial fresh mass (M_0) and sex in the experiment testing the effect of desiccation on salinity tolerance.

Species	Variable	Predictors	Slope ± SE	df	F-statistic	Explained deviance (%)
E. jesusarribasi	WLR	Intercept	-0.136 ± 0.068*			71.1
		Trat (RD)	0.246 ± 0.021***	2	68.252***	
		Trat (SD)	0.125 ± 0.021***			
		M_0	0.023 ± 0.008**	1	8.966**	
		Sex (male)	0.004 ± 0.018	1	0.059	
	WC_d	Intercept	0.618 ± 0.367			84.8
		Trat (RD)	-0.446 ± 0.113***	2	71.626***	
		Trat (SD)	-1.524 ± 0.166***			
		M_0	0.543 ± 0.043***	1	184.731***	
		Sex (male)	-0.005 ± 0.100	1	0.003	
N. baeticus	WLR	Intercept	-0.150 ± 0.110			53.4
		Trat (RD)	0.303 ± 0.034***	2	38.531***	
		Trat (SD)	0.171 ± 0.033***			
		M_0	0.048 ± 0.016**	1	8.464**	
		Sex (male)	-0.014 ± 0.026	1	0.292	
	WC_d	Intercept	1.314 ± 0.493**			56.1
		Trat (RD)	-0.746 ± 0.147***	2	28.524***	
		Trat (SD)	-0.771 ± 0.147***			
		M_0	0.450 ± 0.073***	1	39.990***	
		Sex (male)	-0.158 ± 0.119	1	1.770	

Significance levels: *P < 0.05;**; P < 0.01; ***P < 0.001
RD: rapid desiccation, SD: slow desiccation

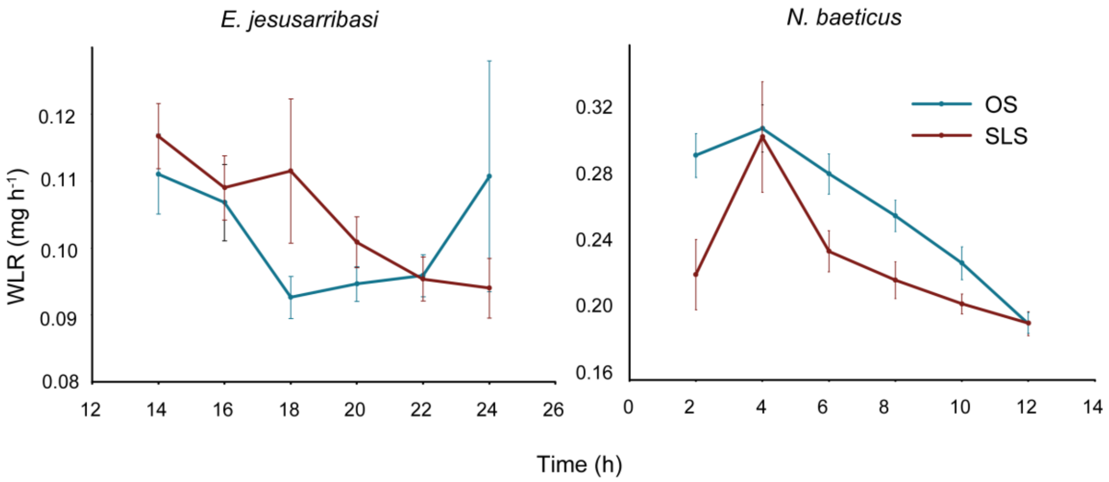

Figure S1. Change in water loss rate (WLR) with time along desiccation exposure in the experiment testing the effect of salinity on desiccation resistance. OS: optimum salinity, SLS: sublethal salinity.

CHAPTER 5

Adaptation to desiccation and salinity tolerance in a lineage of water beetles.

INTRODUCTION

How organisms acquire novel traits or undergo adaptive trait divergence are central questions in evolutionary ecology, as these processes facilitate niche shifts and the colonisation of novel environments (Heard & Hauser, 1995; Hunter, 1998; Moczek, 2008). In the aquatic realm, the evolution of mechanisms for ion regulation in hyposmotic media was a key innovation allowing the initial colonisation of freshwater habitats by marine species (e.g. Faria *et al.*, 2011; McNamara & Faria 2012; Schultz & McCormick, 2012). In a number of lineages of inland water organisms, there appear to have been multiple transitions between fresh and saline habitats over the course of their evolutionary history (e.g. Bradley *et al.*, 2011), these sometimes extending into hypersaline environments. Most interestingly, such transitions appear to be much more frequent in some taxa than others, with closely related genera either being entirely restricted to freshwaters, or spanning the fresh-hypersaline gradient (e.g. Arribas *et al.*, 2014 – beetles; Carbonell *et al.* 2012 – water bugs; Herbst, 1999 – flies). The physiological and evolutionary processes that may facilitate the colonisation of extreme habitats such as saline waters remain poorly understood, however, and require the study of relevant organismal traits within a phylogenetic context (Cheng & Chen, 1999; Tobler *et al.*, 2011).

In insects, the main osmoregulatory adaptations are a highly impermeable cuticle and a rectum capable of producing hyperosmotic excreta. These are ancestral characters, found in virtually all insect lineages and are clearly essential to their success on land, where desiccation can be a major physiological stress factor (Bradley *et al.*, 2009). In contrast, tolerance to the osmotic stress produced by a saline aquatic medium (hyperosmotic stress) seems to be a very specialized secondary adaption, only present in a few insect orders (Bradley *et al.*, 2009). In general, insect species that show tolerance to salinities above that of seawater are efficient hyporegulators, being able to maintain the concentration of haemolymph within a narrow range regardless of the concentration of the external medium (e.g. Tones & Hammer, 1975; Herbst *et al.*, 1988; Pallarés *et al.*, 2015). Ultimately, hyporegulation capacity has the same physiological basis as mechanisms dealing with dehydration in air, as both desiccation and hyperosmotic stress alter ionic and water balance, with similar effects at the cellular level (Bradley, 2009; Cohen, 2012; Evans, 2008). Indeed, changes in excretory organs such as

Malpighian tubules and the rectum, including their morphological specialisation (e.g. the two-part rectum of saline-water culicid larvae, Bradley, 1987, 1994), underlie the success of some insects in highly saline waters (e.g. Tones, 1977; Grueber & Bradley, 1994). Given the physiological similarities between mechanisms to cope with salinity and desiccation stress and the frequent spatial and temporal co-occurrence of both stressors, tolerance to them may be evolutionarily linked in some insect lineages. In such cases, selection on the osmoregulatory system to deal with desiccation stress could have secondarily facilitated hyporegulation at high salinities, or vice-versa.

Recent studies on salinity tolerance in aquatic insects support this association. Firstly, beetle adults (Pallarés *et al.*, 2017) and dipteran larvae (Elnitsky *et al.*, 2009) sequentially exposed to salinity and desiccation showed cross-tolerance responses (Sinclair *et al.*, 2013; Todgham & Stillman, 2013), suggesting a mechanistic link between the response to both stressors. Secondly, a recent study reconstructing the colonisation of saline waters by *Enochrus* water beetles (Hydrophilidae) suggested that salinity tolerance arose during periods of global aridification, when multiple independent transitions from fresh to saline waters apparently occurred (Arribas *et al.*, 2014). These authors also found a positive correlation between the optimum salinity of species and the aridity of their distribution range. Finally, in agreement with this ecological correlation, Pallarés *et al.* (2016) revealed a positive relationship between desiccation resistance and salinity tolerance in *Enochrus* in the laboratory.

Despite multiple lines of evidence suggesting an evolutionary link between tolerance to salinity and desiccation in water beetles, the temporal sequence of these adaptations - and hence their evolutionary origin - is still not well established. Arribas *et al.* (2014) hypothesized that the development of drought tolerance during periods of global aridification could have secondarily increased hyporegulation capacity, facilitating the colonisation of saline waters in the *Lumetus* subgenus of *Enochrus*. In this case, hyporegulation capacity would represent an exaptation of increased tolerance to desiccation. The inverse exaptation sequence is also plausible, however, as the enhancement of osmoregulatory mechanisms for salinity tolerance would also facilitate aridity tolerance (Lee *et al.*, 2011). Tolerance to salinity and desiccation could have also evolved as a joint response to aridification, as this process typically results in a simultaneous decrease of precipitation and increase in the mineralization of surface waters.

The relationship between aridity and salinity demonstrated by Arribas *et al.* (2014) was based on ecological data alone (species habitat occupancies and regional climates), which do not always fully reflect the potential physiological tolerance of species (Carbonell *et al.*, 2012; Céspedes *et al.*, 2013). Mismatches between realized and fundamental niches may result if physiological tolerance has evolved as a result of prior exposure to different stressors, since in such cases species may retain the ability to deal with conditions absent in their current habitats. Disentangling the evolution of salinity and desiccation tolerance in organisms spanning the fresh-saline spectrum is clearly complex, and requires an integrative approach, based on the measurement of ecological and organismal traits within a sound phylogenetic context – something which has not been attempted to date in any lineage.

Here, we combine experimental, ecological and molecular data to track the evolution of desiccation resistance, hyporegulation ability and habitat transitions across the saline gradient in the water beetle subgenus *Lumetus*. This lineage includes species in all habitat types from fresh to hypersaline waters, with differing hyporegulation capacities (Pallarés *et al.*, 2015). We provide a well-resolved phylogeny of the lineage, together with experimental data on desiccation resistance and hyporegulation capacity across its constituent taxa, and go on to use ancestral trait reconstruction and phylogenetic comparative methods to test the following alternative hypotheses:

1) The hyporegulation capacity allowing the colonisation of saline waters in these beetles was co-opted from physiological mechanisms evolved originally for desiccation resistance.

2) The development of hyporegulation capacity in saline waters was the primary adaptation, secondarily leading to an increase in desiccation resistance.

3) Desiccation resistance and hyporegulation capacity evolved in correlation.

In the first case, all species living in meso- or hypersaline waters should be efficient hyporegulators and tolerant to desiccation, but the reverse needs not be true (i.e. there may be desiccation resistant species with low hyporegulation capacity). In addition, there could be species with high desiccation resistance and hyporegulation capacity primarily living in fresh - hyposaline waters (i.e. able to tolerate higher salinities even if they -or their ancestors- have never occupied this type of habitat). In the phylogeny, increases in hyporegulation capacity may be expected to be preceded by increases in desiccation resistance.

Under the second hypothesis, the situation would be the reverse of that in the first, with all species living in saline habitats able to tolerate desiccation even if they (of their ancestors) are not found in areas with high aridity. Again, if resistance to desiccation results from a physiological mechanism derived from hyporegulation capacity, we could expect all species that are resistant to desiccation will be good hyporegulators, but not necessarily vice-versa. In this case an increase in desiccation resistance should be preceded by an increase in hyporegulation capacity across the phylogeny.

Finally, if desiccation resistance and hyporegulation capacity evolved in correlation, enhanced values of these traits should coincide phylogenetically. All species with high hyporegulation capacity should then be tolerant to desiccation, and vice-versa. This would be observed under an exaptation process (hypothesis i or ii) if both tolerances are governed by essentially identical physiological mechanisms and gene pathways.

MATERIAL AND METHODS

Taxon sampling

A total of 220 specimens representing 18 of the 23 known species of the subgenus were used to obtain the phylogeny of *Lumetus* (Table S1). Molecular data were obtained from *de novo* sequencing of 64 specimens plus sequences from previous work (Arribas *et al.*, 2012, 2013, 2014). Several *Enochrus* species of the subgenera *Methydrus*, *Enochrus* and *Hugoscottia* and a related genus (*Helochares*) were used as outgroups, with two more distantly related genera of Hydrophilidae (*Hydrobius* and *Arabhydrus*) (Short & Fikácek, 2013) being used to root the tree, resulting in a phylogeny of 43 species.

Data on osmoregulatory ability and desiccation resistance were obtained experimentally from a representative subset of species (Table S2). Studied species

included at least one from each of the main *Lumetus* clades obtained in preliminary phylogenetic analyses and one outgroup species from the subgenus *Methydrus* (*Enochrus coarctatus*).

Phylogeny of *Lumetus*

DNA from the new collected specimens was extracted and sequenced following the methodology of Arribas *et al.* (2013, 2014). We sequenced five mitochondrial genes: two non-overlapping fragments of the cytochrome c oxidase I gene corresponding to the 5′ (cox1–A) and the 3′ end (cox1–B); an internal fragment of the cytochrome b gene (cyt b); and a fragment spanning three genes (5′ end of the large ribosomal subunit plus Leucine transferase and the 5′ end of NADH dehydrogenase subunit 1; rrnL+trnL+ nad1). From nuclear DNA we sequenced an internal fragment of the large ribosomal unit, 28S rRNA, (LSU) and an internal fragment of the internal transcribed spacer 2 (ITS2) (Table S3).

Sequences were assembled and edited with Geneious 5.5.9 (Biomatters Ltd. Auckland, New Zeland), using Ns (missing data) for ambiguous positions. Alignments were obtained with the online version of MAFFT v.7 (Katoh & Toh, 2008) using the *auto* option for protein coding and *QINS-i* for ribosomal genes, with other parameters set as defaults. For protein coding genes, the correct translation to amino acids was checked to ensure there were no stop codons or frame shifts.

Bayesian phylogenetic analyses on the concatenated DNA matrix were implemented in BEAST 1.8.0 (Drummond *et al.*, 2012). The concatenated data set was divided into 3 partitions: the three protein-coding genes, the mitochondrial ribosomal gene and the two nuclear sequences. Analyses were conducted by applying the best fitting substitution model to each partition, as previously estimated with Partition Finder (Lanfear *et al.*, 2012). We applied a Yule speciation tree prior. To calibrate the tree, we used as a prior for the age of *Lumetus* (time to most recent common ancestor, tMRCA) the age distribution of this node obtained by Arribas *et al.* (2014) – i.e. ≈45 Ma (Gamma distribution shape: 56.84, scale: 0.74). An uncorrelated lognormal clock was applied for the nuclear partition, with a uniform prior distribution for the rate of substitutions set between 0.0001 – 0.01 substitutions per site per time unit (subs/s/Ma) and an initial value of 0.001, together with a strict clock for each of the mitochondrial partitions with a uniform prior distribution for the rate with 0.01 (0.001 – 0.1) subst/s/Ma.

We set two independent runs of 100 million generations each, sampling one tree every 10,000 generations. LogCombiner (Drummond *et al.,* 2012) was used to combine trees from both runs andto obtain 1,000 randomly resampled postburnin trees. The consensus tree was estimated with Treeanotator (Drummond *et al.*, 2012).The 25 % initial trees were discarded as a burnin fraction, after checking for convergence in Tracer v1.6 (Drummond *et al.*, 2012).

Ecological data, osmoregulatory capacity and desiccation resistance

To track habitat transitions across the salinity gradient, each *Lumetus* species was assigned a category according to our field data on the salinity of their most frequently occupied habitats, following the same criteria and categorization made previously by Arribas *et al.* (2014), i.e. freshwater (≤0.5 g/L), mineralized (0.5–5 g/L), hyposaline (5–20 g/L), mesosaline (20–40 g/L), hypersaline (40–80 g/L) and extreme hypersaline (>80 g/L).

To determine the osmoregulatory capacity of the selected species (Table S2), haemolymph osmolalities were measured in individuals exposed to different salinities within their specific tolerance ranges (as determined by pilot trials or previous work - Pallarés *et al.*, 2015) for 48 h. All species were exposed to at least two common hypoosmotic treatments (0.3 and 12 g L^{-1}) and a hyperosmotic one (35 g L^{-1}) to obtain comparable osmolality measurements. For each species, the treatment in which mortality exceeded 50% of the tested individuals was considered as the upper lethal limit (e.g. Faria *et al.*, 2017) (Table S4). Experimental procedures were conducted as described in Pallarés *et al.* (2015). From each treatment we obtained samples from a minimum of three exposed individuals (Table S4); pilot trails showed low intraspecific variation within salinity treatments. Osmolality of the haemolymph and the saline media were measured using a calibrated nanolitre osmometer (Otago Osmometers, Dunedin, New Zealand). Osmotic capacity (i.e. the difference between the osmotic concentration of the haemolymph and the external medium - Charmantier *et al.*, 1984; Calosi *et al.*, 2005) was estimated for each treatment. The hyposmotic capacity at 35 g L^{-1} (hyposmotic capacity hereafter) and the maximum hyposmotic capacity (i.e. that measured at the highest salinity tolerated by each species) showed the highest variation amongst species and were therefore used for subsequent analyses.

Controlled desiccation experiments were conducted as described by Pallarés *et al.* (2016). For each specimen we measured the initial water content (%wet mass to fresh mass prior to desiccation exposure) and water loss rate (% of water lost per initial fresh mass, per hour) at 20±5 % RH, 20±1ºC for 6 h. These variables, and in particular water loss rates, have previously been shown to be relevant for desiccation resistance in *Lumetus* species (Pallarés *et al.*, 2016, 2017). Specimens were allowed to recover at freshwater conditions for 24 h after desiccation. Mortality was assessed after both desiccation and the recovery period. These estimates were obtained individually for 20-30 specimens per species (Table S4).

Habitat transitions, evolution of desiccation resistance and osmoregulatory capacity

Ancestral trait reconstruction. We tested different models of trait evolution (Brownian motion – BM and Ornstein-Uhlenbeck – OU) (Kaliontzopoulou *et al.*, 2016) to reconstruct ancestral values of habitat salinity (considered as a semi-continuous variable), hyposmotic capacity and desiccation resistance traits. Intraspecific variation, missing observations and small tree size can profoundly affect the performance of such models (Boettiger *et al.*, 2012; Cooper *et al.*, 2016). To account for this, we used a Monte-Carlo based approach to assess the ability of our data to distinguish between the models tested. We compared the distribution of δ (i.e. the difference in log likelihood of observing the data under the two maximum likelihood estimates) from Monte Carlo simulations (n= 1,000 replicates) using *pmc* (Phylogenetic Monte Carlo) in R (Boettiger *et al.*, 2012). When there was insufficient power to distinguish between models, the simplest (i.e. BM) was used. Ancestral trait reconstructions were made using the R function *phylopars* (package Rphylopars, Bruggeman *et al.*, 2009; Goolsby *et al.*, 2016), which uses a maximum likelihood-based method to estimate trait covariance across (phylogenetic covariance) and within species (phenotypic covariance) for datasets with missing data and multiple within-species observations (e.g. Pollux *et al.*, 2014). This method provides predicted trait values and variances for ancestral nodes and unmeasured extant species, offering a valuable alternative to removing missing observations in phylogenetic comparative analyses (Penone *et al.*, 2014). Trees were pruned to keep one representative specimen per species (or lineage in the *E. quadripunctatus* species complex); for those used in experiments, specimens from the same or nearest population (Table S2) were chosen. Outgroup species with missing physiological and ecological data were excluded.

Rates of evolution. Using the reconstructed ancestral values, we examined the rates of phenotypic change of each trait on individual branches across the phylogeny. For this we regressed the absolute phenotypic change of each branch (i.e. the absolute value of the difference between the reconstructed values of the corresponding initial and final node) against branch length (Ma) for each trait. Generalized Linear Models (GLMs) were used for this, assuming a Poisson distribution (or quasi-Poisson when overdispersion was detected) and the log link function. We identified outlier branches (beyond the upper 99% confidence interval of the regression line) which can be considered to show accelerated rates of evolution. We also compared the global rate of evolutionary change between maximum hyposmotic capacity, water loss rate and water content using Adam's method (Adams, 2013). For simplicity, only the maximum hyposmotic capacity was used for these analyses as it was significantly positively correlated with hyposmotic capacity ($R^2 = 0.37$, $P < 0.001$). This method compares a model that allows rates to vary amongst traits to one in which the rates are constrained to be equal, using a likelihood ratio test and AICc.

Phylogenetic signal. To determine whether the traits show a significant phylogenetic signal, we calculated the maximum likelihood value of Pagel's lambda (λ; Pagel, 1999) using *phylosig* (R package phytools, Revell, 2012). For those species with missing data, the predicted species means estimated from ancestral reconstruction analyses were employed. We used a likelihood ratio test to compare the fitted maximum likelihood value of λ with i) a model assuming no phylogenetic signal, i.e. an evolution of the character independent of phylogenetic relationships ($\lambda = 0$) and ii) a model entirely in agreement with BM, i.e. the probability of shared inheritance is strictly proportional to relatedness ($\lambda = 1$) (Freckleton *et al.*, 2002).

Relationships between traits. Phylogenetic generalized least squares (PGLS) were applied, using the R function *pgls* (caper), to explore the relationships between i) habitat

salinity and hyposmotic capacity, ii) habitat salinity and desiccation resistance, iii) desiccation resistance and hyposmotic capacity. Proportional data (% water content and % water loss rate) were arcsine transformed and hyposmotic capacity was log-transformed prior to analyses to improve fit to a normal distribution. Again, for simplicity, only the maximum hyposmotic capacity was used for these analyses (see above). We also traced the relative order of appearance of changes in desiccation resistance and maximum hyposmotic capacity across the entire tree (i.e. from root to the tip) for species for which data were obtained experimentally by plotting the reconstructed value of the variable at each of the nodes against the time of the node.

Topological uncertainty

To account for topological uncertainty we repeated all analyses using 1,000 randomly resampled post-burnin trees from the BEAST output where this was feasible (estimation of phylogenetic signal, PGLS and comparison of rates of phenotypic change).

RESULTS

Phylogeny of *Lumetus*

We obtained a well-resolved phylogeny of *Lumetus,* with strong support for most of the main nodes within the subgenus (Fig 1). The origin of *Lumetus* was estimated to have been in the Late Eocene (44 Ma, 95% HPD 32–57), followed by a split of the lineage containing *E. ochropterus* and *E. salomonis* at 38 (28–49) Ma and the lineage containing only *E. testaceus* at 36 (26–46) Ma. Within the remaining *Lumetus* species, the next split, at 32 (23–42) Ma, separated a clade of saline species (the *E. bicolor* group) from one including three subclades of Nearctic and Palaearctic species. Within these groups, both short branches and node age estimations suggest rapid diversification in the Oligocene-Miocene, around 27–5 Ma. The *E. quadripunctatus* group was formed of 6 recently diverged lineages (the *E. quadripunctatus* complex) with well characterised geographical distributions. These included (A) a coastal Mediterranean clade; (B) another containing a single specimen from Canada; two Eurasian clades, one (C) widely distributed and another (D) restricted to Bulgaria and Turkey; (E) a clade apparently restricted to Italy and (F) an Ibero-Moroccan clade.

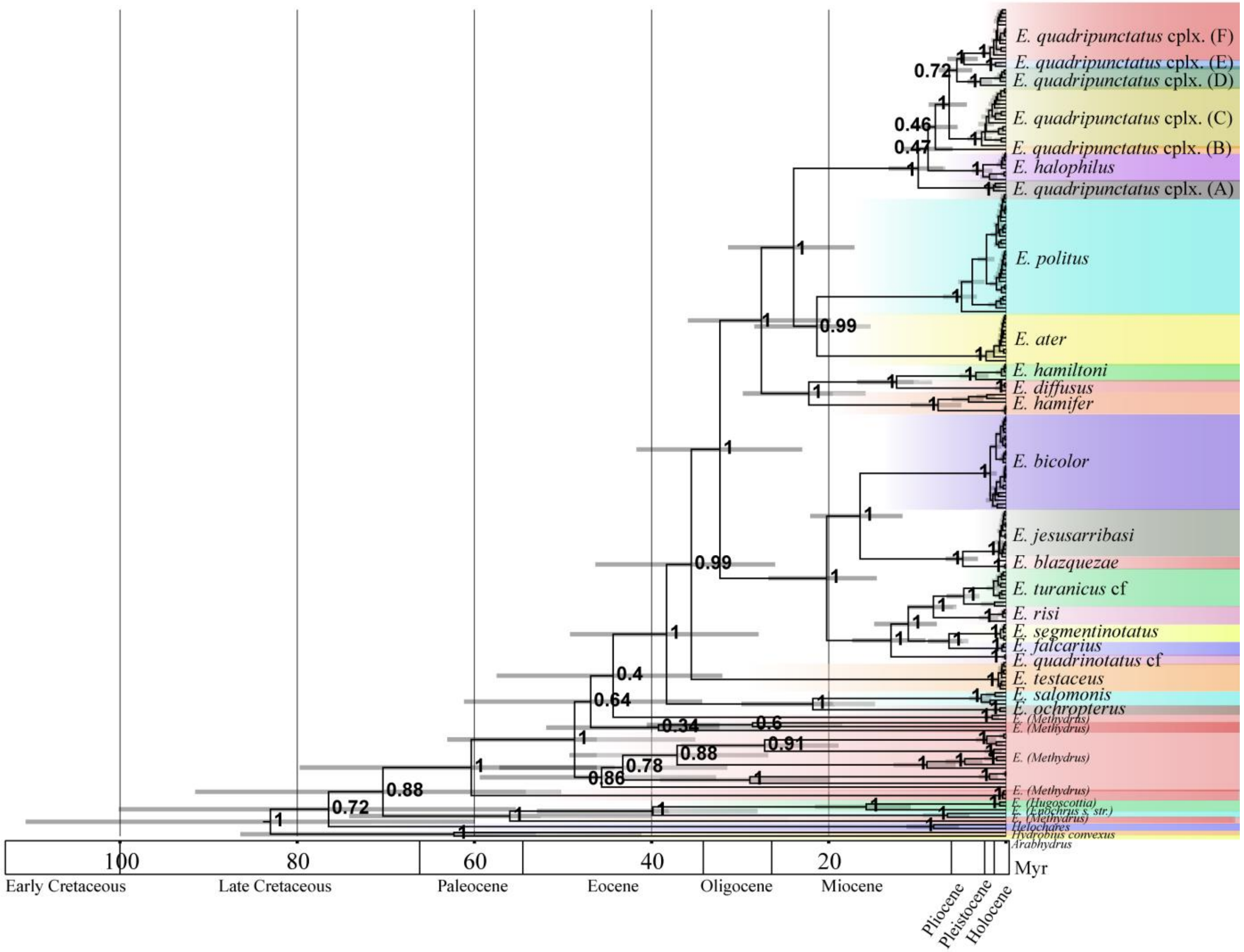

Figure 1. Dated phylogeny of *Lumetus*. Node numbers are posterior probabilities, bars on nodes are 95% confidence intervals for node ages.

Hyposmotic capacity and desiccation resistance

All species were hyperegulators at salinities above the isosmotic point. Under hyperosmotic conditions, all the species showed hyporegulation capacity within specific salinity ranges, except for one freshwater species, *E. salomonis*, which did not survive exposure to hyperosmotic conditions (> 35 g L^{-1}) (Fig. S1a, Table S4). In desiccation experiments, *E. halophilus* was the least desiccation resistant species (highest mortality and lowest recovery capacity), followed by *E. coarctatus* and *E. salomonis*, all living in fresh-mineralized waters. Amongst the remaining species, most exposed specimens survived, and were able to recover after desiccation (Fig. S1b). Water loss rates were highly correlated with survival (Fig. S1c) but not water content (Fig. S1c).

Habitat transitions, evolution of desiccation resistance and osmoregulatory capacity

Ancestral traits reconstruction and rates of evolution. For all traits studied, the distributions of δ under BM and OU models showed a high degree of overlap, indicating limited power to distinguish between evolutionary models (Fig. S2). Ancestral state reconstruction was therefore made assuming the simplest model. i.e. BM. The reconstructed values of all traits were within the range of the mean values obtained experimentally for extant species (Fig. 2). All measures of absolute phenotypic change (showed in Table S5) were significantly related to branch length ($P < 0.05$), except for water loss rate ($P = 0.07$). Accelerated rates of phenotypic evolution of all traits were identified in several branches across the tree (Fig. 2, Fig. S3).

The ancestor of *Lumetus* was inferred to be a species which lived in mineralized waters (Fig. 2a) with some degree of hyporegulation capacity (423 mOsmol kg^{-1} at 35 g L^{-1}, Fig. 2b), but within a limited salinity range (maximum estimated hyposmotic capacity of 1,000 mOsmol kg^{-1}, Fig. 2c). A rapid, direct transition to mesosaline waters took place at the origin of the *E. bicolor* group, as well as other independent transitions to mineralized-hyposaline waters (e.g. at the origin of *E. diffusus*-*E. hamiltoni* or *E. politus*) and accelerated reversions to freshwater habitats in the Nearctic-Palaearctic clades (Fig. 2a). In the *E. bicolor* group, transitions to meso and hypersaline waters were preceded by rapid increases in hyposmotic capacity, whilst a shift to freshwater habitats in *E. salomonis* was associated with the loss of hyporegulation ability.

The reconstructed ancestral values of water loss rate and water content varied little across *Lumetus* (13.6 – 16.5 % of fresh mass h^{-1} and 61.7 – 66.2 % of water to fresh mass, respectively). Water loss rates progressively decreased after the split of *E. testaceus* and within the *E. bicolor* group, alongside occupation of meso- and hypersaline waters. In the clades occupying fresh to hyposaline waters, desiccation rates remained almost constant, although some accelerated changes were identified within these, mostly on terminal branches (Fig. 2d). Water content showed accelerated increases on several branches, in some cases coinciding with rapid increases in hyposmotic capacity and transition to saline waters (*E. bicolor* group) and also accelerated and significant decreases in the *E. quadripunctatus* group (Fig. 2e).

Likelihood ratio tests indicated that the global rate of evolution for maximum osmotic capacity was significantly higher than for water loss rate and water content (Table 1).

Phylogenetic signal. For all traits, except from water loss rate, estimates of Pagel's λ were close to 1 (for habitat salinity λ was < 1 in 14% of resampled trees) and significantly better than those obtained when the phylogenetic structure was erased ($\lambda = 0$), indicating a significant phylogenetic signal (Table 2). For maximum osmotic capacity and water content, estimated λs were also better than those from a model in which the distribution of trait values across the phylogeny was as expected under BM (i.e. $\lambda = 1$). Water loss rate was the only trait showing no phylogenetic signal (Table 2).

Relationships between traits. In PGLS analyses (Table S6) habitat salinity showed no significant relationships either with maximum hyposmotic capacity or desiccation traits (Fig. 3a-c). Variability in maximum hyposmotic capacity and desiccation traits was higher amongst freshwater species than saline ones (i.e. mineralized-hypersaline taxa). In saline species, hyposmotic capacity and desiccation resistance tended to increase with habitat salinity (Fig. 3a-c).

Maximum hyposmotic capacity was negatively related to water loss rate and water content. However, these relationships were strongly influenced by the outlier values that

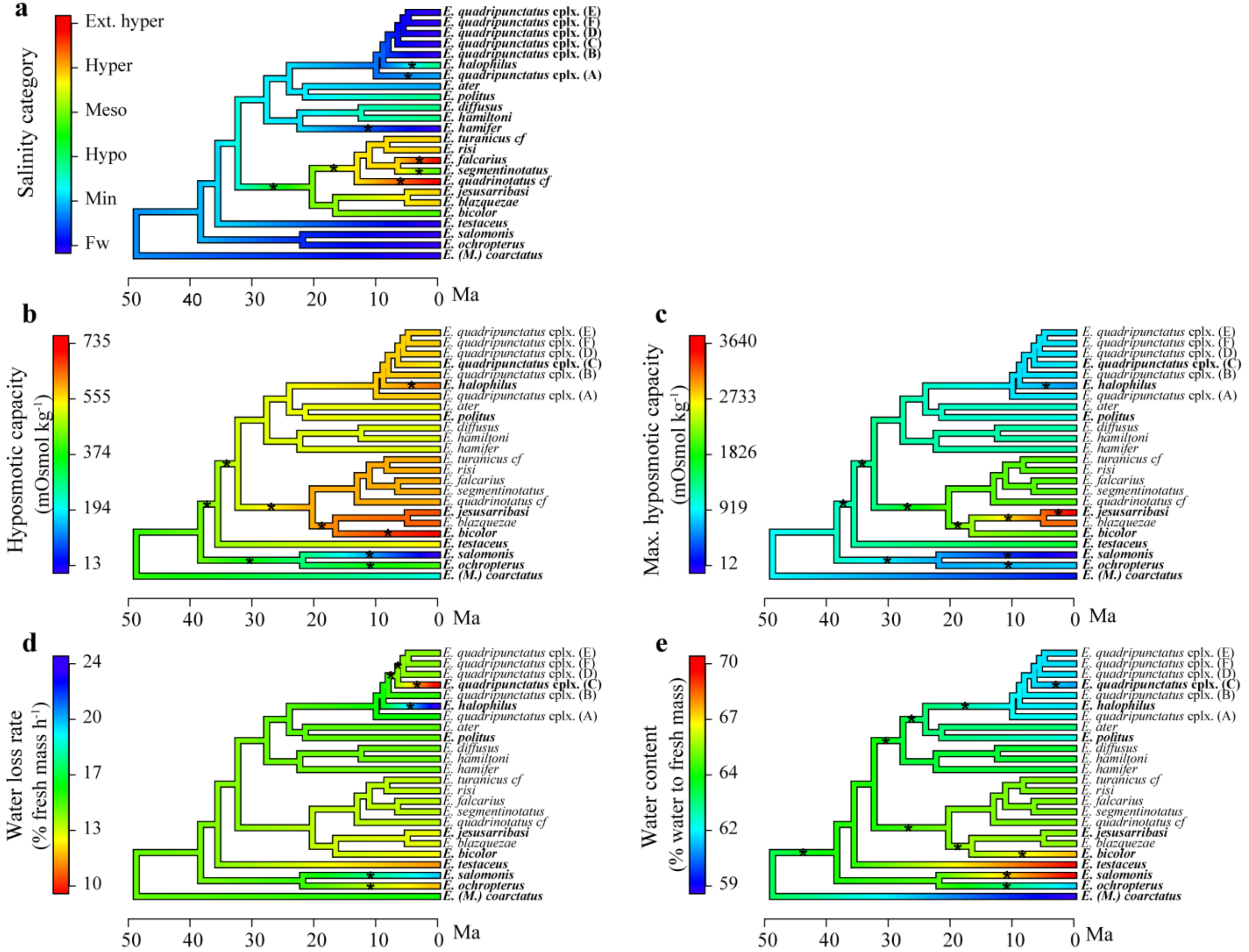

Figure 2. Ancestral reconstruction of desiccation and osmoregulation traits. The warmer (red) colours indicate higher resistance to desiccation or salinity than cooler (blue) colours. Branches where significantly accelerated increases or decreases in the rate of phenotypic change were identified are indicated by asterisks. Species for which ecological or experimental data were available are indicated in bold.

one species, *E. salomonis*, showed for these variables. After removing this species from PGLS, the relationship with water loss rate was not significant and the relationship with water content became stronger and positive (Table S6, Fig. 3d-e).

When the relative order of appearance of changes in desiccation resistance and maximum hyposmotic capacity was traced across individual branches of the phylogeny (Figs. 4 and 5), increases in hyposmotic capacity were coupled with decreases in water loss rate and increases in water content (*E. testaceus, E. bicolor* and *E. jesusarribasi*). However, increases in hyposmotic capacity were not clearly preceded by an increase in desiccation resistance. On the other hand, increases in desiccation resistance were not always associated with an increase in osmoregulatory capacity (e.g. *E. ochopterus* and *E. quadripunctatus* in Fig. 4, *E. salomonis* in Fig. 5).

Table 1. Comparison of evolutionary rates (log scale) for maximum osmotic capacity (Max. HC), water loss rate (WLR) and water content (WC). AIC$_C$ scores for a model that allows rates to vary amongst traits (observed) and a model that constrains rates of evolution to be equal amongst traits (constrained) as well as likelihood ratio tests for pairwise comparisons of evolutionary rates between trait pairs are given.

trait	σ^2	pairwise comparison	LRT$_{df=1}$	P	AICc
Max. HC	0.021 – 0.049				
WLR	0.001 – 0.004	Max. HC *vs*. WLR	27.4 – 36.4	< 0.001	obs = 54.2 – 67.4 cons = 82.5 – 100.9
WC	0.00003 – 0.00007	Max. HC *vs*. WC	121.1 – 125.5	< 0.001	obs = -40.3 – -25.2 cons = 78.8 – 97.9 cons = 80.9 – 99.2

Table 2. Ranges of the estimated Pagel's λ (for the randomized sample of 1,000 post-burnin trees) and P-values for the likelihood ratio test comparing estimated λ with a model assuming λ = 0 or λ = 1 (for the consensus tree).

Variable	Pagel's λ	P ($_{\lambda = 0}$)	P ($_{\lambda = 1}$)
Habitat salinity	0.96 – 1.13	< 0.001	0.697
Hyposmituc capacity	1.07 – 1.14	< 0.001	< 0.001
Max. hyposmotic capacity	1.04 – 1.13	< 0.001	0.051
Water loss rate	< 0.001	1	< 0.001
Water content	1.07 – 1.14	< 0.001	< 0.001

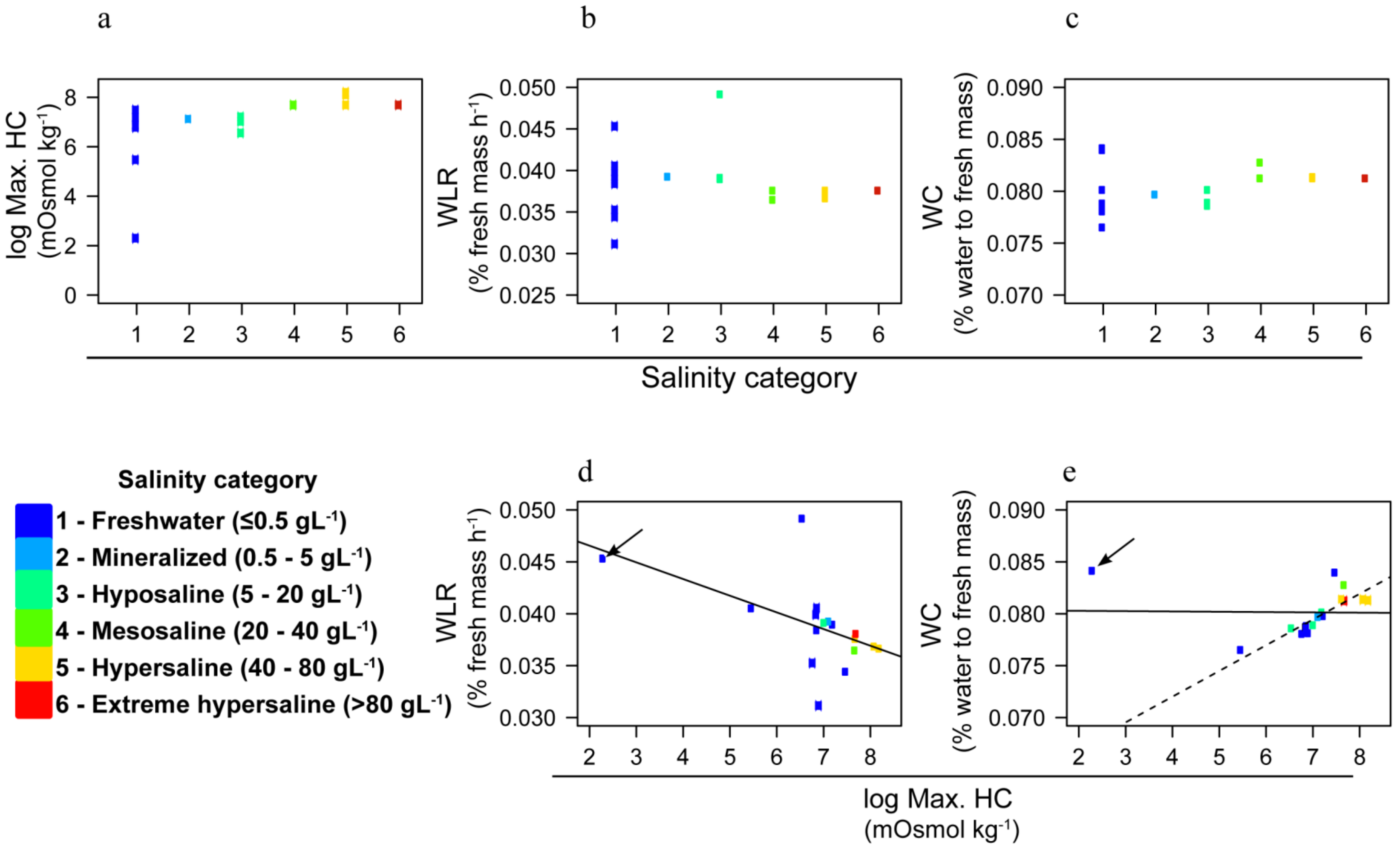

Figure 3. Relationships between habitat salinity, hyposmotic capacity and desiccation traits. Regression lines are shown for significant relationships in PGLS (see Table S6). Dashed line for regressions exluding *E. salomonis* (indicated by arrow). Max. HC: maximum hyposmotic capacity, WLR: water loss rate, WC: water content.

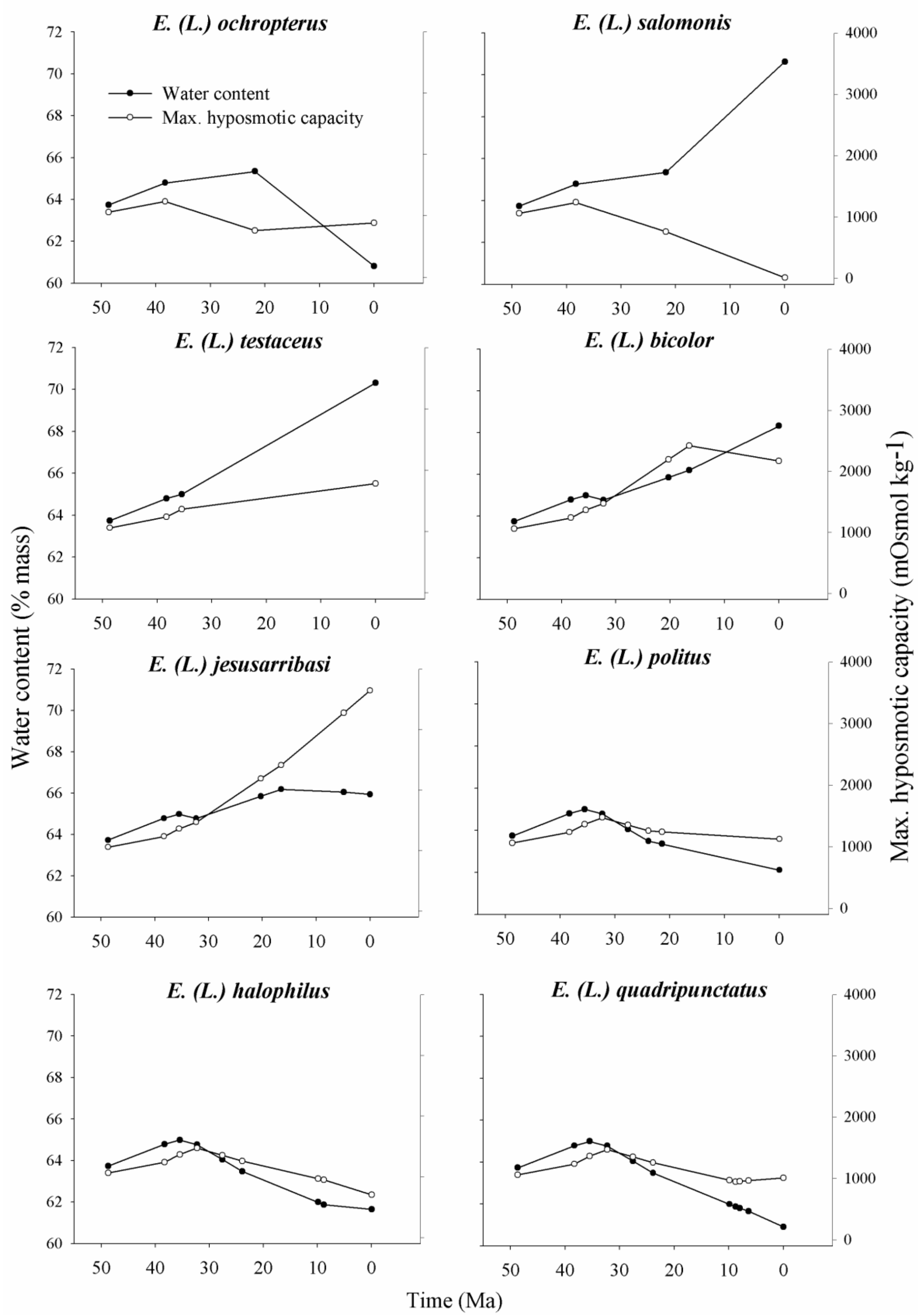

Figure 4. Values of water loss rate and maximum hyposmotic capacity through the full evolutionary path of the *Lumetus* species used in desiccation and osmoregulation experiments.

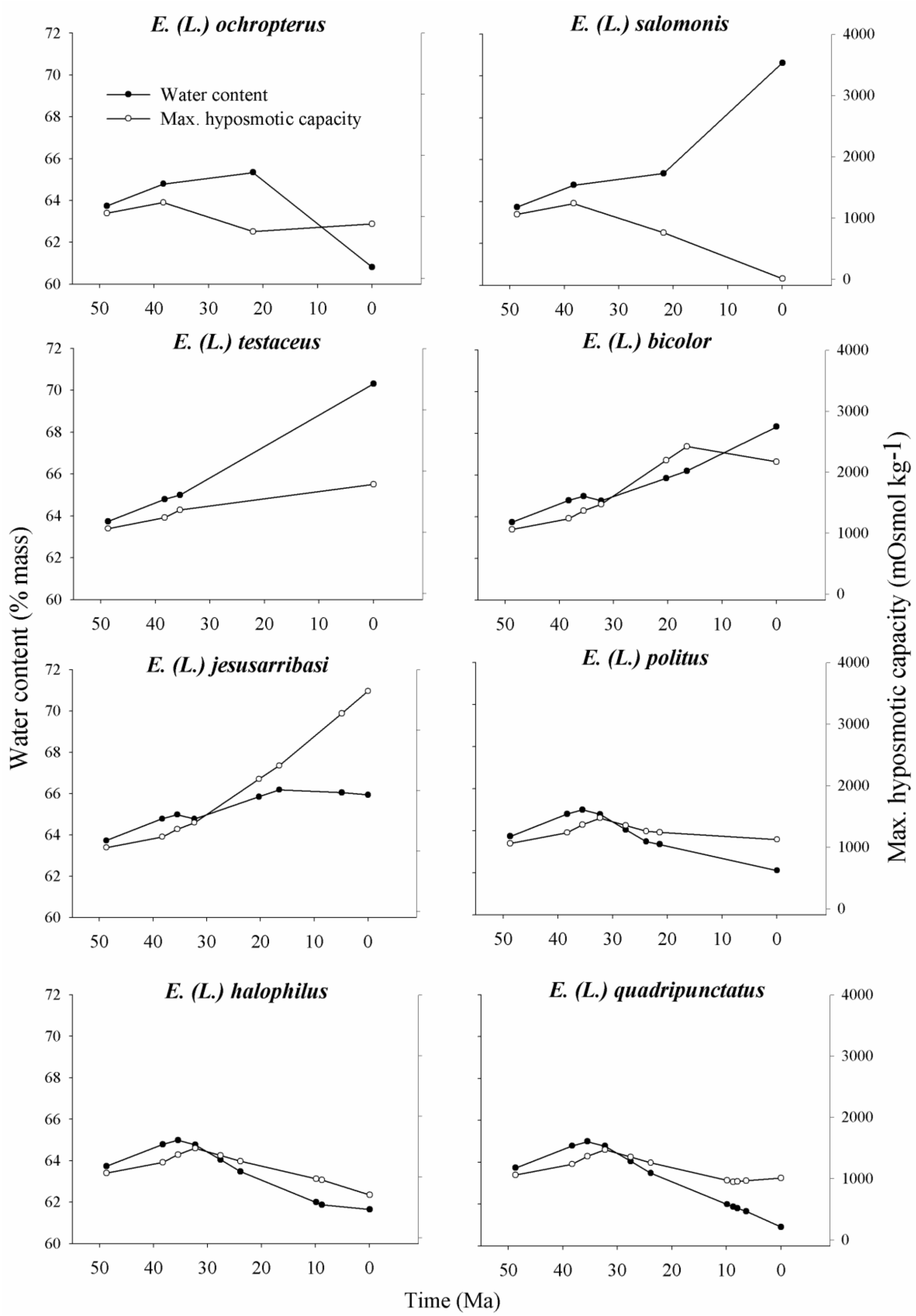

Figure 5. Values of water content and maximum osmotic capacity trough the full evolutionary path of the *Lumetus* species used in desiccation and osmoregulation experiment

DISCUSSION

The reconstruction of habitat transitions, desiccation and osmoregulatory traits in *Lumetus* species suggest that hyporegulation capacity, an essential trait for the colonisation of hyperosmotic media by aquatic insects, arose as a mechanism derived from those originally developed to deal with desiccation stress in this lineage, supporting our first hypothesis.

The ancestral reconstruction of water loss rates suggests that the most common recent ancestor of *Lumetus* had similar desiccation resistance to extant species of the subgenus. Water loss rates did not change abruptly through the evolutionary history of the lineage, but have instead apparently remained relatively stable, as suggested by the lack of phylogenetic signal in this trait. The control of water loss has been previously reported as essential for survival in some *Lumetus* species (Pallarés *et al.*, 2016), and lower water loss rates in *E. jesusarribasi* have been shown to be associated with the relatively high survorship of desiccation in this species, when compared with co-occuring hypersaline diving beetles (Pallarés *et al.*, 2017). Our data suggest high resistance to desiccation in the whole *Lumetus* subgenus, something which could be a plesiomorphic character present in the wider genus *Enochrus*, or even the Hydrophilidae. Despite the lack of data on desiccation resistance of other hydrophilids, there have been multiple transitions between terrestrial and aquatic environments within this family (Bernhard *et al.*, 2006; Short & Fikacek, 2013), something which would be in agreement with this hypothesis.

The ancestor of *Lumetus* was inferred to had lived in mineralized waters, and to have had moderate hyporegulation capacity. In contrast to the low variation in water loss rates, hyporegulation capacity has undergone large and, in some cases, accelerated changes trough the evolutionary history of *Lumetus*, most of these being associated with habitat transitions across the salinity gradient. Arribas *et al.* (2014) found that transitions to saline habitats in the *E. bicolor* group occurred more rapidly than in the rest of the lineage. In agreement with this result, we found that transitions from fresh-mineralized to mesosaline waters and the subsequent diversification of these beetles in saline habitats were associated with rapid increases in the efficiency of their hyporegulation.

Species living in the most saline conditions showed high hyposmotic capacity, but also an increased desiccation resistance (i.e. lower water loss rates). In the case of species living in fresh to hyposaline waters, we found i) some with comparable or even higher desiccation resistance than their saline water relatives, but relatively low hyposmotic capacity (e.g. *E. ochropterus* and *E. quadripunctatus*) and ii) others which had both high desiccation resistance and hyporegulation capacity. For example, *E. testaceus* and *E. politus* were able to hyporegulate at salinities well above those encountered by these beetles in nature. A lack of association between habitat salinity and osmoregulatory ability is also seen in some Crustacea (e.g. McNamara & Faria, 2012; Faria *et al.*, 2017). In light of this, Faria *et al.* (2017) proposed that the evolution of osmoregulatory ability in semi-aquatic crabs may be mediated by selection on gill function to reduce water loss (Takeda *et al.*, 1996), meaning that desiccation resistance (i.e. degree of terrestrialization) and osmoregulation capacities are positively associated.

Overall, our findings are consistent with an evolutionary sequence in which improved desiccation resistance in *Lumetus* provided the physiological basis for the development of efficient hyporegulation mechanisms, which in some cases allowed them to colonize and diversify in the meso- and hypersaline habitats. The accelerated increases of hyporegulation capacity in some parts of the phylogeny are consistent with the hypothesis that such capacity is based on a derived mechanism (i.e. in agreement with our first hypothesis). Accelerated evolution of complex mechanisms such as those involved in hyporegulation (Bradley, 2009) are more likely to occur when such a mechanistic basis is already present (Barrett & Schluter, 2008; Roesti *et al.*, 2014).

Our assumption of a Brownian-motion model of evolution for ancestral trait reconstruction constrains reconstructed values to within the range of measured variation of each trait (Finarelly & Goswami, 2013). This could underestimate the real interspecific variation of some traits in *Lumetus*. However, the water contents of the species studied were close to typical values seen in most beetles (i.e. 60% of body mass - Hadley, 1994) and hyporegulation capacity covered the full physiological range (i.e. from no hyporegulation ability to a very high capacity under extreme hyperosmotic conditions). Species that inhabit the most extreme hypersaline habitats (e.g. *E. quadrinotatus* and *E. falcarius*), for which no experimental data were available, may possess higher hyporegulation capacities than those inferred in our ancestral reconstructions. Such high hyporegulation capacities would result from accelerated evolution of this trait in some branches within the *E. bicolor* clade, providing additional weight to our conclusions.

Due to the high ancestral tolerance to desiccation in the subgenus *Lumetus* it was not possible to reconstruct the hypothesised increase in desiccation resistance preceding any improvements in hyporegulation capacity. Notwithstanding this limitation, rapid increases in hyporegulation capacity were associated with weak decreases in water loss rates across the evolutionary path of the species. Water content and hyposmotic capacity evolved in parallel in *Lumetus*, being positively associated across the phylogeny. This is consistent with a recent study demonstrating that water beetles acclimated at high salinities have higher body water content than those acclimated at lower salinities (Pallarés *et al.*, 2017). Despite these parallel changes, correlated evolution of both tolerances constrained by identical genes and mechanisms (genetic correlation *sensu* Kellermann *et al.*, 2013) (i.e. our third hypothesis), is incompatible with the occurrence of species resistant to desiccation but with reduced hyporegulation capacities such as *E. ochropterus* and the studied species of the *E. quadripunctatus* group. Although control of water loss under desiccation and responses to salinity stress share a common physiological basis (e.g. ion transport and cell volume regulation processes, Beyenbach, 2016), insects have also evolved other specific mechanisms to deal with either desiccation or salinity, which will determine their overall tolerance to these stressors. Some transcriptomic studies have characterised changes in gene expression patterns elicited by either dehydration (López-Martínez *et al.*, 2009) or salinity (Uyhelhi *et al.*, 2016) in insect taxa. Further research identifying potential gene expression pathways common to both stressors would shed light on their degree of mechanistic overlap.

Parallel increases in desiccation resistance and salinity tolerance could have been strengthened as a response to aridification during the radiation of *Lumetus*. According to Arribas *et al.* (2014) and in agreement with our results, desiccation resistance and hyporegulation capacity in the *E. bicolor* group started to increase in parallel in the Late Eocene, a period of global aridification (Mosbrugger *et al.*, 2005; Bosboom *et al.*, 2014). The simultaneous decrease of precipitation and an increase in the mineralization of the surface waters in some populations of these *Lumetus* species could have posed a strong selective pressure on the further development of existing mechanisms for water and ionic balance. Other studies have proposed that global aridification events promoted diversification of several aquatic taxa (e.g. Pinceel *et al.*, 2013; Dorn *et al.*, 2014). Aridification, by enhancing the linked tolerance of desiccation and salinity could have also been a key driver in the diversification of *Lumetus*.

Euryhalinity is an important source of evolutionary diversity (Schultz & McCormick, 2012; Brauner *et al.*, 2013). However, the process of adaption to saline inland waters seems to be a unidirectional path, likely reflecting trade-offs between competitive ability and tolerance to osmotic stress (Dunson & Travis, 1991; Herbst, 2001; Latta *et al.*, 2012). Species of *Lumetus* (and other beetle genera) typical of saline waters, are almost absent from freshwater habitats, despite been able to hyperregulate (Tones, 1977; Céspedes *et al.*, 2013; Pallarés *et al.*, 2015). Such a situation also holds for saline Hemiptera (corixids, Tones & Hammer, 1975), coastal and estuarine decapods (McNamara &Faria, 2012; Faria *et al.*, 2017) and fish (Schultz & McCormick, 2012). The maintenance of hyperosmotic capacity, despite it having apparently lost its ecological role, may reflect positive pleiotropies or functional correlations between hypo- and hyperregulatory mechanisms (e.g. Smith *et al.*, 2008, 2010) or that there is little cost to maintaining functional osmoregulatory responses outside conditions commonly encountered in nature (Divino *et al.*, 2016).

The fundamental salinity tolerance niche of some fresh-hyposaline species is also much broader than their realized niches (e.g. in *E. testaceus*), something which also supports the view that hyporegulation capacity arose as a co-opted mechanism. The osmoregulatory physiology of water beetles is still poorly explored, so it is not known if euryhalinity is common in freshwater species of other genera, but at least two dytiscid species typical of freshwater habitats are unable to osmoregulate at salinities above their isosmotic point (*Nebrioporus,* Family Dytiscidae, Pallarés *et al.*, 2015). The absence of species of *Lumetus* able to osmoregulate in saline habitats may be due to multiple factors, amongst them biological interactions, ecological requirements of juvenile stages, or physiological traits other than osmoregulation (e.g. Dowse *et al.*, 2017).

In short, our results demonstrate how a combination of ecological, experimental and phylogenetic data can offer powerful insights into the origin and evolution of traits underlying ecological transitions and the diversification of lineages into previously unavailable areas of niche space. Further research is still needed to understand why only some insect taxa have colonized the naturally stressful inland saline waters, but we show here that linked evolution of stress resistance traits could have been key for development of tolerance to extreme salinities.

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Table S1. Studied specimens of *Enochrus* and outgroup species.

Specimen	Species	Locality	Colection date	Colector	Voucher
ARAsp_OMA_RA106	*Arabhydrus sp*	Oman, Al Rija, Al Mayb wady	10-04-10	Ribera, Cieslak & Hernando Leg	IBE-RA106
ENO_BRA_PAR_IGUA_AB321	*Enochrus sp*	Brazil, Parana, Fos do Iguassu	25-08-00	Ribera Leg	IBE-AB321
ENO_CAN_NOV_BRET_AN352	*Enochrus (Lumetus) quadripunctatus* cplx.	Canada, Nova Scotia, Cape Breton, freshwater stream	20-08-07	Ribera & Cieslak Leg	IBE-AN352
ENO_SAF_SP33	*Enochrus sp*	South Africa		Hidalgo Leg	IBE-SP33
ENO_SAF_SP34	*Enochrus sp*	South Africa		Hidalgo Leg	IBE-SP34
ENO_SAF_SP35	*Enochrus sp*	South Africa	2007	Leschen via Abellán	IBE-SP35
ENO_USA_CAL_POST_AN387	*Enochrus sp*	United States, California, Post Office Spring	10-04-08	Abellán Leg	IBE-AN387
ENOaffi_DEN_ROM_POND_AB315	*Enochrus (Methydrus) affinis*	Denmark, Romo Island, Romo Island pond	23-08-01	Ribera & Cieslak Leg	IBE-AB315
ENOaffi_SCH_SUTH_BED_AB311	*Enochrus (Methydrus) affinis*	Scotland, Sutherland, Strath of Kildonan, Bed river	15-07-01	Ribera & Foster Leg	IBE-AB311
ENOater_CYP_ARK_AN444	*Enochrus (Lumetus) ater*	Cyprus, Akrotiri, Fassouri reedbeds	26-04-16	Millán, Ribera, Velasco & Villastrigo Leg	IBE-AN444
ENOater_CYP_ORO_AN456	*Enochrus (Lumetus) ater*	Cyprus, Oroklini, saline wetland	25-04-16	Millán, Ribera, Velasco & Villastrigo Leg	IBE-AN456
ENOater_AZE_KAT_ABSE_SP1	*Enochrus (Lumetus) ater*	Azerbaijan, Kathai, pond in Abseron pen.	2014	Rudoy Leg	IBE-SP1
ENOater_AZE_QOB_SP13	*Enochrus (Lumetus) ater*	Azerbaijan, Qobustan, ponds in beach	2014	Ribera & Rudoy Leg	IBE-SP13
ENOater_FRA_SIG_PALM2_AB235	*Enochrus (Lumetus) ater*	France, Sigean, Arroyo hiposalino Salines de Lapalme	16-10-09	Andújar & Arribas Leg	IBE-AB235
ENOater_MOR_MOU_DRAD_AB2	*Enochrus (Lumetus) ater*	Morocco, Moulay, Bousselahm, Oued Drader	12-04-07	Ribera, Aguilar, Hernando Leg	IBE-AB2
ENOater_OMA_BAMA_AB267	*Enochrus (Lumetus) ater*	Oman, Bamah, Bamah marsh	10-04-10	Ribera, Cieslak & Hernando Leg	IBE-AB267
ENOater_SPA_ALB_ARQU_AB192	*Enochrus (Lumetus) ater*	Spain, Albacete, Robledo, Laguna del Arquillo	02-06-02	Ribera & Cieslak Leg	IBE-AB192
ENOater_SPA_BAL_FONT_AB263	*Enochrus (Lumetus) ater*	Spain, Baleares, Mallorca, Salines de Ses Fontanelles. Can Pastilla	13-12-09	Andújar & Lencina Leg	IBE-AB263
ENOater_SPA_CAS_TORR_AB234	*Enochrus (Lumetus) ater*	Spain, Castellón, Torreblanca, Laguna Costera Torreblanca	24-10-09	Andújar & Arribas Leg	IBE-AB234

ENOater_SPA_TAR_TORR_AB254	*Enochrus (Lumetus) ater*	Spain, Tarragona, Torredembarra, Estany El Saler. Marítima Residencial	22-10-09	Andújar & Arribas Leg	IBE-AB254
ENOater_SPA_ZAR_SMAR_AB179	*Enochrus (Lumetus) ater*	Spain, Zaragoza, San Marcos, Chiprana pond	20-07-04	Ribera & Cieslak Leg	IBE-AB179
ENOater_TUR_IZM_ILDI_SP37	*Enochrus (Lumetus) ater*	Turkey, Izmir prov, saline ditch	24-07-14	Ribera & Cieslak	IBE-SP37
ENObico_CYP_LAR_AN450	*Enochrus (Lumetus) bicolor*	Cyprus, Larnaka, saline coastal pond	25-07-14	Millán, Ribera,Velasco & Villastrigo Leg	IBE-AN450
ENObico_ALG_MHAD_AB328	*Enochrus (Lumetus) bicolor*	Algeria, Oran, Mer el Hadja, Charca playa Mer el Hadja	27-05-10	Lencina & Serrano Leg	IBE-AB328
ENObico_ALG_MHAD_AB329	*Enochrus (Lumetus) bicolor*	Algeria, Oran, Mer el Hadja, Charca playa Mer el Hadja	27-05-10	Lencina & Serrano Leg	IBE-AB329
ENObico_AZE_ACI_SP9	*Enochrus (Lumetus) bicolor*	Azerbaijan, Acidere	2014	Ribera & Rudoy Leg	IBE-SP9
ENObico_FRA_ADG_ONGL_AB228	*Enochrus (Lumetus) bicolor*	France, Adge, Les Onglous	16-10-09	Andújar & Arribas Leg	IBE-AB228
ENObico_IRE_CLA_FINA_AB303	*Enochrus (Lumetus) bicolor*	Ireland, Clare, Finavarra, Lough Muree brackish pond	23-05-10	Ribera Leg	IBE-AB303
ENObico_ITA_SIC_TRAP_AB39	*Enochrus (Lumetus) bicolor*	Italy, Sicily, Trapani, Salinas di Trapani	11-06-07	Abellán & Picazo Leg	IBE-AB39
ENObico_POR_VIL_SP20	*Enochrus (Lumetus) bicolor*	Portugal, Vilanova de Milfontes, pond in grassland	24-01-08	Ribera Leg	IBE-SP20
ENObico_SPA_ALA_CARR_AB108	*Enochrus (Lumetus) bicolor*	Spain, Vitoria, La Guardia, Laguna de Carralogroño	22-07-04	Ribera & Cieslak Leg	IBE-AB108
ENObico_SPA_ALB_CORR_AB227	*Enochrus (Lumetus) bicolor*	Spain, Albacete, Corralrubio, Laguna de Corralrubio	07-07-09	Millán & Arribas Leg	IBE-AB227
ENObico_SPA_ALM_GATA_AB232	*Enochrus (Lumetus) bicolor*	Spain, Almería, Cabo de Gata, Salinas de Cabo de Gata	01-02-10	Andújar & Arribas Leg	IBE-AB232
ENObico_SPA_BAL_CODO_AB184	*Enochrus (Lumetus) bicolor*	Spain, Baleares, Ibiza, Salinas de Platja Codolar	13-10-04	Palmer & Jaume Leg	IBE-AB184
ENObico_SPA_BAL_FONT_AB286	*Enochrus (Lumetus) bicolor*	Spain, Baleares, Mallorca, Salines de Ses Fontanelles. Can Pastilla	13-12-09	Andújar & Lencina Leg	IBE-AB286
ENObico_SPA_BAL_POLL_AB29	*Enochrus (Lumetus) bicolor*	Spain, Baleares, Mallorca, Port de Pollensa pozas	11-11-00	Ribera & Cieslak Leg	IBE-AB29
ENObico_SPA_BAR_AVIN_SP26	*Enochrus (Lumetus) bicolor*	Spain, Barcelona, Avinyo, Torrent Salat	15-07-13	Ribera, Sánchez, Picazo Leg	IBE-SP26
ENObico_SPA_GUA_ALCO_AB93	*Enochrus (Lumetus) bicolor*	Spain, Guadalajara, Alcolea de la Peñas,arroyo salino	21-05-05	Millán et col. Leg	IBE-AB93
ENObico_SPA_GUA_BELI_AB78	*Enochrus (Lumetus) bicolor*	Spain, Guadalajara, Belinchón, Arroyo en las Salinas de Belinchón	08-10-06	Millán et col. Leg	IBE-AB78
ENObico_SPA_GUA_IMON_AB16	*Enochrus (Lumetus) bicolor*	Spain, Guadalajara, Imón, Arroyo en las Salinas de Imón	22-05-05	Ribera & Cieslak Leg	IBE-AB16

ENObico_SPA_HUV_PINA_AB287	*Enochrus (Lumetus) bicolor*	Spain, Huelva, Marismas del Pinar	30-01-10	Andújar & Arribas Leg	IBE-AB287
ENObico_SPA_JAE_BRUJ_AB59	*Enochrus (Lumetus) bicolor*	Spain, Jaén, Brujuelo, Arroyo en las Salinas de Brujuelo	27-07-07	Millán et col. Leg	IBE-AB59
ENObico_SPA_MUR_REVE_AB58	*Enochrus (Lumetus) bicolor*	Spain, Murcia, Rambla del Reventón	18-09-07	Millán et col. Leg	IBE-AB58
ENObico_SPA_NAV_MEND_AB7	*Enochrus (Lumetus) bicolor*	Spain, Navarra, Barranco Salado de Mendavia	29-07-07	Millán et col. Leg	IBE-AB7
ENObico_SPA_NAV_YUGO_AB176	*Enochrus (Lumetus) bicolor*	Spain, Navarra, El Yugo, Bárdenas Blancas	21-07-04	Ribera & Cieslak Leg	IBE-AB176
ENObico_SPA_TAR_GERR_AB170	*Enochrus (Lumetus) bicolor*	Spain, Lérida, Gerry de la Sal, Arroyo en Gerry de la Sal	27-09-08	Abellán Leg	IBE-AB170
ENObico_SPA_ZAR_MAGA_SP28	*Enochrus (Lumetus) bicolor*	Spain, Magallón, Lagunas de Magallón	2014	Ribera & Cieslak Leg	IBE-SP28
ENObico_SPA_ZAR_SMAR_AB43	*Enochrus (Lumetus) bicolor*	Spain, Zaragoza, San Marcos, Chiprana pond	20-07-04	Ribera & Cieslak Leg	IBE-AB43
ENOblaz_MOR_JOR_MGHA_AB246	*Enochrus (Lumetus) blazquezae*	Morocco, Jorf El Melha, Sidi Kacem, Oued Mghassem	18-04-06	Millán et col. Leg	IBE-AB246
ENOblaz_MOR_TIS_TISA_AB247	*Enochrus (Lumetus) blazquezae*	Morocco, Tissa, Taounate, Salines de Tissa	19-04-06	Millán et col. Leg	IBE-AB247
ENOcoar_ENG_NOR_BROA_AB312	*Enochrus (Methydrus) coarctatus*	England, Norfolk, The Broads, Catfield Fen 4	07-05-06	Ribera Leg	IBE-AB312
ENOcoar_ENG_NOR_HOWH2_AB37	*Enochrus (Methydrus) coarctatus*	England, Norfolk, Lufham, How Hill Marsh 2	06-05-06	Ribera Leg	IBE-AB37
ENOcoar_IRE_CLA_RINE_AB305	*Enochrus (Methydrus) coarctatus*	Ireland, Clare, Rinecaha, Rinecaha fen	22-05-10	Ribera Leg	IBE-AB305
ENOcoar_ITA_BRE_SANA_AB236	*Enochrus (Methydrus) coarctatus*	Italy, Brescia, Santa Anna, Santa Anna pond	17-10-02	Ribera, Cieslak, Toledo & Mazzoldi Leg	IBE-AB236
ENOdiff_USA_CAL_AMAR_AN378	*Enochrus (Lumetus) diffusus*	California, Amargosa River in Tecopa	2008	Abellán Leg	IBE-AN378
ENOdiff_USA_CAL_BALD_AN372	*Enochrus (Lumetus) diffusus*	California, Baldwin Lake	2008	Abellán Leg	IBE-AN372
ENOdiff_USA_CAL_SODA_AB49	*Enochrus (Lumetus) diffusus*	United States, California, Baker, Soda lake	07-04-08	Abellán Leg	IBE-AB49
ENOfalc_ITA_SIC_SALS_AB224	*Enochrus (Lumetus) falcarius*	Italy, Sicily, Villadoro, Afluente mesosalino del Salso	27-07-09	Gutiérrez-Cánovas Leg	IBE-AB224
ENOfalc_ITA_SIC_TURV_AB223	*Enochrus (Lumetus) falcarius*	Italy, Sicily, Cianciana, Fiume Tùrvoli	26-07-09	Gutiérrez-Cánovas Leg	IBE-AB223
ENOfalc_ITA_SIC_VACC_AB23	*Enochrus (Lumetus) falcarius*	Italy, Sicily, Caltanissetta, Torrente Vaccarizzo, Castello	12-06-07	Abellán & Picazo Leg	IBE-AB23
ENOhalo_ENG_SHE_NHM3	*Enochrus (Lumetus) halophilus*	England, Sheppey Island	2016		
ENOhalo_MOR_AZR_AFEN_AB323	*Enochrus (Lumetus) halophilus*	Morocco, Azrou, Afenourir, Lac Afenourir	29-04-00	Ribera, Aguilar, Hernando & Millán Leg	IBE-AB323

ENOhalo_MOR_IFR_AZOL_AB41	*Enochrus (Lumetus) halophilus*	Morocco, Ifrane, Hachlaf, Azolla	11-04-07	Ribera, Aguilar, Hernando Leg	IBE-AB41
ENOhalo_SPA_ALA_CARR_AB32	*Enochrus (Lumetus) halophilus*	Spain, Vitoria, La Guardia, Laguna de Carralogroño	22-07-04	Ribera & Cieslak Leg	IBE-AB32
ENOhalo_SPA_ALB_PET_NHM10	*Enochrus (Lumetus) halophilus*	Spain, Albacete, Pétrola, Laguna de Pétrola	2016		
ENOhalo_SPA_HUV_LUCI_AB193	*Enochrus (Lumetus) halophilus*	Spain, Huelva, Doñana, Lucio del Palacio	22-04-03	Ribera, Aguilar, Hernando, Cieslak & Millán Leg	IBE-AB193
ENOhalo_SPA_NAV_PITI_AB64	*Enochrus (Lumetus) halophilus*	Spain, Navarra, Pitillas, Pitillas poza	21-07-04	Ribera & Cieslak Leg	IBE-AB64
ENOhalo_SPA_NAV_PURG_AB63	*Enochrus (Lumetus) halophilus*	Spain, Navarra, Tudela, Purguer poza	20-07-04	Ribera & Cieslak Leg	IBE-AB63
ENOhamf_CYP_ARK_AN443	*Enochrus (Lumetus) hamifer*	Cyprus, Akrotiri, Fassouri reedbeds	26-04-16	Millán, Ribera, Velasco & Villastrigo Leg	IBE-AN443
ENOhamf_CYP_LAR_AN453	*Enochrus (Lumetus) hamifer*	Cyprus, Larnaka, saline wetland	25-04-16	Millán, Ribera, Velasco & Villastrigo Leg	IBE-AN453
ENOhamf_CYP_ORO_AN457	*Enochrus (Lumetus) hamifer*	Cyrpus, Oroklini, saline wetland	25-04-16	Millán, Ribera, Velasco & Villastrigo Leg	IBE-AN457
ENOhamf_BUL_KOT_ARDA_SP38	*Enochrus (Lumetus) hamifer*	Bulgaria, Kotlari, residual pools in river Arda	2015	Ribera Leg	IBE-SP38
ENOhamf_GRE_ARK_SP2	*Enochrus (Lumetus) hamifer*	Greece, Arkadia, pond	2013	Ribera & Cieslak Leg	IBE-SP2
ENOhamf_TUR_ERZ_SENY_RA690	*Enochrus (Lumetus) hamifer*	Turkey, Erzurum, Senyurtköyu, stream	12-06-11	Ribera et col. Leg	IBE-RA690
ENOhami_USA_CAL_AMAR_AB48	*Enochrus (Lumetus) hamiltoni*	United States, California, Tecopa, Amargosa River	08-04-08	Abellán Leg	IBE-AB48
ENOhami_USA_CAL_CARP_AB47	*Enochrus (Lumetus) hamiltoni*	United States, California, Carpinteria Salt Marsh	26-03-08	Abellán Leg	IBE-AB47
ENOhami_USA_CAL_OIL_AN375	*Enochrus (Lumetus) hamiltoni*	California, Coal Oil Point Reserve	26-03-08	Abellán Leg	IBE-AN375
ENOhami_USA_CAL_POST_AN388	*Enochrus (Lumetus) hamiltoni*	California, Post Office Spring	10-04-08	Abellán Leg	IBE-AN388
ENOhami_USA_CAL_SPRI_AN376	*Enochrus (Lumetus) hamiltoni*	California, Salt Creek in Spring Hills	08-04-08	Abellán Leg	IBE-AN376
ENOjesu_SPA_CAD_HORT_AB221	*Enochrus (Lumetus) jesusarribasi*	Spain, Cádiz, El Bosque, Salinas de Hortales	29-01-10	Andújar & Arribas Leg	IBE-AB221
ENOjesu_SPA_COR_MATU_AB369	*Enochrus (Lumetus) jesusarribasi*	Spain, Córdoba, Baena, Salinas de la Maturra	28-07-98	Ribera Leg	IBE-AB369
ENOjesu_SPA_COR_PRIE_AB81	*Enochrus (Lumetus) jesusarribasi*	Spain, Córdoba, Priego de Córdoba, Río Salado de Priego	01-02-08	Millán et col. Leg	IBE-AB81
ENOjesu_SPA_CUE_MING_AB139	*Enochrus (Lumetus) jesusarribasi*	Spain, Cuenca, Minglanilla, Arroyo Salado en Minglanilla	31-01-08	Millán et col. Leg	IBE-AB139
ENOjesu_SPA_JAE_BRUJ_AB80	*Enochrus (Lumetus) jesusarribasi*	Spain, Jaén, Brujuelo, Arroyo en las	21-07-06	Millán et col. Leg	IBE-AB80

		Salinas de Brujuelo			
ENOjesu_SPA_JAE_PORC_AB387	*Enochrus (Lumetus) jesusarribasi*	Spain, Jaén, Porcuna, Arroyo en las Salinas de Porcuna	28-07-98	Ribera Leg	IBE-AB387
ENOjesu_SPA_JAE_SILE_AB222	*Enochrus (Lumetus) jesusarribasi*	Spain, Jaén, Siles, Arroyo Salado en Siles	24-10-09	Andújar & Arribas Leg	IBE-AB222
ENOjesu_SPA_MUR_AMAR_AB457	*Enochrus (Lumetus) jesusarribasi*	Spain, Murcia, Rambla de Agua Amarga	27-11-10	Andújar & Arribas Leg	IBE-AB457
ENOjesu_SPA_MUR_CHIC_AB386	*Enochrus (Lumetus) jesusarribasi*	Spain, Murcia, Abanilla, Río Chícamo	21-09-07	Millán et col. Leg	IBE-AB386
ENOjesu_SPA_MUR_REVE_AB463	*Enochrus (Lumetus) jesusarribasi*	Spain, Murcia, Rambla del Reventón	27-11-10	Andújar & Arribas Leg	IBE-AB463
ENOjesu_SPA_MUR_RSAL_AB79	*Enochrus (Lumetus) jesusarribasi*	Spain, Murcia, Fortuna, Rambla Salada en las Salinas	21-09-07	Millán et col. Leg	IBE-AB79
ENOjesu_SPA_MUR_SANG_AB9	*Enochrus (Lumetus) jesusarribasi*	Spain, Murcia, Sangonera, Arroyo de las Salinas de Sangonera	22-01-08	Millán et col. Leg	IBE-AB9
ENOjesu_SPA_SEV_MONT_AB82	*Enochrus (Lumetus) jesusarribasi*	Spain, Sevilla, Montellano, Arroy Montero	21-07-06	Millán et col. Leg	IBE-AB82
ENOjesu_SPA_SEV_PINT_AB393	*Enochrus (Lumetus) jesusarribasi*	Spain, Sevilla, Osuna, Arroyo El Pintado	27-07-98	Ribera Leg	IBE-AB393
ENOmela_ENG_SHE_NHM1	*Enochrus (Enochrus) melanocephalus*	England, Sheppey Island	2016		
ENOmela_ENG_SHE_NHM2	*Enochrus (Enochrus) melanocephalus*	England, Sheppey Island	2016		
ENOmore_POR_ALG_CABE_AB317	*Enochrus (Methydrus) morenae*	Portugal, Algarbe, Cabezo Gordo	01-05-09	Andújar, Arribas & Sánchez-Gea Leg	IBE-AB317
ENOmore_SPA_CAC_VID_AB62	*Enochrus (Methydrus) morenae*	Spain, Cáceres, Arroyo de la Vid, Arroyo de la Vid	14-05-05	Ribera Leg	IBE-AB62
ENOmore_SPA_CAC_VISE_AB318	*Enochrus (Methydrus) morenae*	Spain, Cáceres, Villarreal, PN Monfragüe, Villarreal y Serradilla	2009	Ribera & Abellán Leg	IBE-AB318
ENOnata_MOR_GHA_KHOU_AB308	*Enochrus (Methydrus) natalensis*	Morocco, Gharviya, Oued Khoulj	21-04-06	Millán et col. Leg	IBE-AB308
ENOnata_MOR_KENI_DICH_AB60	*Enochrus (Methydrus) natalensis*	Morocco, Kenitra, ditch Kenitra	04-04-02	Millán et col. Leg	IBE-AB60
ENOnata_MOR_MOU_DRAD_AB3	*Enochrus (Methydrus) natalensis*	Morocco, Moulay, Bousselahm, Oued Drader	12-04-07	Ribera, Aguilar, Hernando Leg	IBE-AB3
ENOnigr_ITA_SIC_UBRI_AB314	*Enochrus (Methydrus) nigritus*	Italy, Sicily, Parco dei Nebrodi, Lago Ubrio Quattrocchi	12-06-07	Abellán & Picazo Leg	IBE-AB314
ENOnigr_SPA_MAD_PENA_AB44	*Enochrus (Methydrus) nigritus*	Spain, Madrid, Rascafría, Laguna Grande, PN Peñalara	02-06-07	Ribera & Hidalgo Leg	IBE-AB44
ENOochr_BEL_LUX_REGN_RA995	*Enochrus (Lumetus) ochropterus*	Belgium, Luxembourg, nr Regné	31-07-12	Foster Leg	IBE-RA995
ENOochr_SWE_OLA_MOK_RA817	*Enochrus (Lumetus) ochropterus*	Sweden, Öland, Möckelmossen, ponds in limestone	22-05-11	Ribera Leg	IBE-RA817
ENOpoli_ITA_SIC_MAND_AB27	*Enochrus (Lumetus) politus*	Italy, Sicily, Nicosia, Villadoro, Torrente Mandre	12-06-07	Abellán & Picazo Leg	IBE-AB27

ENOpoli_MOR_AKN_LARB_AB251	*Enochrus (Lumetus) politus*	Morocco, Aknoul, Oued Larbaa	23-03-08	Millán et col. Leg	IBE-AB251
ENOpoli_MOR_ALH_PNAC_RA889	*Enochrus (Lumetus) politus*	Morocco, PN Alhucemas	29-08-12	Alonso Leg	IBE-RA889
ENOpoli_MOR_MOUL_SP39	*Enochrus (Lumetus) politus*	Morocco, Morocco, Delta du Moulouya			IBE-SP39
ENOpoli_MOR_TIS_TISS_AB20	*Enochrus (Lumetus) politus*	Morocco, Tissint, Oued Tissint	18-04-01	Ribera & Cieslak Leg	IBE-AB20
ENOpoli_SPA_ALA_AÑANA_AB175	*Enochrus (Lumetus) politus*	Spain, Álava, Salinas de Añana, Arroyo de las Salinas de Añana	23-07-04	Ribera & Cieslak Leg	IBE-AB175
ENOpoli_SPA_ALA_CARR_AB107	*Enochrus (Lumetus) politus*	Spain, Vitoria, La Guardia, Laguna de Carralogroño	22-07-04	Ribera & Cieslak Leg	IBE-AB107
ENOpoli_SPA_ALB_CENA_AB255	*Enochrus (Lumetus) politus*	Spain, Albacete, Hellín, Presa del Cenajo.Trampa de luz	16-10-09	Andújar & Arribas Leg	IBE-AB255
ENOpoli_SPA_ALB_CORD_AB242	*Enochrus (Lumetus) politus*	Spain, Albacete, Hellín, Saladar de Cordovilla	10-11-09	Andújar Leg	IBE-AB242
ENOpoli_SPA_ALB_PINI_AB262	*Enochrus (Lumetus) politus*	Spain, Albacete, Pinilla, Salinas de Pinilla	21-11-09	Arribas, Arribas & Reolid Leg	IBE-AB262
ENOpoli_SPA_ALM_VERA_AB252	*Enochrus (Lumetus) politus*	Spain, Almería, Vera, Salar de los Carros	01-02-10	Andújar & Arribas Leg	IBE-AB252
ENOpoli_SPA_BAL_MOND_AB42	*Enochrus (Lumetus) politus*	Spain, Baleares, Mallorca, Cala Mondragó, s'Amarador	11-10-04	Ribera Leg	IBE-AB42
ENOpoli_SPA_BAL_PUDE_AB173	*Enochrus (Lumetus) politus*	Spain, Baleares, Formentera, Es Brolls, Estany Pudent	14-10-04	Palmer & Jaume Leg	IBE-AB173
ENOpoli_SPA_BAR_AVIN_SP25	*Enochrus (Lumetus) politus*	Spain, Barcelona, Avinyo, Torrent Salat	2013	Ribera, Sánchez, Picazo	IBE-SP25
ENOpoli_SPA_CAD_HORT_AB253	*Enochrus (Lumetus) politus*	Spain, Cádiz, El Bosque, Salinas de Hortales	29-01-10	Andújar & Arribas Leg	IBE-AB253
ENOpoli_SPA_CUE_MANZ2_AB117	*Enochrus (Lumetus) politus*	Spain, Cuenca, Arroyo hiposalino antes de Salinas del Manzano	28-07-07	Millán et col. Leg	IBE-AB117
ENOpoli_SPA_CUE_VALS_AB72	*Enochrus (Lumetus) politus*	Spain, Cuenca, Molina de Aragón, Salinas de Valsalobre	28-07-07	Millán et col. Leg	IBE-AB72
ENOpoli_SPA_GCA_AZUA_AB28	*Enochrus (Lumetus) politus*	Spain, Gran Canaria, Moya, Barranco de Azuaje, arroyo y pozas	15-04-01	Ribera & Cieslak Leg	IBE-AB28
ENOpoli_SPA_GUA_ARCO_AB68	*Enochrus (Lumetus) politus*	Spain, Guadalajara, Arcos de las Salinas, Arroyo hipersalino	19-09-07	Millán et col. Leg	IBE-AB68
ENOpoli_SPA_HUC_ROLD_AB265	*Enochrus (Lumetus) politus*	Spain, Huesca, Naval, Salinas de la Rolda	17-10-09	Andújar & Arribas Leg	IBE-AB265
ENOpoli_SPA_HUV_PINA_AB264	*Enochrus (Lumetus) politus*	Spain, Huelva, Huelva, Marismas del Pinar	30-01-10	Andújar & Arribas Leg	IBE-AB264
ENOpoli_SPA_MUR_CALB_AB244	*Enochrus (Lumetus) politus*	Spain, Murcia, Calblanque, Calblanque poza	12-04-09	Millán & Bilton Leg	IBE-AB244
ENOpoli_SPA_MUR_MAJA_AB177	*Enochrus (Lumetus) politus*	Spain, Murcia, Mazarrón, Rambla Majada	29-07-98	Ribera Leg	IBE-AB177
ENOpoli_SPA_MUR_REST_AB266	*Enochrus (Lumetus) politus*	Spain, Murcia, Lorca, Rambla del	15-11-09	Abellán Leg	IBE-AB266

		Estrecho			
ENOpoli_SPA_MUR_SANG_AB56	*Enochrus (Lumetus) politus*	Spain, Murcia, Sangonera, Arroyo de las Salinas de Sangonera	22-01-08	Millán et col. Leg	IBE-AB56
ENOpoli_SPA_MUR_ZACA_AB256	*Enochrus (Lumetus) politus*	Spain, Murcia, Moratalla, Salinas del Zacatín	03-08-09	Velasco & Millán Leg	IBE-AB256
ENOpoli_SPA_NAV_MEND_AB92	*Enochrus (Lumetus) politus*	Spain, Navarra, Barranco Salado de Mendavia	29-07-07	Millán et col. Leg	IBE-AB92
ENOpoli_SPA_TAR_TRAB_AB6	*Enochrus (Lumetus) politus*	Spain, Tarragona, Sant Jaume Enveja, Cami Trabucador pond	21-02-09	Ribera & Cieslak Leg	IBE-AB6
ENOpoli_SPA_ZAR_CAST_AB268	*Enochrus (Lumetus) politus*	Spain, Zaragoza, Alfajarín, Barranco Hermita del Castillo	01-11-09	Ribera & Cieslak Leg	IBE-AB268
ENOpoli_SPA_ZAR_MAGD_AB76	*Enochrus (Lumetus) politus*	Spain, Zaragoza, Mediana de Aragón, Arroyo de la Ermita de la Magdalena	24-07-06	Millán et col. Leg	IBE-AB76
ENOpoli_TUN_GAB_EREB_AB95	*Enochrus (Lumetus) politus*	Tunisia, Gabes, Kebili, Oued Erebaieb	26-10-01	Ribera & Cieslak Leg	IBE-AB95
ENOpoli_TUN_TOZ_MELA_AB34	*Enochrus (Lumetus) politus*	Tunisia, Tozeur, Gafsa, Oued El Melah	26-10-01	Ribera & Cieslak Leg	IBE-AB34
ENOquad_AUS_VOR_RHEI_AB307	*Enochrus (Lumetus) quadripunctatus* cplx	Austria, Voralberg, Rheintal, Bodensee, Hard Rheinvorland	02-07-01	Ribera Leg	IBE-AB307
ENOquad_AZE_ALI_SP11	*Enochrus (Lumetus) quadripunctatus* cplx	Azerbaijan, Alishanli, ponds	2014	Ribera & Rudoy	IBE-SP11
ENOquad_AZE_YEV_SP10	*Enochrus (Lumetus) quadripunctatus* cplx	Azerbaijan, Yevlakh, ponds	2014	Ribera & Rudoy	IBE-SP10
ENOquad_BUL_KOT_ARDA_SP31	*Enochrus (Lumetus) quadripunctatus* cplx	Bulgaria, Kotlari, residual pools in river Arda	2015	Ribera Leg	IBE-SP31
ENOquad_ENG_PLY_AB238	*Enochrus (Lumetus) quadripunctatus* cplx	England, Plymouth	06-09-09	Bilton & Arribas Leg	IBE-AB238
ENOquad_ENG_PLY_AB322	*Enochrus (Lumetus) quadripunctatus* cplx	England, Plymouth	06-09-09	Bilton & Arribas Leg	IBE-AB322
ENOquad_ENG_WRE_HEAT_RA994	*Enochrus (Lumetus) quadripunctatus* cplx	England, West Norfolk, Weast Wretham Heath	22-08-12	Foster Leg	IBE-RA994
ENOquad_FRA_SIG_PALM2_AB239	*Enochrus (Lumetus) quadripunctatus* cplx	France, Sigean, Arroyo hiposalino Salines de Lapalme	16-10-09	Andújar & Arribas Leg	IBE-AB239
ENOquad_GER_LUN_WALS_AB300	*Enochrus (Lumetus) quadripunctatus* cplx	Germany, Luneburger Heide, Walsrode, Walsrode pond 1	13-07-02	Ribera, Cieslak & Spieck Leg	IBE-AB300
ENOquad_GRE_ARK_MOUS_SP3	*Enochrus (Lumetus) quadripunctatus* cplx	Greece, Arkadia, Astros, lake Moustou and nearby wetland	2013	Ribera & Cieslak	IBE-SP3
ENOquad_GRE_ATT_ARTE_SP5	*Enochrus (Lumetus) quadripunctatus* cplx	Greece, Attika, Artemida, ditch in beach next to wetland	2013	Ribera & Cieslak	IBE-SP5
ENOquad_IRA_MAZ_ALAN_AB122	*Enochrus (Lumetus) quadripunctatus* cplx	Iran, Mazandaran, Sari, Lac Alandan	16-09-07	Ponel Leg	IBE-AB122
ENOquad_IRA_MAZ_ALAN_AB97	*Enochrus (Lumetus) quadripunctatus* cplx	Iran, Mazandaran, Sari, Lac Alandan	16-09-07	Ponel Leg	IBE-AB97
ENOquad_ITA_CONT_SP29	*Enochrus (Lumetus) quadripunctatus* cplx	Italy, Lago de Contorno, marsh N of lake	2015	Ribera & Cieslak	IBE-SP29
ENOquad_ITA_COR_MONA_RA929	*Enochrus (Lumetus) quadripunctatus* cplx	Italy, Corsica, Monacia d'Aullène	10-08-08	Fresneda Leg	IBE-RA929

ENOquad_KAZ_EAS_BALG_RA493	*Enochrus (Lumetus) quadripunctatus* cplx	Kazakhstan, East Kazakhstan, Balgyn, Balgyn	31-05-11	Vila Leg	IBE-RA493
ENOquad_MOR_AZR_AFEN_SP49	*Enochrus (Lumetus) quadripunctatus* cplx	Morocco, Aïn Leu, Azrou, lac Afenourir and side pond	14-07-11		IBE-SP49
ENOquad_POR_ALG_FOIA_AB237	*Enochrus (Lumetus) quadripunctatus* cplx	Portugal, Algarbe, Monchite, Alto da Foia	02-05-09	Andújar, Arribas & Sánchez-Gea Leg	IBE-AB237
ENOquad_POR_AZO_TERC_RA913	*Enochrus (Lumetus) quadripunctatus* cplx	Portugal, Azores, Terceira, Lagoa do Negro, Pico Gordo	03-09-12	Ribera & Cieslak Leg	IBE-RA913
ENOquad_POR_AZO_TERC_SP48	*Enochrus (Lumetus) quadripunctatus* cplx	Portugal, Azores, Terceira	04-09-12		IBE-SP48
ENOquad_POR_AZO_TERC_SP8	*Enochrus (Lumetus) quadripunctatus* cplx	Portugal, Azores, Terceira, Guinjal, euthrophic lagoon	05-09-12	Ribera & Cieslak Leg	IBE-SP8
ENOquad_POR_MAN_ESTR_SP43	*Enochrus (Lumetus) quadripunctatus* cplx	Portugal, Serra Estrela, Manteigas, ponds	12-05-05		IBE-SP43
ENOquad_ROM_MAR_POIE_RA564	*Enochrus (Lumetus) quadripunctatus* cplx	Romania, Maramures, Poienile Izei, arroyo	20-07-11	Fresneda Leg	IBE-RA564
ENOquad_SLO_HAM_SP22	*Enochrus (Lumetus) quadripunctatus* cplx	Slovakia, Hámske tŕstie, ditch near Číčov	07-06-09	Ribera Leg	IBE-SP22
ENOquad_SPA_ALA_GAZE_SP45	*Enochrus (Lumetus) quadripunctatus* cplx	Spain, Alava, Gazeo Laku pond 3	18-03-07		IBE-SP45
ENOquad_SPA_ALA_LAKU_AB241	*Enochrus (Lumetus) quadripunctatus* cplx	Spain, Álava, Gaceo, Laku pond 2	18-03-07	Ribera, Aguilar, Hernando Leg	IBE-AB241
ENOquad_SPA_AST_REGU_AB302	*Enochrus (Lumetus) quadripunctatus* cplx	Spain, Asturias, Lagos de Covadonga, Río de los Reguerones	05-09-09	Millán et col. Leg	IBE-AB302
ENOquad_SPA_CAC_DEHE_AB1	*Enochrus (Lumetus) quadripunctatus* cplx	Spain, Cáceres, Casas de Miravete, Dehesa Boyal poza ganado	14-05-05	Ribera & Hernando Leg	IBE-AB1
ENOquad_SPA_GUA_DUEÑ_SP30	*Enochrus (Lumetus) quadripunctatus* cplx	Spain, Guadalajara, El Pobo de Dueñas, pond	01-04-15	Ribera & Cieslak	IBE-SP30
ENOquad_SPA_GUA_POBO_AB4	*Enochrus (Lumetus) quadripunctatus* cplx	Spain, Guadalajara, El Pobo de Dueñas, pond	03-06-06	Ribera Leg	IBE-AB4
ENOquad_SPA_HUC_ANET_RA923	*Enochrus (Lumetus) quadripunctatus* cplx	Spain, Huesca, Aneto, Estany Anglios	15-08-12	Fresneda Leg	IBE-RA923
ENOquad_SPA_LEO_ANCA_SP46	*Enochrus (Lumetus) quadripunctatus* cplx	Spain, León, Pto Ancares			IBE-SP46
ENOquad_SWE_OLA_MOK_RA818	*Enochrus (Lumetus) quadripunctatus* cplx	Sweden, Öland, Möckelmossen, ponds in limestone	22-05-11	Ribera Leg	IBE-RA818
ENOquad_TUR_DUZ_SP44	*Enochrus (Lumetus) quadripunctatus* cplx	Turkey, Düzce, pools in mountain pass	23-04-06		IBE-SP44
ENOquad_TUR_ERZ_CIGD_RA688	*Enochrus (Lumetus) quadripunctatus* cplx	Turkey, Erzurum, Cigdemli, pond	11-06-11	Ribera et col. Leg	IBE-RA688
ENOquad_TUR_ERZ_SENY_SP24	*Enochrus (Lumetus) quadripunctatus* cplx	Turkey, Erzurum, Senyurtköyu, stream	12-06-11	Ribera Leg	IBE-SP24
ENOquad_TUR_ERZ_TOPR_RA547	*Enochrus (Lumetus) quadripunctatus* cplx	Turkey, Erzurum, Toprakkaleköyü, stream	11-06-11	Ribera et col. Leg	IBE-RA547
ENOquad_TUR_KAY_ERCI_AN58	*Enochrus (Lumetus) quadripunctatus* cplx	Turkey, Kayseri, Hisarcik, Erciyes Mountain	2010	via Polat	IBE-AN58
ENOquad_TUR_ORD_KULA_RA715	*Enochrus (Lumetus) quadripunctatus* cplx	Turkey, Ordu, Kulakköy, stream	17-06-11	Ribera et col. Leg	IBE-RA715

ENOquad_TUR_SIN_YESI_AB163	*Enochrus (Lumetus) quadripunctatus* cplx	Turkey, Sinop, Sarayduzu, Boyabat, Yesilyurt stream	27-04-06	Ribera, Cieslak, Aguilar, Hernando Leg	IBE-AB163
ENOquan_OMA_MUQ_LOTI_RA490	*Enochrus (Lumetus) quadrinotatus cf*	Oman, Wadi Muqshin	2011	Carranza Leg	IBE-RA490
ENOquan_OMA_MUQ_LOTI_RA553	*Enochrus (Lumetus) quadrinotatus cf*	Oman, Wadi Muqshin	2011	Carranza Leg	IBE-RA553
ENOrisi_MOR_AGUI_AB280	*Enochrus (Lumetus) risii*	Morocco, Oued El-Aguig	03-04-07	Millán et col. Leg	IBE-AB280
ENOrisi_MOR_AOUD_AB229	*Enochrus (Lumetus) risii*	Morocco, Oued Aoudrei	05-04-07	Millán et col. Leg	IBE-AB229
ENOrisi_MOR_KHN_KHAN_AB248	*Enochrus (Lumetus) risii*	Morocco, Khniffis, Oued Khanoi Naâm	04-04-07	Millán et col. Leg	IBE-AB248
ENOrisi_TUN_TOZ_MELA_AB40	*Enochrus (Lumetus) risii*	Tunisia, Tozeur, Gafsa, Oued El Melah	26-10-01	Ribera & Cieslak Leg	IBE-AB40
ENOsalo_SPA_ALB_PET_NHM8	*Enochrus (Lumetus) salomonis*	Spain, Albacete, Pétrola, Laguna de Pétrola	2016		
ENOsalo_SPA_ALB_PET_NHM9	*Enochrus (Lumetus) salomonis*	Spain, Albacete, Pétrola, Laguna de Pétrola	2016		
ENOsalo_SPA_NAV_BARD_AB240	*Enochrus (Lumetus) salomonis*	Spain, Navarra, Bárdenas Reales, Arroyo Salado en las Bárdenas Reales	18-10-09	Andújar & Arribas Leg	IBE-AB240
ENOsalo_SPA_ZAR_MAGA_SP27	*Enochrus (Lumetus) salomonis*	Spain, Zaragoza, Magallón, Lagunas de Magallón	2014	Ribera & Cieslak Leg	IBE-SP27
ENOsegm_FRA_MON_PCAM_AB288	*Enochrus (Lumetus) segmentinotatus*	France, Montpellier, Mauguio, Petit Camargua. Etang d'Or	11-10-09	Andújar & Arribas Leg	IBE-AB288
ENOsegm_FRA_SIG_PALM_AB289	*Enochrus (Lumetus) segmentinotatus*	France, Sigean, Salin de Lapalme	16-11-09	Andújar & Arribas Leg	IBE-AB289
ENOsegm_MOR_BRI_AZIL_AB274	*Enochrus (Lumetus) segmentinotatus*	Morocco, Briech, Azilah, Salines Azilah	21-04-06	Millán et col. Leg	IBE-AB274
ENOsegm_SPA_BAL_POLL_AB162	*Enochrus (Lumetus) segmentinotatus*	Spain, Baleares, Mallorca, Port de Pollensa pozas	11-11-00	Ribera & Cieslak Leg	IBE-AB162
ENOsegm_SPA_CAD_SMAR_AB231	*Enochrus (Lumetus) segmentinotatus*	Spain, Cádiz, Puerto de Santa María, Salinas de Santa María	30-01-10	Andújar & Arribas Leg	IBE-AB231
ENOsp_ARG_BAI_LORO_AB326	*Enochrus (Hugoscottia) sp*	Argentina, Buenos Aires, Sierra de la Ventana, Arroyo el Loro	14-12-00	Villalobos Leg	IBE-AB326
ENOsp_CAN_ALB_WATE2_AB87	*Enochrus (Methydrus) sp*	Canada, Alberta, Waterton lakes park limit	28-06-00	Ribera & Cieslak Leg	IBE-AB87
ENOsp_CHI_CHI_CUCA_AB88	*Enochrus (Hugoscottia) sp*	Chile, Chiloé, Chiloé, Laguna de Cucao	03-11-99	Ribera & Guerrero Leg	IBE-AB88
ENOsp_CHI_CHI_TRIN_AB89	*Enochrus (Hugoscottia) sp*	Chile, Chiloé, Chiloé, Arroyo en Quellón Trincao	05-11-99	Ribera & Guerrero Leg	IBE-AB89
ENOsp_SAF_CAP_FRAN_AB310	*Enochrus (Methydrus) sp*	South Africa, Cape, Franschhoek, Franschhoek river	26-03-01	Ribera & Cieslak Leg	IBE-AB310
ENOsp_SAF_CAP_HOUT_AB85	*Enochrus (Enochrus) sp*	South Africa, Cape, Hout Bay, Hout river	19-03-01	Ribera & Cieslak Leg	IBE-AB85
ENOsp_SAF_CAPE_HARO_AB84	*Enochrus (Methydrus) sp*	South Africa, Cape, Pringle Bay, Harold Porter pond	21-03-01	Ribera & Cieslak Leg	IBE-AB84
ENOsp_USA_CAL_COAL_AB55	*Enochrus (Methydrus) sp*	United States, California, Coal Oil Point Reserve	26-03-08	Abellán Leg	IBE-AB55

ENOtest_AUS_VOR_RHEI_AB306	*Enochrus (Lumetus) testaceus*	Austria, Voralberg, Rheintal, Bodensee, Hard Rheinvorland	02-07-01	Ribera Leg	IBE-AB306
ENOtest_AZE_QOB_SP36	*Enochrus (Lumetus) testaceus*	Azerbaijan, Qobustan, ponds in beach	2014	Ribera & Rudoy	IBE-SP36
ENOtest_ENG_NOR_BROA_AB36	*Enochrus (Lumetus) testaceus*	England, Norfolk, The Broads, Catfield Fen 4	07-05-06	Ribera Leg	IBE-AB36
ENOtest_ENG_SHE_NHM7	*Enochrus (Lumetus) testaceus*	England, Sheppey Island	2016		
ENOtest_IRE_CLA_RINE_AB304	*Enochrus (Lumetus) testaceus*	Ireland, Clare, Rinecaha, Rinecaha fen	22-05-10	Ribera Leg	IBE-AB304
ENOtest_POL_ZAC_SP42	*Enochrus (Lumetus) testaceus*	Poland, Zachodniopomorsky, Dygowo, garden pond	16-08-04		IBE-SP42
ENOtest_SLO_HAM_SP21	*Enochrus (Lumetus) testaceus*	Slovakia, Hámske tŕstie, ditch near Číčov	07-06-09	Ribera Leg	IBE-SP21
ENOtest_SPA_GER_CAMP_AB45	*Enochrus (Lumetus) testaceus*	Spain, Gerona, Capmany, Estany inferior	09-05-98	Ribera & Foster Leg	IBE-AB45
ENOtura_CYP_ORO_AN458	*Enochrus (Lumetus) turanicus cf*	Cyrpus, Oroklini, saline wetland	25-04-16	Millán, Ribera, Velasco & Villastrigo Leg	IBE-AN458
ENOtura_GRE_ARK_MOUS_SP4	*Enocrhus (Lumetus) turanicus cf*	Greece, Arkadia, Astros, lake Moustou and nearby wetland	2013	Ribera & Cieslak Leg	IBE-SP4
ENOtura_GRE_ATT_ARTE_SP6	*Enocrhus (Lumetus) turanicus cf*	Greece, Attika, Artemida, ditch in beach next to wetland	2013	Ribera & Cieslak Leg	IBE-SP6
ENOtura_ISR_ATL_COAS_RA194	*Enochrus (Lumetus) turanicus cf*	Israel, Atlit	29-07-10	Rudoy Leg	IBE-RA194
ENOtura_TUR_BAL_KUCU_SP15	*Enocrhus (Lumetus) turanicus cf*	Turkey, Balikesir prov.. Küçükköy, salty marsh	22-07-14	Ribera & Cieslak	IBE-SP15
ENOtura_TUR_BAL_SARI_SP16	*Enocrhus (Lumetus) turanicus cf*	Turkey, Balikesir prov., Sarimsakli, salty ditch	23-07-14	Ribera & Cieslak	IBE-SP16
ENOtura_TUR_CAN_SP14	*Enocrhus (Lumetus) turanicus cf*	Turkey, Çanakkale prov., Dalyan, salty stream	21-07-14	Ribera & Cieslak	IBE-SP14
ENOtura_TUR_IZM_GEDI_SP17	*Enochrus (Lumetus) turanicus cf*	Turkey, Izmir prov., Gediz Nehri Delta, saline pond	24-07-14	Ribera & Cieslak	IBE-SP17
ENOtura_TUR_IZM_KUCU_SP19	*Enochrus (Lumetus) turanicus cf*	Turkey, Izmir prov., Selçuk, delta Küçükmenderes	25-07-14	Ribera & Cieslak	IBE-SP19
HEL_MOR_AZR_AFEN_SP40	*Helochares sp*	Morocco, Azrou, Lac Afenourir			IBE-SP40
HELlivi_SPA_ZAR_MAGD_AB83	*Helochares lividus*	Spain, Zaragoza, Mediana de Aragón, Arroyo de la Ermita de la Magdalena	29-07-07	Millán et col. Leg	IBE-AB83
HYDconv_ENG_EWC_POND3_AB31	*Hydrobius convexus*	England, East Walton Common, Pingos, pond 3	06-06-99	Ribera & Berendonk Leg	IBE-AB31

Table S2. Species used for osmoregulation and desiccation experiments and collection sites.

Species	Localities	Collector(s)
E. (M.) coarctatus (Gredler 1863)	Carrick ponds, Kirkcudbright, Scotland, U.K.	D.T. Bilton & G.N. Foster; G. Robinson & S. Routledge
E. (L.) salomonis (J. Sahlberg, 1900)	Pétrola ponds, Pétrola, Albacete, Spain	A. Millán, J. Velasco & S. Pallarés
E. (L.) ochropterus (Marsham 1802)	Carrick ponds, Kirkcudbright, Scotland, U.K.	D.T. Bilton & G.N. Foster; G. Robinson & S. Routledge
E. (L.) politus (Küster, 1849)	Chícamo stream, Abanilla, Murcia, Spain	A. Millán, J. Velasco & S. Pallarés
E. (L.) quadripunctatus (Herbst, 1797)	Drakeland Corner, Plymouth (Devon), UK	D.T. Bilton
	Knockewart moss, Ayrshire, Scotland, UK.	G.N. Foster
E. (L.) testaceus (Fabricius, 1801)	Carrick ponds, Kirkcudbright, Scotland, U.K.	D.T. Bilton & G.N Foster; G. Robinson & S. Routledge
E. (L.) halophilus (Bedel, 1878)	Pétrola ponds, Pétrola, Albacete, Spain	A. Millán, J. Velasco & S. Pallarés
E. (L.) bicolor (Fabricius, 1792)	Mojón Blanco pond, Albacete, Spain	A. Millán, J. Velasco & S. Pallarés
E. (L.) jesusarribasi Arribas y Millán, 2013	Rambla Salada stream, Fortuna, Murcia, Spain	A. Millán, J. Velasco & S. Pallarés

Table S3. Primers used for PCR and sequencing.

Marker	Primer		Sequence (5’–3’)	Reference
cox1-A	Jerry	F	CAACATTTATTTTGATTTTTTGG	Simon *et al.* (1994)
	Pat	R	TCCAATGCACTAATCTGCCATATTA	
cyt b	CB3	F	GAGGAGCAACTGTAATTACTAA	Barraclough *et al.* (1999)
	CB4	R	AAAAGAAA(A/G)TATCATTCAGGTTGAAT	
*rrn*L+*trn*L+nd1	16SaR	F	CGCCTGTTTATCAAAAACAT	Simon *et al.* (1994)
	ND1A	R	GGTCCCTTACGAATTTGAATATATCCT	
LSU	Ka	F	ACACGGACCAAGGAGTCTAGCATG	Monaghan *et al.* (2007)
	Kb	R	CGTCCTGCTGTCTTAAGTTAC	
cox1-B	lco1490	F	GGTCAACAAATCATAAAGATATTGG	Folmer *et al.*(1994)
	hco2198	R	TAAACTTCAGGGTGACCAAAAAATCA	
ITS2	5.8sF	F	GTGAATTCTGTGAACTGCAGGACACATGAAC	Porter & Collins (1991)
	28sR	R	ATGCTTAAATTTAGGGGGTA	

F, forward; R, reverse

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Table S4. Details of osmoregulation and desiccation experiments. For osmoregulation experiments, the number of specimens exposed and dead and the number of observations analyzed (i.e. replicates) for each salinity treatment (N) is provided; the lethal limit of each species (treatment with mortality ≥ 50% of exposed individuals) is indicated by an asterisk. For desiccation experiments, the number of specimens used for water loss rate and water content measurements (N) is given.

	E. (M.) coarctatus			***E. (L.) salomonis***			***E. (L.) ochropterus***			***E. (L.) politus***			***E. (L.) quadripunctatus***			***E. (L.) testaceus***			***E. (L.) halophilus***			***E. (L.) bicolor***			***E. (L.) jesusarribasi***		
Salinity treatments (g L^{-1})	Exp	Dead	N	Exp	Dead	N	Exp	Dead	N	Exp	Dead	N	Exp	Dead	N	Exp	Dead	N	Exp	Dead	N	Exp	Dead	N	Exp	Dead	N
0.3	15	0	4	14	2	3	8	1	3	16	0	3	10	1	3	4	0	3	6	0	4	16	0	3	10	0	3
3	15	1	4	14	1	3				16	0	3	10	0	3												
6	15	0	4	14	1	3	8	0	4				10	0	3	4	1	3									
12	15	2	3	14	0	3	8	0	4	16	0	3	10	0	3	4	0	4	6	0	4	16	1	3	10	0	3
35	20	4	3	14	9*		8	0	4	16	1	3	16	2	4	10	1	3	8	0	4	16	2	3	10	0	3
50	20	15*					8	4*		16	1	3	16	7	4	10	2	4	10	2	4						
75										16	9*		16	16*		10	4	6	10	6*		18	3	3	10	0	3
100																10	10*					18	12*		10		
140																									18	3	3
Desiccation treatment			29			25			22			23			35			24			24			20			23

Table S5. Branch measurements for the analyses of rates of phenotypic evolution of water loss rate (WLR), water content (WC), hyposmotic capacity (Hypo), maximum hyposmotic capacity (Max HC) and habitat salinity (Hab Sal).

Branch Nº	Initial node	Final node	Branch length (Ma)	Node values										Absolute phenotypic change				
				WLR (% fresh mass h^{-1})		WC (% water to fresh mass)		Hypo (mOsmol kg^{-1})		Max HC (mOsmol kg^{-1})		Hab sal (category)						
				initial	final	initial	final	initial	final	initial	final	initial	final	WLR	WC	HYPO	MAX HC	HAB SAL
1	26	*E. coarctatus*	48.698	15.27	16.41	63.73	58.42	423	238	1060	238	1.91	1.00	1.14	5.31	185	822	0.91
2	25	26	10.391	15.27	15.03	63.73	64.79	423	462	1060	1236	1.91	2.10	0.24	1.06	39	176	0.19
3	26	27	16.511	15.03	15.89	64.79	65.34	462	319	1236	761	2.10	1.44	0.86	0.55	143	475	0.66
4	27	*E. ochropterus*	21.796	15.89	12.42	65.34	60.81	319	441	761	883	1.44	1.00	3.47	4.53	122	122	0.44
5	27	*E. salomonis*	21.796	15.89	20.52	65.34	70.62	319	0	761	10	1.44	1.00	4.63	5.28	318.9	751	0.44
6	26	28	2.787	15.03	14.82	64.79	64.99	462	496	1236	1363	2.10	2.26	0.21	0.2	34	127	0.16
7	28	*E. testaceus*	35.521	14.82	11.84	64.99	70.32	496	569	1363	1782	2.26	1.00	2.98	5.33	73	419	1.26
8	28	29	3.237	14.82	14.84	64.99	64.77	496	530	1363	1472	2.26	2.57	0.02	0.22	34	109	0.31
9	29	41	12.042	14.84	14.11	64.77	65.85	530	619	1472	2191	2.57	4.17	0.73	1.08	89	719	1.6
10	41	46	3.774	14.11	13.88	65.85	66.19	619	647	2191	2416	4.17	4.30	0.23	0.34	28	225	0.13
11	46	*E. bicolor*	16.468	13.88	13.29	66.19	68.32	647	744	2416	2171	4.30	4.00	0.59	2.13	97	245	0.3
12	46	47	11.595	13.88	13.56	66.19	66.05	647	671	2416	3278	4.30	4.88	0.32	0.14	24	862	0.58
13	47	*E. blazquezae*	4.873	13.56	13.56	66.05	66.05	671	671	3278	3278	4.88	5.00	0	0	0	0	0.12
14	47	*E. jesusarribasi*	4.873	13.56	13.42	66.05	65.94	671	673	3278	3650	4.88	5.00	0.14	0.11	2	372	0.12
15	41	42	7.257	14.11	14.11	65.85	65.85	619	619	2191	2191	4.17	4.91	0	0	0	0	0.74
16	42	*E. quadrinotatus*	12.985	14.11	14.11	65.85	65.85	619	619	2191	2191	4.91	6.00	0	0	0	0	1.09
17	42	43	1.954	14.11	14.11	65.85	65.85	619	619	2191	2191	4.91	4.94	0	0	0	0	0.03
18	43	44	4.599	14.11	14.11	65.85	65.85	619	619	2191	2191	4.94	4.97	0	0	0	0	0.03
19	44	*E. segmentinotatus*	6.432	14.11	14.11	65.85	65.85	619	619	2191	2191	4.97	4.00	0	0	0	0	0.97
20	44	*E. falcarius*	6.432	14.11	14.11	65.85	65.85	619	619	2191	2191	4.97	6.00	0	0	0	0	1.03
21	43	45	2.842	14.11	14.11	65.85	65.85	619	619	2191	2191	4.94	4.96	0	0	0	0	0.02
22	45	*E. risi*	8.189	14.11	14.11	65.85	65.85	619	619	2191	2191	4.96	5.00	0	0	0	0	0.04

23	45	*E. turanicus*	8.189	14.11	14.11	65.85	65.85	619	619	2191	2191	4.96	5.00	0	0	0	0	0.04
24	29	30	4.669	14.84	15.16	64.77	64.05	530	543	1472	1351	2.57	2.39	0.32	0.72	13	121	0.18
25	30	39	5.361	15.16	15.16	64.05	64.05	543	543	1351	1351	2.39	2.30	0	0	0	0	0.09
26	39	*E. hamifer*	22.254	15.16	15.16	64.05	64.05	543	543	1351	1351	2.30	1.00	0	0	0	0	1.3
27	39	40	9.912	15.16	15.16	64.05	64.05	543	543	1351	1351	2.30	2.73	0	0	0	0	0.43
28	40	*E. hamiltoni*	12.342	15.16	15.16	64.05	64.05	543	543	1351	1351	2.73	3.00	0	0	0	0	0.27
29	40	*E. diffusus*	12.342	15.16	15.16	64.05	64.05	543	543	1351	1351	2.73	3.00	0	0	0	0	0.27
30	30	31	3.655	15.16	15.41	64.05	63.48	543	554	1351	1257	2.39	2.30	0.25	0.57	11	94	0.09
31	31	32	2.607	15.41	15.39	63.48	63.35	554	554	1257	1242	2.30	2.34	0.02	0.13	0	15	0.04
32	32	*E. politus*	21.353	15.39	15.29	63.35	62.11	554	551	1242	1124	2.34	3.00	0.1	1.24	3	118	0.66
33	32	*E. ater*	21.353	15.39	15.39	63.35	63.35	554	554	1242	1242	2.34	2.00	0	0	0	0	0.34
34	31	33	14.054	15.41	16.43	63.48	61.99	554	598	1257	970	2.30	1.76	1.02	1.49	44	287	0.54
35	33	*E. quadripunctatus cplx. A*	9.905	16.13	16.43	61.99	61.99	598	598	970	970	1.76	1.00	0.3	0	0	0	0.76
36	33	34	1.121	16.43	16.51	61.99	61.87	598	601	970	947	1.76	1.69	0.08	0.12	3	23	0.07
37	34	*E. halophilus*	8.785	16.51	24.14	61.87	61.64	601	663	947	705	1.69	3.00	7.63	0.23	62	242	1.31
38	34	35	0.804	16.51	15.92	61.87	61.80	601	599	947	953	1.69	1.52	0.59	0.07	2	6	0.17
39	35	*E. quadripunctatus cplx. B*	7.981	15.92	15.92	61.80	61.80	599	599	953	953	1.52	1.00	0	0	0	0	0.52
40	35	36	1.578	15.92	14.74	61.80	61.66	599	594	953	964	1.52	1.29	1.18	0.14	5	11	0.23
41	36	*E. quadripunctatus cplx. C*	6.403	14.74	9.70	61.66	60.91	594	572	964	1009	1.29	1.00	5.04	0.75	22	45	0.29
42	36	37	0.858	14.74	14.74	61.66	61.66	594	594	964	964	1.29	1.20	0	0	0	0	0.09
43	37	*E. quadripunctatus cplx. D*	5.543	14.74	14.74	61.66	61.66	594	594	964	964	1.20	1.00	0	0	0	0	0.2
44	37	38	0.812	14.74	14.74	61.66	61.66	594	594	964	964	1.20	1.15	0	0	0	0	0.05
45	38	*E. quadripunctatus cplx. E*	4.733	14.74	14.74	61.66	61.66	594	594	964	964	1.15	1.00	0	0	0	0	0.15
46	38	*E. quadripunctatus cplx. F*	4.733	14.74	14.74	61.66	61.66	594	594	964	964	1.15	1.00	0	0	0	0	0.15

Table S6. Correlation between traits in PGLS analyses.

			Max. hyposmotic capacity	**Water loss rate**	**Water content**	**Cuticle content**
Habitat salinity		Range R^2	-0.027 – 0.0001	-0.034 – -0.034	-0.045 – -0.040	-0.042 – -0.013
		% trees				
Max. hyposmotic capacity	including *E. salomonis*	Range R^2		**0.263 – 0.263**	**0.051 – 0.259**	**0.100 - 0.315**
		% trees		100	58	99
	excluding *E. salomonis*	Range R^2		-0.045 – -0.045	**0.334 – 0.434**	**0.320 – 0.399**
		% trees			100	100
Fresh mass		Range R^2		-0.034 – -0.034	**0.335 – 0.377**	0.089 – 0.121
		% trees			100	

The range of adjusted R^2 for 1000 resampled postburnin trees is presented. Significant correlations are highlighted in bold and the percentage of trees showing significant relationships ($P<0.05$) is indicated.

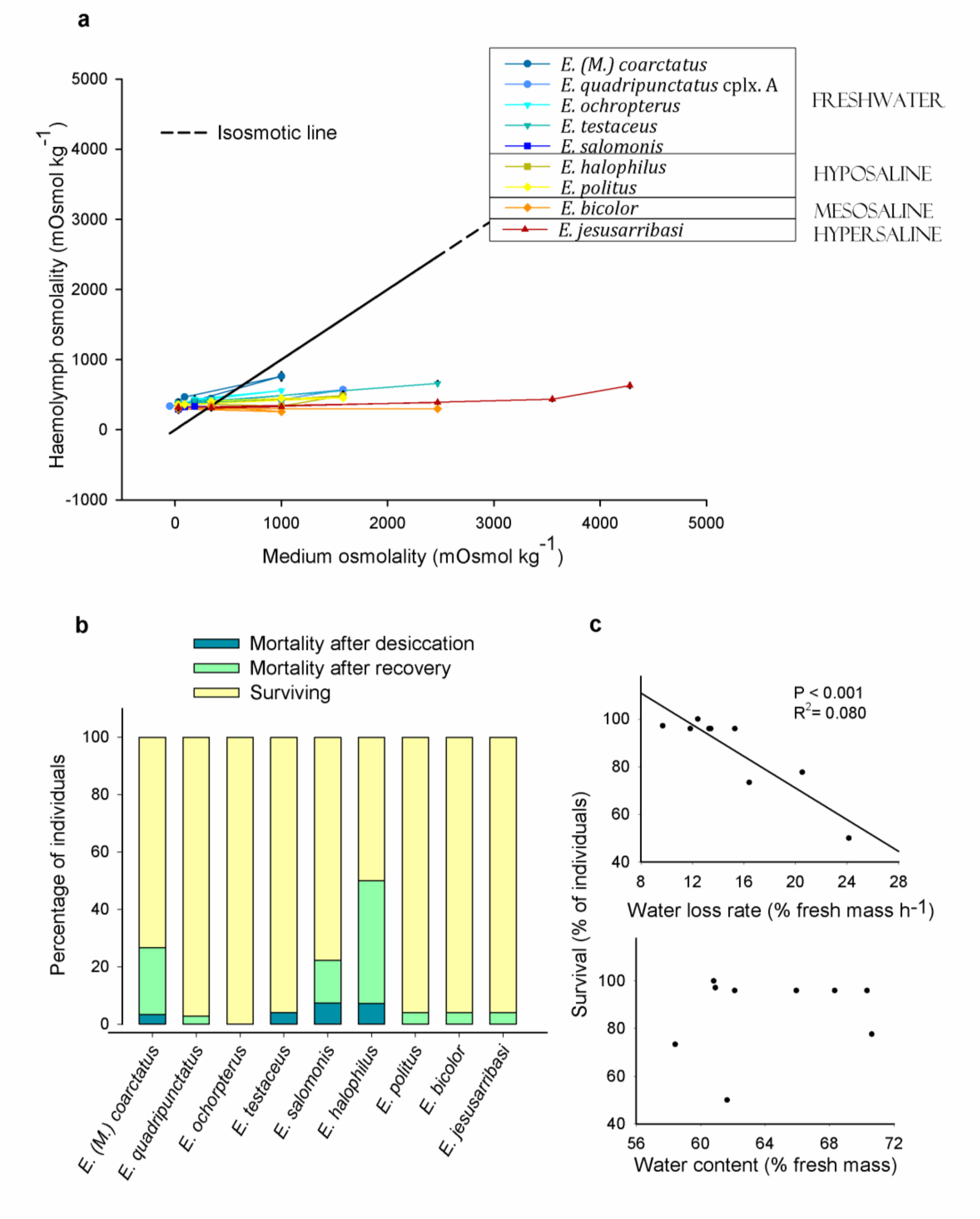

Figure S1. Summary of results of desiccation resistance experiments: a) mortality, recovery and survival percentages; relationships between survival to desiccation and b) water loss, c) water content.

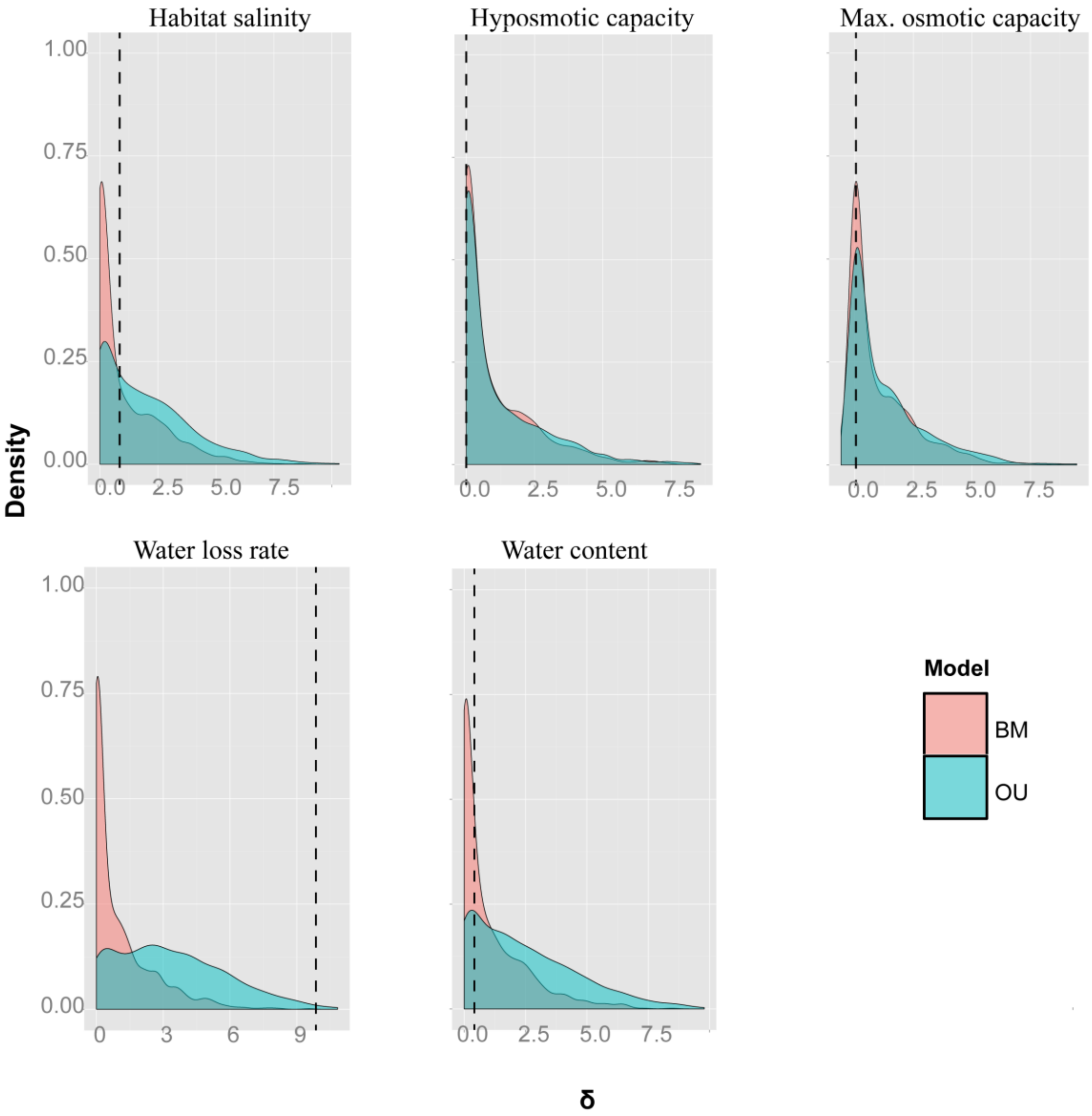

Figure S2. Distributions of the likelihood ratio statistic δ for model comparisons with Monte-Carlo simulations (n=1000 replicates). The dashed vertical lines indicate the observed value of δ when the models are fit to the *Lumetus* dataset. BM: Brownian Motion; OU: Ornstein–Uhlenbec

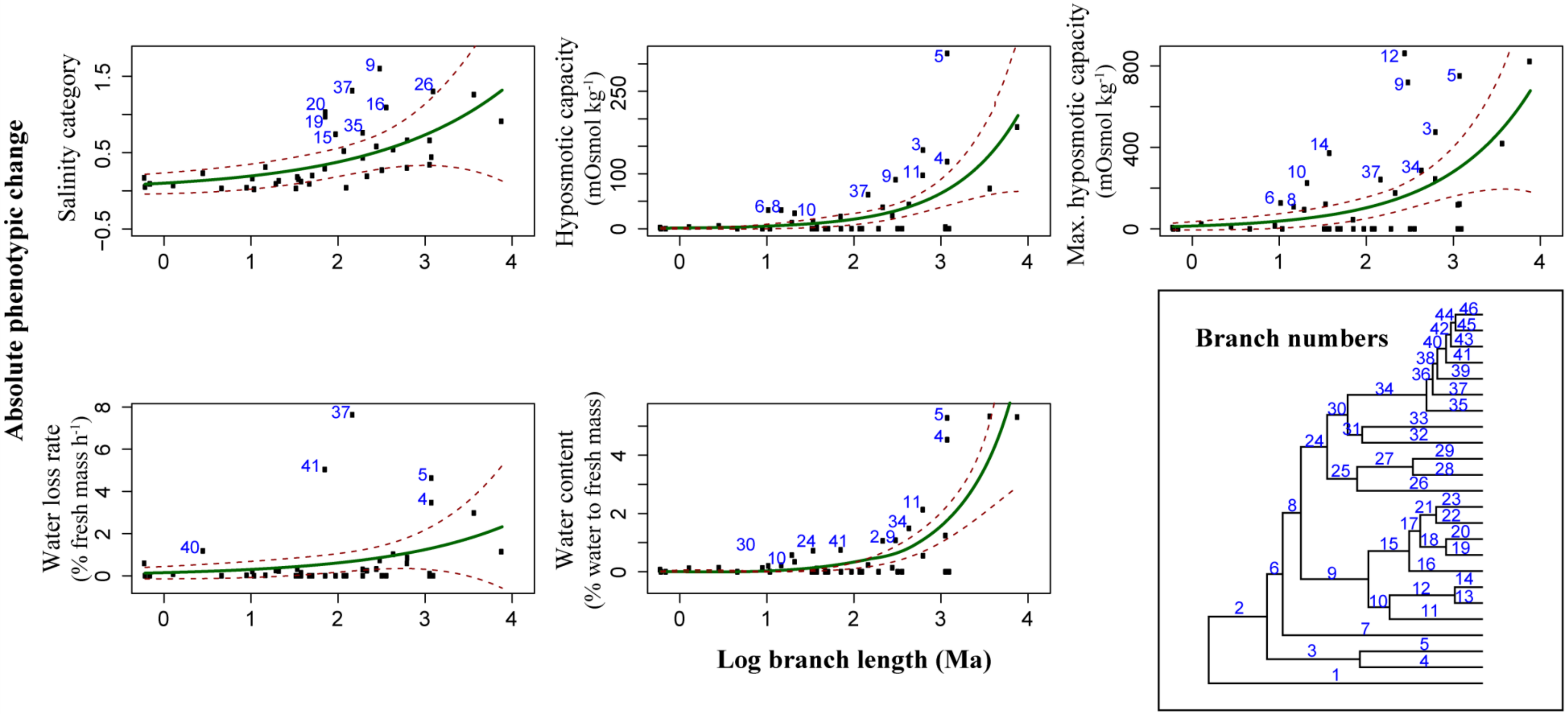

Figure S3. Plot of the branch length with the absolute phenotypic change of each trait. Green line: regression line; red dashed lines: 99% confidence intervals; numbers: branch numbers

SYNTHESIS AND FUTURE PERSPECTIVES

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SYNTHESIS AND FUTURE PERSPECTIVES

Aquatic beetles, one of the few insect groups specialised in inland saline waters, have been traditionally neglected in the physiological literature, despite it being an ideal model group to address transversal topics on adaption to environmental stress by aquatic fauna. The results of this thesis cover part of the knowledge gap on ecophysiology in this group. This information has contributed to understand patterns of diversification and habitat occupation in inland waters and has provided insights into the evolution of salinity tolerance, a key trait for the colonisation of stressful saline waters. In an effort to integrate the main findings that derive from this thesis and to contextualise them within a broad and updated context, several new pending questions have emerged.

Predicting physiological and behavioural traits within the *habitat templet* framework.

This thesis has explored physiological and behavioural responses of water beetles to multiple natural stressors in inland waters, as essential components of the species' colonization capacity, with special focus on understanding the relationship of such responses with the main constraints in aquatic habitats.

Results of this thesis show some generalities about the set of traits primarily related to habitat stability (the lotic/lentic divide) and those that seem to be constrained mainly by abiotic stress gradients (salinity-desiccation). Species traits can, to some extent, be predicted in the *habitat templet* concept. However, the outcomes from this thesis and related publications which have addressed these issues highlight the complexity of the relationships between species traits and habitat constraints, which are mediated by the interactive effects of different stressors and the mechanistic links between the physiological responses to some of them.

Our results strongly support the *habitat constraint* hypothesis (Ribera, 2008); the lotic/lentic specialisation is associated with differences in several essential traits between closely related species that ultimately result in a higher colonisation capacity for the species that occupy the lentic systems. However, the specific combination of traits shaped by habitat stability seems to differ across water beetle lineages.

Differences have been found in survival and behavioural responses under acute heat stress or the combination of heat and salinity stress between lotic and lentic species pairs in two of the studied genera (*Nebrioporus* and *Ochthebius,* respectively) (Chapter 2), in agreement with the *habitat constraint* hypothesis. Another study that used a dynamic method to measure thermal tolerance has also reported that the lentic *N. ceresyi* shows higher tolerance to low temperatures than the lotic *N. baeticus*, which results in a broader fundamental thermal niche (Sánchez-Fernández *et al.*, 2010). Regarding the studied *Enochrus* species pair, neither the approach followed in this thesis nor the dynamic method used in Arribas et al. (2012) have found differences in thermal tolerances between these sister species. In this case, differences in range size between the lentic-widespread *E. bicolor* and the lotic-geographically restricted *E. jesusarribasi* have been pointed to be driven by the lentic species' greater dispersal ability, according to wing morphometry measurements (Arribas *et al.* 2012[1]). The same pattern in dispersal capacity has been found between lentic and lotic corixid species (genus *Sigara*, Carbonell *et al.*, 2016). Intriguingly, wing measurements made on *Nebrioporus* and *Ochthebius* species showed no consistent differences between lentic and lotic species (Pallarés *et al.*, unpublished data).

Taken together, these results suggest that natural selection in lentic systems drives two apparently non-overlapping strategies: improved dispersal capacity or the widening of the thermal fundamental niches of lentic species. However, comparisons of thermal tolerance measurements have to be interpreted with caution. When comparing thermal tolerances measured by different approaches (e.g. short *vs.* long-term exposure or dynamic *v.s* static methods, Lutterschmidt & Hutchison, 1997; Terblanche *et al.*, 2007) it must be taken into account that acute exposure to sublethal stress and longer-term exposure may trigger different physiological responses (e.g. hardening *vs.* acclimation processes; Bowler, 2005). In the light of this, measurements of stress thresholds for behavioural responses could be a complementary approach to define species' tolerance limits. In adult water beetles, avoidance behavior, such as emersion and flight activity (examined in Chapter 2), showed to be good proxies of species' physiological

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[1] Arribas *et al.* (2012) compared *E. bicolor* and *E. falcarius. Enochrus jesusarribasi* was later described as a cryptic species within the *E. falcarius* complex (Arribas *et al.*, 2013)

sensitivity to stressful conditions, and could then be employed as useful sublethal measures.

On the other hand, further research on many other aspects of stress physiology, not directly explored in this thesis, may help us to better understand the ecological and evolutionary responses to the constraints imposed by habitat and to predict species responses to global change. Phenotypic variation stemming from developmental plasticity and phenotypic flexibility strongly affects different aspects of population-level performance (Forsman, 2015). It has been suggested the acclimatory plasticity of species or populations from highly variable environments might be limited compared with those from moderately variable environments (reviewed for marine invertebrates in Tomanek, 2010). In this context, a question that arises is to what extent the contrasting ecological and geological stability between lentic and lotic habitats might constrain acclimatory plasticity in inland aquatic species. Several studies have also suggested an evolutionary or functional trade-off between acquiring extreme thermal tolerance limits and retaining a high level of plasticity of such limits (e.g. Stillmann, 2003; Hoffmann *et al.* 2005; Mitchell *et al.*, 2011; Esperk *et al.* 2016), although such negative correlation has not been found in diving beetles (Calosi *et al.*, 2008). Some studies have addressed acclimatory plasticity in saline water beetles (e.g. Sánchez-Fernández *et al.* 2010; Arribas *et al.*, 2012; Botella-Cruz *et al.*, 2016) and corixids (Carbonell *et al.*, 2017), with interesting outcomes as the influence of salinity on thermal plasticity. However, the role of plasticity in its multiple forms (i.e. physiological, behavioural and developmental) in driving stress responses of aquatic beetles is still not completely understood.

In addition to tolerance to acute heat and osmotic stress, this thesis has also explored other of the multiple dimensions of the physiological fundamental niche of water beetles (salinity tolerance and desiccation resistance). Lentic temporary waters show greater seasonal fluctuations in salinity and water levels than lotic systems and have been more unstable on a geological time scale. So according to the *habitat constraint* hypothesis, lentic species might also be expected to be more tolerant to desiccation and salinity. Consequently, a recent study has shown broader fundamental osmotic niches of lentic than lotic congeneric beetle species (Céspedes *et al.*, 2013). In contrast, these differences were not found in desiccation resistance between the lotic and lentic species compared in this thesis (Chapter 3). Therefore, the lotic/lentic divide seems to play a less important role in shaping these traits related to osmotic stress. Instead, the selective pressures of salinity and aridity were inferred to be the major drivers of the evolution of osmoregulation and desiccation resistance (see below).

From the study of osmoregulatory patterns in congeneric species with different osmotic niches in nature (i.e. different osmotic realised niches), it was concluded that hyporegulation capacity plays a key role in mediating the segregation of species from different lineages along the salinity gradient. Those *Nebrioporus* and *Enochrus* species that inhabit the least saline habitats were hyperegulators, but unable to hyporegulate, whilst species from hypo to hypersaline waters were all euryhaline osmoregulators (Chapter 1). This results in a clear mismatch between the fundamental and realised niches of the studied saline species, i.e. they tend to occupy the upper part of their fundamental osmotic niche. This pattern has been also found in other inland aquatic insects (Carbonell *et al.*, 2012; Céspedes *et al.*, 2013) and in some fish groups (Schultz & McCormick, 2012). The maintenance of hyperregulation capacity in saline beetle species contrasts with what has been found in other taxa; e.g. in several fish, speciation from saltwater to freshwater has been accompanied by loss of hyperosmotic capacity (Whitehead, 2010, 2011; Brennan *et al.*, 2015). Despite maintaining tolerance to a broad osmotic range, no reversals from saline to freshwater systems seem to have occurred in *Lumetus* (Chapter 5). In general, such transitions are infrequent in other insect groups (Albers & Bradley, 2011). This suggests that specialisation in saline waters may result in trade-offs with other biological traits that would promote fitness in freshwater habitats (Herbst, 2001), as demonstrated in other aquatic taxa with competitive ability or fecundity (Florencio *et al.*, 2013; Carbonell *et al.*, 2016). Therefore, the colonization of inland saline waters could represent an evolutionary irreversible process that would reduce opportunities for transitions across aquatic habitats.

On the other side of the osmotic spectrum, some species that live in fresh-hyposaline habitats show hyporegulation capacity, which was discovered when the study of osmoregulation was extended to more species within a lineage of *Enochrus* (subgenus *Lumetus*) (Chapter 5). So, although hyporegulation capacity is a necessary trait for transitions into physiologically more stressful habitats (i.e. from fresh to saline waters) in beetles, it is not likely the only one. Salinity might act as an environmental filter for other physiological or non-physiological traits, preventing the colonisation of these systems by physiologically suitable species. These might include traits related with feeding or reproductive strategies, physiological tolerances or ecological requirements of larvae and eggs (Herbst, 2006).

Overall, the experimental studies that have compared biological, physiological and behavioural traits between species specialised in different habitat types over the wide spectrum of inland water habitats suggest that lotic species, especially those restricted to freshwaters, might be more vulnerable to environmental changes than their lentic relatives. However, the real capacity of aquatic species to deal with global change will depend on the combination of multiple traits as well as on the interactive effects of multiple stressors (Boggs, 2016; Arribas *et al.*, 2017).

Insights into the evolution of salinity tolerance in water beetles.

To date, most efforts made to understand the physiological basis salinity tolerance in aquatic insects have centered on only one of the insect orders present in inland saline waters: the Diptera (Bradley, 1987, 2009). Osmoregulation patterns have been extensively studied for Coleoptera for the first time by the work undertaken in this thesis. One of the main findings is the generalisation of the osmoregulatory strategy in adult aquatic beetles or, in other words, absence of osmoconformity. Although the specific osmoregulatory processes may differ among species (e.g. Patrick *et al.*, 2002a,b) or life stages (e.g. Tones, 1977), the fact that osmoregulation is apparently the only osmotic strategy used by saline aquatic beetles contrasts with the diversity of strategies found across dipteran species (e.g. Neumann, 1976; Patrick & Bradley, 2000; Herbst *et al.*, 1988). Osmoregulation has been one of the key adaptations that have enabled the colonisation of the full saline spectrum in inland systems by aquatic insects. The osmoconformity strategy appears to confer salinity tolerance to insects only up to moderate salinity levels because, in general, no osmoconforming species are found at salinities higher than that of seawater (Griffith, 2017). Under such extreme hyperosmotic conditions, efficient excretory adaptations are required.

This thesis reports evidence for mechanistic and evolutionary links between osmoregulatory ability and desiccation resistance in some water beetle lineages. Results show that both tolerances have played an essential role in driving the habitat segregation of beetle species along the salinity gradient, but also demonstrate cross-tolerance between them. These findings provide a novel framework to understand osmotic stress physiology in aquatic insects and the processes associated with habitat transitions in inland water fauna. From a mechanistic perspective, further investigation on the genetic and mechanistic bases of osmotic stress in insects would shed light into the physiological links between these stressors. However, research on these issues for insects is still in a very early stage (Uyhelji *et al.* 2016). Investigating the common and specific physiological and transcriptomic responses involved in desiccation and salinity stress would be especially illuminating. Recent studies have been conducted in this sense on two related, but distinct, osmoregulatory processes, hyper- and hyporegulation, mainly in fishes. While exposure to hyper- *vs*. hyposmotic environments in fishes requires different physiological responses to maintain homeostasis, a core set of compensatory responses is common to general osmotic stress (Bonga, 1997). Transcriptional analyses have allowed to identify some molecular pathways that are common in both hypo- and hyperosmotic salinity challenges as well as specific responses to each stress type (Brennan *et al.*, 2015). Some insect groups also rely on similar mechanisms for hyper- and hyporegulation; e.g. anopheline mosquito have rectal cells that shift from a resorptive function in freshwater to a secretive function in saline water (Smith *et al.*, 2008, 2010). Similar plastic mechanisms could explain the maintenance of hyperegulation capacity in saline beetle species that do not frequently experience hyposmotic conditions in nature (see above). Organisms need to control the intra- and extracelullar volume and osmotic concentration under both aerial desiccating conditions or hyperosmotic stress (Griffith, 2017; Beyenbach, 2016). Therefore, the mechanistic and genetic links between physiological responses to salinity and desiccation in aquatic insects likely lie in the processes involved in ion transport and cell volume regulation.

From an evolutionary perspective, it would be interesting to examine the evolution of tolerance to desiccation and salinity in other saline insect lineages, in order to determine whether similar processes to those described herein for the beetle subgenus *Lumetus* have promoted their diversification in saline waters. Although not enough data are available to propose any consistent hypothesis, preliminary data suggest that dytiscid lineages that have colonised saline waters were not as pre-adapted to osmotic stress as apparently occurs in *Lumetus*. Species responses were not directly compared in Chapter 4, but the hydrophilid *Enochrus jesusarribasi* showed much higher desiccation resistance than the dytiscid *Nebrioporus baeticus* in cross-tolerance experiments. Consistently with this, differences in the cuticular lipid composition indicate that *E. jesusarribasi* adults have a more impermeable cuticle than that of *N. baeticus* (Botella-Cruz *et al.*, under review). In addition, the fresh-hyposaline *Nebrioporus* species used in the osmoregulation experiments (Chapter 1) showed no hyporegulation capacity, unlike some fresh-hyposaline *Enochrus* species (Chapter 5).

It has been proposed here that the enhancement of desiccation resistance and salinity tolerance observed in *Lumetus* might be related with the evolutionary history of hydrophilids and driven by global aridification events. Secondary colonisations of the terrestrial medium (and back to water again) seem to have been frequent within the family Hydrophilidae (Bernhard *et al.*, 2006; Short & Fikácek, 2013; Bloom *et al.* 2014). While relationships among beetle lineages and habitat transitions remain largely controversial (Ribera & Beutel, 2014), it could be hypothesised that the enhanced desiccation resistance in hydrophilids could have facilitated the colonisations of terrestrial environments in some lineages and transitions to saline waters in others, e.g. *Enochrus*. The origin of the family Hydrophilidae in the Late Triassic (Bloom *et al.*, 2014) coincides with a period of arid climate (Tanner & Lucas, 2006; McKenna *et al.*, 2015), in which selection for improved tolerance to drought could have pre-adapted species for the colonisation of osmotically stressful habitats. In line with this, an examination at a deeper evolutionary scale would be required to track the origin of desiccation resistance in hydrophilids and to determine whether it was a plesiomorphic trait in the family or if it arose independently in different lineages.

In relation to this, it could be hypothesised that the positive link between the mechanisms to deal with salinity and desiccation in saline insect lineages could promote adaption to more amphibious habitats and represent a plausible scenario of habitat shift and diversification for these saline groups. Early literature suggested that aquatic insects are more permeable to water than their terrestrial counterparts (Beament *et al.*, 1961), but such idea is based on comparisons among species from disparate groups, obviating potential phylogenetic constraints on cuticle properties. Recent research on cuticle permeability in saline aquatic beetles has shown surprising patterns, with cuticular hydrocarbon composition apparently possessing more similar characteristics to that of some terrestrial Coleoptera than freshwater ones (Botella-Cruz *et al.*, under review). While the process of adaption to saline inland waters has been traditionally proposed as an evolutionary dead-end preventing diversification to the most abundant freshwater systems (Herbst, 2001), it could be also seen as an effective open window for the 'terrestrialisation' of those aquatic lineages that have linked mechanisms to deal with salinity and desiccation.

Finally, I would like to emphasise once again the need for further research on the ecology, physiology and evolution of saline insects in order to understand the processes that have generated and that maintain the diversity of inland aquatic ecosystems, as well as the importance of including these habitats in protection networks and conservation policies to adequately protect aquatic biodiversity.

Enochrus jesusarribasi. Author: Jesús Arribas.

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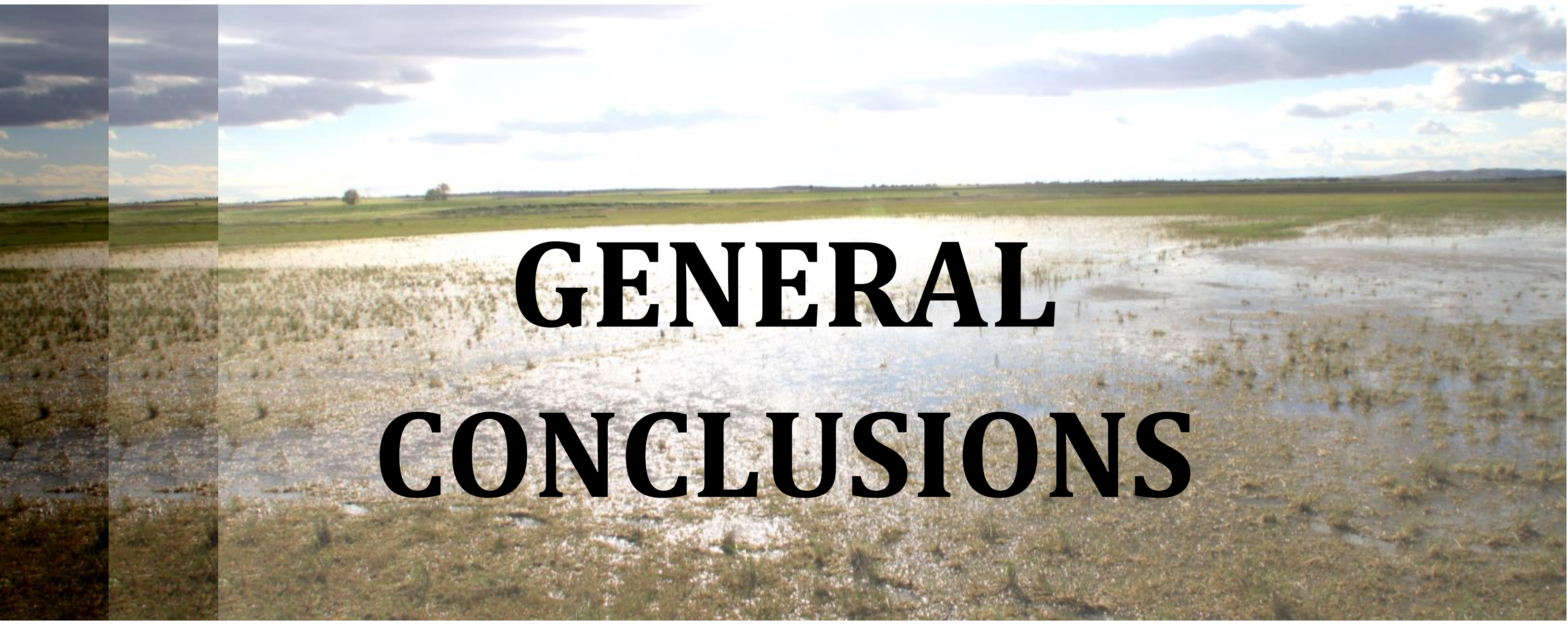
GENERAL CONCLUSIONS



Laguna El Hito, Cuenca (Spain). Author: Abraham Gómez

GENERAL CONCLUSIONS

Chapter 1: Osmoregulation in water beetles

1. Osmoregulation, rather than osmoconformity, is the main osmotic strategy of eight studied aquatic beetle species from two independent lineages (genus *Enochrus*: Hydrophilidae and *Nebrioporus*: Dytiscidae).

2. The species that inhabit freshwater and saline waters of both genera show hyperegulation capacity, while hyporegulation ability is exclusive of species that occur in saline habitats.

3. For both freshwater and saline species, maximum hyposmotic capacity correlates with the upper salinities that each species inhabits in nature. In contrast, for the saline species, there is a clear mismatch between their experimental tolerance to freshwater-low salinity conditions (hyperegulation capacity) and their realised saline niche, as they are commonly absent in freshwaters.

4. Within each studied genus, the differing osmotic capacities of the species mediate their differential tolerances to salinity, and appear to be a key determinant, but not the only one, of their habitat segregation along the salinity gradient.

Chapter 2: Behavioural responses to acute stress

5. Under acute exposure to combined heat and osmotic stress, only temperature has an effect on escape responses (emersion and flight) and survival in saline species of *Enochrus* and *Nebrioporus* genera. The interaction of high temperatures and salinities has a synergistic negative effect on performance only in *Ochthebius* species.

6. *Ochthebius* species are more resistant to acute exposure to heat and salinity than *Enochrus* and *Nebrioporus* species, showing a relatively high survival at the highest tested temperature (45ºC) in some salinity treatments.

7. Differences in survival patterns between the lotic and lentic species are not consistent across the three genera. Within *Nebrioporus* and *Octhebius*, the lotic species are more

sensitive to temperature than their corresponding lentic congeners and also display emersion and flight at lower stress levels, according with the *habitat constraint* hypothesis. In contrast, *Enochrus* species show similar heat tolerance.

Chapter 3: Desiccation resistance in water beetles

8. Desiccation resistance is positively associated with habitat salinity in congeneric water beetle species (genus *Enochrus*). Saline species show higher water content, lower water loss rates and higher survival under desiccation than freshwater and hyposaline congeners. The freshwater species of *Enochrus* may be more vulnerable than saline ones to the drought intensification expected with climate change in semi-arid regions.

9. Interspecific differences in desiccation resistance in these species do not seem to be related with their preference for lentic or lotic habitats, which suggests that differences in habitat stability do not differentially constrain the osmotic dimension of these species niches.

10. At the intraspecific level, variation in water loss rates is positively related with the initial water content of individuals, but not with cuticle content or body mass. Such a relationship suggests that active mechanisms for water conservation are activated when a critical water loss threshold is reached.

Chapter 4: Cross-tolerance in water beetles

11. Exposure to stressful salinities improves water conservation capacity under a subsequent desiccation exposure in two species from distinct genera of water beetles. In *N. baeticus*, such adjustments confer cross-tolerance to salinity, which enhances survival.

12. Exposure to desiccation under different conditions (rapid-extreme and slow-moderate desiccation) decreases performance under a following exposure to high salinity in both studied species.

13. *Enochrus jesusarribasi* shows different osmotic regulation patterns under rapid or slow desiccation, which suggests that the osmotic concentration could be sacrificed in

order to preserve extracellular and intracellular fluid volume under a rapid and extreme desiccation.

14. These results demonstrate mechanistic links between tolerances to salinity and desiccation in water beetles and highlight the central role of adaption to multiple co-occurring stressors in arid inland waters.

Chapter 5: Adaptation to desiccation and salinity

15. Relatively high desiccation resistance and limited hyporegulation capacity are estimated as plesiomorphic traits in the subgenus *Lumetus* (*Enochrus*: Hydrophilidae). Desiccation resistance tended to remain stable through the evolutionary history of the lineage, but hyporegulation capacity undergone large and, in some cases, accelerated increases, which were associated with transitions to saline habitats.

16. Resistance to desiccation and hyporegulation capacity are positively associated in meso-hypersaline species. All species that display good hyporegulation capacity are also resistant to desiccation, but not vice versa. This suggests that hyporegulation capacity was a secondary adaption in this lineage, co-opted from mechanisms derived from those evolved originally for desiccation resistance.

17. On the evolutionary scale of this study, it was not possible to reconstruct the increase in desiccation resistance preceding the improvement in hyporegulation capacity, which would be expected if tolerances were co-opted. However, the accelerated increases in hyporegulation capacity were, to some extent, simultaneous with weak increases in desiccation resistance across the full evolutionary path of the species, which supports a linked evolution.

18. The parallel increases in desiccation resistance and osmoregulation capacity were associated with periods of global aridification in some clades. Aridification, by imposing strong selective pressures on linked mechanisms for desiccation resistance and salinity tolerance, could have been a key driver in the diversification of *Lumetus* along inland waters.

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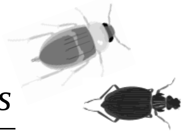

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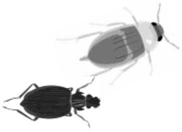

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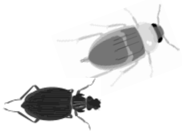

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