


ORIGINAL ARTICLE

The chicken or the egg? Adaptation to desiccation and salinity tolerance in a lineage of water beetles

Susana Pallarés¹  | Paula Arribas² | David T. Bilton³ | Andrés Millán¹ | Josefa Velasco¹ | Ignacio Ribera⁴

¹Department of Ecology and Hydrology, Facultad de Biología, University of Murcia, Murcia, Spain

²Island Ecology and Evolution Research Group, IPNA-CSIC, Santa Cruz de Tenerife, Spain

³Marine Biology and Ecology Research Centre, School of Biological and Marine Sciences, Plymouth University, Plymouth, UK

⁴Institute of Evolutionary Biology (CSIC-Universitat Pompeu Fabra), Barcelona, Spain

Correspondence

Susana Pallarés, Department of Ecology and Hydrology, Facultad de Biología, University of Murcia, Murcia, Spain.
Email: susana.pallares@um.es

Funding information

Royal Society London; Universidad de Murcia; Ministerio de Economía y Competitividad (co-financed by FEDER funds), Grant/Award Number: CGL2013-48950-C2-1&2-P

Abstract

Transitions from fresh to saline habitats are restricted to a handful of insect lineages, as the colonization of saline waters requires specialized mechanisms to deal with osmotic stress. Previous studies have suggested that tolerance to salinity and desiccation could be mechanistically and evolutionarily linked, but the temporal sequence of these adaptations is not well established for individual lineages. We combined molecular, physiological and ecological data to explore the evolution of desiccation resistance, hyporegulation ability (i.e., the ability to osmoregulate in hyperosmotic media) and habitat transitions in the water beetle genus *Enochrus* subgenus *Lumetus* (Hydrophilidae). We tested whether enhanced desiccation resistance evolved before increases in hyporegulation ability or vice versa, or whether the two mechanisms evolved in parallel. The most recent ancestor of *Lumetus* was inferred to have high desiccation resistance and moderate hyporegulation ability. There were repeated shifts between habitats with differing levels of salinity in the radiation of the group, those to the most saline habitats generally occurring more rapidly than those to less saline ones. Significant and accelerated changes in hyporegulation ability evolved in parallel with smaller and more progressive increases in desiccation resistance across the phylogeny, associated with the colonization of meso- and hypersaline waters during global aridification events. All species with high hyporegulation ability were also desiccation-resistant, but not vice versa. Overall, results are consistent with the hypothesis that desiccation resistance mechanisms evolved first and provided the physiological basis for the development of hyporegulation ability, allowing these insects to colonize and diversify across meso- and hypersaline habitats.

KEYWORDS

ancestral reconstruction, aquatic insects, habitat transitions, hyporegulation ability, inland saline waters, water loss

1 | 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 colonization of novel

environments (Heard & Hauser, 1995; Hunter, 1998; Moczek, 2008). In the aquatic realm, the evolution of hydric and osmotic regulation mechanisms was a key innovation allowing transitions from marine to freshwater habitats in some animal groups such as fishes or crustaceans (e.g., Faria, Augusto, & McNamara, 2011; McNamara & Faria,

2012; Schultz & McCormick, 2012). Similarly, but in the opposing direction, the evolution of these mechanisms in inland aquatic lineages has allowed for transitions from fresh to saline inland waters, a recurrent phenomenon in a number of aquatic insect orders (e.g., Albers & Bradley, 2011). Most interestingly, such transitions to saline waters seem 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 (see, e.g., Arribas et al., 2014 for beetles; Carbonell, Millán, & Velasco, 2012 for water bugs; or Herbst, 1999 for flies). The physiological and evolutionary processes that may facilitate the colonization 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 & Plath, 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 is a major physiological stress factor. In contrast, tolerance to the osmotic stress produced by a saline aquatic medium seems to be a very specialized secondary adaptation, 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; that is, they are able to maintain the concentration of haemolymph below that of the external medium and within a narrow range regardless of the external osmotic concentration (e.g., Herbst, Conte, & Brookes, 1988; Pallarés, Arribas, Bilton, Millán, & Velasco, 2015; Tones & Hammer, 1975). Ultimately, hyporegulation 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). Their common physiological basis likely lies in ion transport and cell volume regulation processes (Beyenbach, 2016; Griffith, 2017), which in most insects involve the activity of excretory organs, such as Malpighian tubules and the rectum, and the control of cuticular permeability (Dow & Davies, 2006; Gibbs & Rajpurohit, 2010; Larsen et al., 2014). 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 the other way around.

The relationship between tolerance to salinity and desiccation has been mostly studied in plants (e.g., Barrieu et al., 1999; Cayuela et al., 2007; Hossain, Mostofa, & Fujita, 2013) and to a lesser extent in animal taxa (Faria, Provete, Thurman, & McNamara, 2017; Gómez-Mestre & Tejedo, 2005). Despite the relevance of such relationship, to our knowledge, no previous studies have addressed the potential evolutionary links between mechanisms to deal with salinity and desiccation. However, recent studies on salinity tolerance in aquatic insects point to their close association. First, beetle adults (Pallarés, Botella-Cruz, Arribas, Millán, & Velasco, 2017) and dipteran larvae

(Elnitsky, Benoit, Lopez-Martinez, Denlinger, & Lee, 2009) sequentially exposed to salinity and desiccation showed cross-tolerance responses (Sinclair, Ferguson, Salehipour-shirazi, & MacMillan, 2013; Todgham & Stillman, 2013), suggesting a mechanistic link between the response to both stressors. Second, a recent study reconstructing the colonization 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 salinity of the preferred habitat of a species and the aridity of the region over which it is distributed. Finally, in agreement with this ecological correlation, Pallarés, Velasco, Millán, Bilton, & Arribas, (2016) revealed a positive relationship between desiccation resistance and salinity tolerance in species of *Enochrus* in the laboratory.

Despite multiple lines of evidence suggesting an evolutionary link between hyporegulation ability and desiccation resistance 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 ability, facilitating the colonization of saline waters in the *Lumetus* subgenus of *Enochrus*. In this case, hyporegulation ability 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, Kiergaard, Gelembiuk, Eads, & Posavi, 2011). Mechanisms for tolerance to salinity and desiccation could have also evolved as a joint response to aridification, as this process typically results in a simultaneous decrease in precipitation and increase in the mineralization of surface waters.

The relationship between aridity and salinity demonstrated by Arribas et al. (2014) was based only on ecological data (species habitat occupancies and regional climates), which do not always fully reflect the potential physiological tolerance of species (Carbonell et al., 2012; Céspedes, Pallarés, Arribas, Millán, & Velasco, 2013). Mismatches between realized and fundamental niches may result when physiological tolerance evolved as a result of prior exposure to different stressors, as in such cases species may retain the ability to deal with conditions different from those in their current habitats. Disentangling the evolution of hyporegulation and desiccation resistance in organisms spanning the fresh–saline spectrum is thus not straightforward, 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 adults of the water beetle subgenus *Lumetus*. This lineage includes species in all habitat types from fresh to hypersaline waters, with differing hyporegulation abilities (Pallarés et al., 2015). We provide a comprehensive and generally well-resolved phylogeny of the subgenus,

together with experimental data on desiccation resistance and hyporegulation ability across its constituent taxa, and use ancestral trait reconstruction and phylogenetic comparative methods to test the following alternative hypotheses:

1. The hyporegulation ability allowing the colonization of saline waters was co-opted from physiological mechanisms evolved originally for desiccation resistance.
2. The development of hyporegulation ability in saline waters was the primary adaptation, secondarily leading to an increase in desiccation resistance.
3. Desiccation resistance and hyporegulation ability 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 to be true (i.e., there may be desiccation-resistant species with low or no hyporegulation ability). In addition, there could be species with high desiccation resistance and hyporegulation ability 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 ability may be expected to be preceded by increases in desiccation resistance.

Under the second hypothesis, the situation would be the reverse, and we could expect that all species that are resistant to desiccation will be good hyporegulators, but not necessarily vice versa (i.e., there could be hyporegulator species with low desiccation resistance). In this case, an increase in desiccation resistance should be preceded by an increase in hyporegulation ability across the phylogeny.

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

There could be a fourth possibility, namely that there was an independent evolution of desiccation resistance and hyporegulation ability. There is, however, ample evidence for the association between tolerance to desiccation and salinity in *Lumetus* (Arribas et al., 2014; Pallarés et al., 2016, 2017), allowing this possibility to be discarded a priori.

2 | MATERIAL AND METHODS

2.1 | 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, 2014; Arribas, Andújar, Sánchez-Fernández, Abellán, & Millán,

2013). Several *Enochrus* species of the subgenera *Methydrus*, *Enochrus* and *Hugoscottia* and a related genus (*Helochaeres*) were used as outgroups, with two more distantly related genera of Hydrophilidae, *Hydrobius* and *Arabhydrus* (Short & Fikáček, 2013) used to root the tree, resulting in a phylogeny of 43 species.

Data on hyporegulation ability and desiccation resistance were obtained experimentally from adults of a representative subset of nine 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*).

2.2 | 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 nonoverlapping 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 Zealand), 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, Suchard, Xie, & Rambaut, 2012) and run in the CIPRES Science Gateway (Miller, Pfeiffer, & Schwartz, 2010). The concatenated data set was divided into three partitions: the three protein-coding genes, the mitochondrial ribosomal gene and the two nuclear sequences. Analyses were conducted by applying a GTR + I + G substitution model for each partition, which was the best-fitting model previously estimated with PARTITION FINDER (Lanfear, Calcott, Ho, & Guindon, 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)—that is, ≈ 45 Ma (gamma distribution shape: 56.84, scale: 0.74). An uncorrelated lognormal clock was applied for the nuclear partition, with an uniform prior distribution for the rate of substitutions set between 0.0001 and 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 an uniform prior distribution for the rate with 0.01 (0.001–0.1) subst/s/Ma. The ranges set as priors for the substitution rates cover the range of rates usually reported for

Coleoptera, which are faster for the mitochondrial than for the nuclear genes used in this study (e.g., Andújar, Serrano, & Gómez-Zurita, 2012; Papadopoulou, Anastasiou, & Vogler, 2010; Ribera et al., 2010).

We set two independent runs of 100 million MCMC steps each, sampling one tree every 10,000 generations. LogCombiner (Drummond et al., 2012) was used to combine trees from both runs and to obtain 1,000 randomly resampled post-burnin trees. The consensus tree was estimated with TREEANNOTATOR (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).

2.3 | Ecological data, hyporegulation ability and desiccation resistance

To track habitat transitions across the salinity gradient, each *Lumetus* species was assigned a qualitative salinity category according to our field data or bibliographic data on the salinity of their most frequently occupied habitats. We followed the same criteria and categorization done by Arribas et al. (2014), with special attention to the records of populations in habitats with the highest salinities, as these may better reflect species' tolerance limits (Carbonell et al., 2012; Céspedes et al., 2013). Six categories were used as follows: 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 hyporegulation ability of the nine selected species (Table S2), haemolymph osmolalities were measured in individuals exposed for 48 hr to different salinities within their specific tolerance ranges (as determined by pilot trials or previous work, Pallarés et al., 2015). All species were exposed to at least two common hyposmotic treatments (0.3 and 12 g/L) and a hyperosmotic one (35 g/L) 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). From each treatment, we obtained haemolymph samples from a minimum of three of the exposed individuals (Table S4), as pilot trials 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). For each treatment, we estimated the hyper- or hyposmotic capacity, that is, the difference between the osmotic concentration of the haemolymph and the external medium, which represents an integrated measure of the physiological ability to compensate for the osmotic gradient between internal and external media (Calosi, Ugolini, & Morritt, 2005; Charmantier, Charmantier-Daures, & Aiken, 1984). The hyposmotic capacity at 35 g/L (hyposmotic capacity hereafter) and the maximum hyposmotic capacity (i.e., that measured at the highest salinity tolerated by each species) showed the highest variation between species and were therefore used for subsequent analyses.

Controlled desiccation experiments were conducted as described by Pallarés et al. (2016). Specimens were exposed to desiccation at

$20 \pm 5\%$ RH (relative humidity), $20 \pm 1^\circ\text{C}$ for 6 hr. For each specimen, we measured the initial and final fresh mass (i.e., specimen mass before and after desiccation treatments) as well as dry mass. From these measurements, we obtained the initial water content as the % wet mass (difference between fresh and dry mass) relative to initial fresh mass and water loss as the % of water lost relative to initial fresh mass. These variables, and in particular water loss, 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 hr after desiccation. Mortality was assessed after both desiccation and the recovery period. These estimates were obtained for 20–30 specimens per species (Table S4).

After each experiment, specimens were sexed by examining genitalia under a Leica M165C stereomicroscope. Further details of the experimental procedures are indicated in the Appendix S1.

2.4 | Habitat transitions, evolution of desiccation resistance and osmoregulatory capacity

2.4.1 | Ancestral trait reconstruction

We tested different models of trait evolution (Brownian motion—BM and Ornstein–Uhlenbeck—OU) (Kaliotzopoulou & Adams, 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, Coop, & Ralph, 2012; Cooper, Thomas, Venditti, Meade, & Freckleton, 2016). To account for this, we used a Monte Carlo-based approach to assess the power 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 estimate models) 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, Heringa, & Brandt, 2009; Goolsby, Bruggeman, & Ane, 2017), which uses a maximum-likelihood-based method to estimate trait covariance across (phylogenetic covariance) and within species (phenotypic covariance) for data sets with missing data and multiple within-species observations (e.g., Pollux, Meredith, Springer, Garland, & Reznick, 2014). This method provides predicted trait values and variances for ancestral nodes and unmeasured extant species (Penone et al., 2014). Trees were pruned to keep one representative specimen per putative species in order to fix the species-level resolution of the physiological traits. Outgroup species with missing physiological and ecological data were excluded. Multiple trait observations per species were included to account for interindividual variation and measurement error (Bruggeman et al., 2009).

2.4.2 | 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 difference between the reconstructed trait values of the corresponding initial and final node) against branch length (Ma) for each trait separately. We identified outlier branches (i.e., those above the upper 99% confidence interval of the regression line), which can be considered to show accelerated rates of evolution. 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 also compared the global rate of evolutionary change between maximum hyposmotic capacity, water loss and water content using Adam's method (Adams, 2013). 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. For simplicity, only the maximum hyposmotic capacity was used for these analyses as it was significantly positively correlated with hyposmotic capacity ($R^2 = 0.37$, $p < .001$).

2.4.3 | 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, that is, an evolution of the character independent of phylogenetic relationships ($\lambda = 0$) and (ii) a model entirely in agreement with BM, that is, the probability of shared inheritance is strictly proportional to relatedness ($\lambda = 1$) (Freckleton, Harvey, & Pagel, 2002).

2.4.4 | 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, and (iii) desiccation resistance and hyposmotic capacity. Proportional data (% water content and % water loss) 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.

2.5 | Topological uncertainty

To account for topological uncertainty, the analyses for estimation of the phylogenetic signal, PGLS and comparison of rates of phenotypic change were repeated using 1,000 randomly resampled post-burnin trees from the *BEAST* output.

3 | RESULTS

3.1 | Phylogeny of *Lumetus*

We obtained a well-resolved phylogeny of the subgenus *Lumetus*, with strong support for most of the main nodes except for some internal nodes in the *Enochrus quadripunctatus* group (Figures 1 and S1). The first splits separated *Enochrus ochropterus* and *Enochrus salomonis* from the rest of the *Lumetus* species at 38 (28–49 95% confidence interval, c.i.) Ma (clade C1) and the lineage containing only *Enochrus testaceus* at 36 (26–46 c.i.) Ma (clade C2). Within the remaining *Lumetus* species, the next split, at 32 (23–42 c.i.) Ma, separated a clade of saline species (the *Enochrus bicolor* group, clade C3) from one including three subclades of Nearctic and Palearctic species (clades C4–C6). 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 (clade C6) was formed of six recently diverged lineages (the *E. quadripunctatus* complex) with well-characterized geographical distributions. These included (i) a coastal Mediterranean clade; (ii) another containing a single specimen from Canada; two Eurasian clades; one (iii) widely distributed and another (iv) restricted to Bulgaria and Turkey; (v) a clade apparently restricted to Italy; and (vi) an Ibero-Moroccan clade. Sequence length, number of variable sites and the estimated substitution rates for each partition are provided in Table S5.

3.2 | Hyporegulation ability and desiccation resistance

All species were hyper-regulators at salinities below the isosmotic point. Under hyperosmotic conditions, all the species showed hyporegulation ability within specific salinity ranges, except for one freshwater species, *Enochrus salomonis*, which did not survive exposure to hyperosmotic conditions (>35 g/L) (Fig. S2a, Table S4). In desiccation experiments, *Enochrus 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. S2b). No significant mortality was observed in control (nondesiccated) individuals. Survival under desiccation was highly correlated with water loss but not with water content (Fig. S2c).

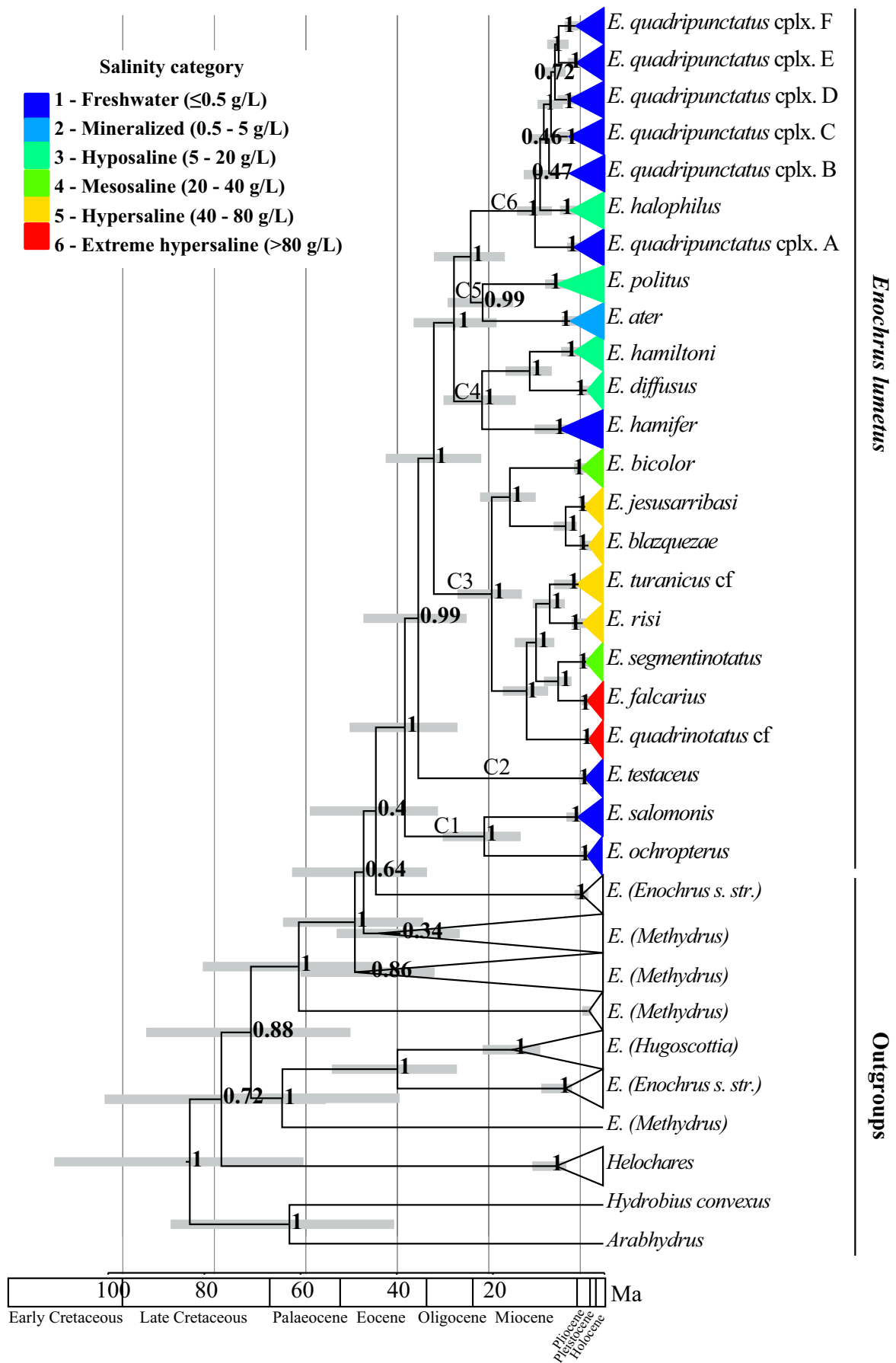


FIGURE 1 Dated phylogeny of *Lumetus*. Node numbers: posterior probabilities; bars on nodes: 95% confidence intervals for node ages; letters: main clades as referred to in the text. Terminals are collapsed to reflect species-level relationships (see Fig. S1 and Table S1 for details on terminals)

3.3 | Habitat transitions, evolution of desiccation resistance and hyporegulation ability

3.3.1 | 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. S3). Ancestral state reconstruction was therefore made assuming the simplest model, that is, BM. All measures of absolute phenotypic change (shown in

Table S6) were significantly related to branch length ($p < .05$), except for water loss ($p = .07$). Accelerated rates of phenotypic evolution of all traits were identified in several branches across the tree (Figures 2 and S4).

The ancestor of *Lumetus* was inferred to be a species which lived in mineralized waters (Figures 2a and S5) with some degree of hyposmotic capacity (423 mOsmol/kg at 35 g/L, Figures 2b and S5), but within a limited salinity range (maximum estimated hyposmotic capacity of 1,000 mOsmol/kg, Figures 2c and S5). A rapid, direct transition to mesosaline waters took place at the origin of the *E. bicolor* group, as well as other independent transitions to hyposaline waters (e.g., at the

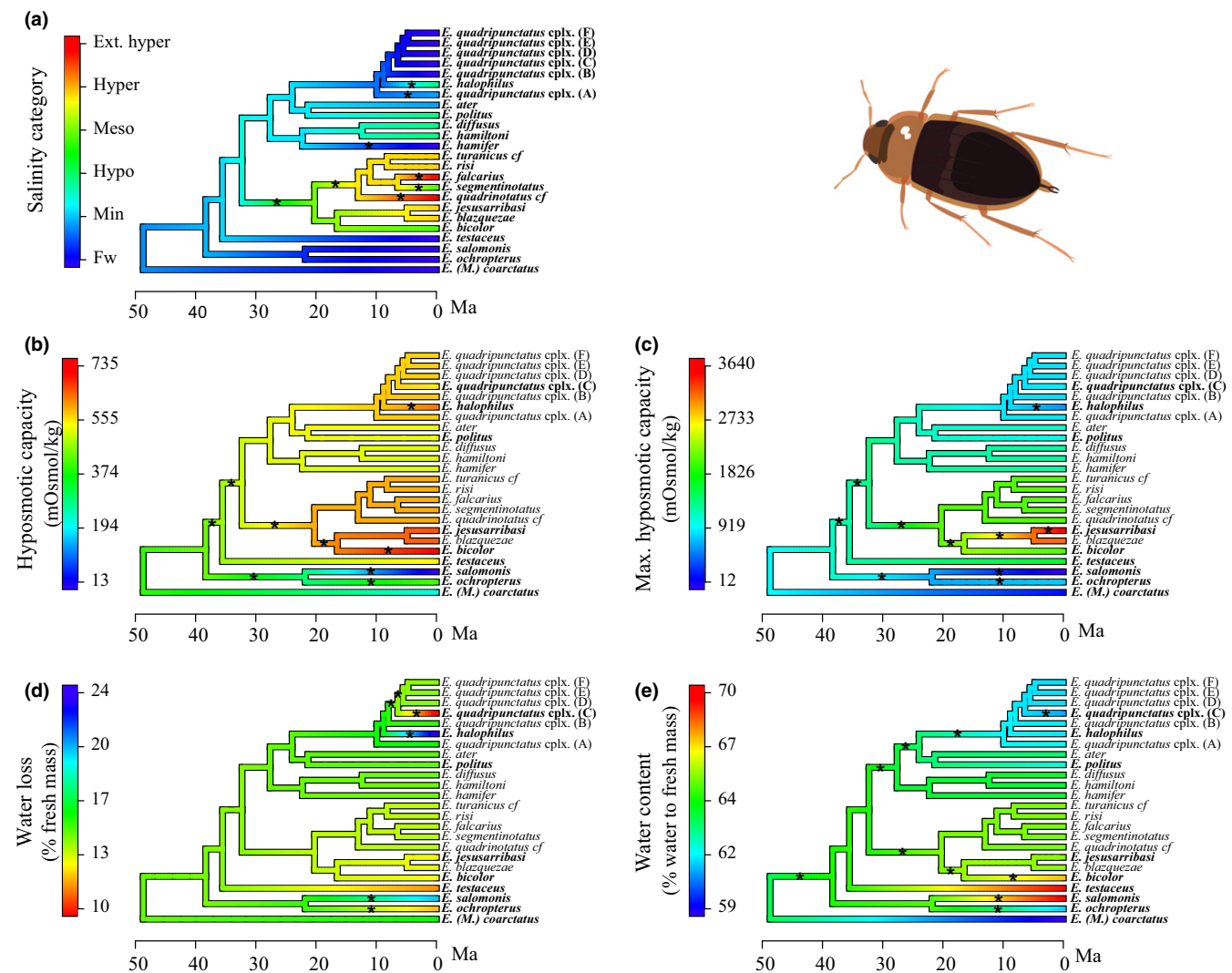


FIGURE 2 Ancestral reconstruction of desiccation and osmoregulation traits. The warmer (red) colours indicate higher resistance to desiccation or salinity than cooler (blue) colours. Branches where significantly accelerated increases or decreases in the rate of phenotypic change were identified (see Fig. S4) are indicated by asterisks. Species for which ecological or experimental data were available are indicated in bold. See reconstructed values in Fig. S5

origin of *Enochrus diffusus*–*Enochrus hamiltoni* or *Enochrus politus*) and accelerated reversions to freshwater habitats in the Nearctic–Palearctic clades (Figure 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 and water content varied little across *Lumetus* (13.6%–16.5% of fresh mass and 61.7%–66.2% of water to fresh mass, respectively, Fig. S5). Water loss 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 (Figure 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 (Figure 2e).

Likelihood ratio tests indicated that the global rate of evolution for maximum hyposmotic capacity was significantly higher than for water loss and water content. These same results were consistently recovered when analysing the 1,000 post-burnin resampled trees (Table 1).

3.3.2 | Phylogenetic signal

For all traits, except for water loss, estimates of Pagel's λ were close to 1 in all the resampled trees (although for habitat salinity λ was <1 in 14% of trees) and significantly better than those obtained when the phylogenetic structure was erased ($\lambda = 0$), indicating a significant phylogenetic signal (Table 2). For hyposmotic capacity and water content, estimated λ s 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., $\lambda = 1$) in all resampled trees. Water loss was the only trait consistently showing no phylogenetic signal in all the analysed trees (Table 2).

3.3.3 | Relationships between traits

In PGLS analyses (Table S7), habitat salinity showed no significant relationships either with maximum hyposmotic capacity or desiccation traits (Figure 3a–c) in any of the analysed trees. Variability in

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 $\lambda = 0$ or $\lambda = 1$ (for the consensus tree)

Variable	Pagel's λ	p ($\lambda = 0$)	p ($\lambda = 1$)
Habitat salinity	0.96–1.13	$<.001$.697
Hyposmotic capacity	1.07–1.14	$<.001$	$<.001$
Max. hyposmotic capacity	1.04–1.13	$<.001$.051
Water loss	$<.001$	1	$<.001$
Water content	1.07–1.14	$<.001$	$<.001$

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 (Figure 3a–c).

Maximum hyposmotic capacity was negatively related to water loss in 100% of the resampled trees and with water content in 58% of the trees. However, these relationships were strongly influenced by the outlier values that one species, *E. salomonis*, showed for these variables. After removing this species from PGLS, the relationship with water loss was not significant and the relationship with water content became stronger and significantly positive for all the analysed trees (Table S7, Figure 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 (Figures 4 and 5), increases in hyposmotic capacity were not clearly preceded by increases in desiccation resistance nor vice versa. Amongst the species with the highest hyporegulation ability (*E. testaceus*, *E. bicolor* and *Enochrus jesuarrubasi*), the increase in hyposmotic capacity along their evolutionary path was coupled with parallel decreases in water loss and increases in water content, suggesting an associated increase in desiccation resistance. On the contrary, increases in desiccation resistance were not always associated with an increase in hyposmotic capacity, as in, for example, *E. ochopterus* and *E. quadripunctatus* in Figure 4, or *E. salomonis* in Figure 5.

4 | DISCUSSION

The reconstruction of habitat transitions, desiccation and osmoregulatory traits in *Lumetus* species suggest that hyporegulation ability,

TABLE 1 Comparison of evolutionary rates (log scale) for maximum hyposmotic capacity (Max. HC), water loss (WL) and water content (WC). AIC_C scores refer to the comparison of a model allowing rates to vary amongst traits (observed, “obs”) and a model constraining rates of evolution to be equal amongst traits (constrained, “cons”); LRT refers to likelihood ratio tests for pairwise comparisons of evolutionary rates between trait pairs. The ranges in parameter values reflect the range of variation in the analyses of 1,000 post-burnin trees

Trait	σ^2	Pairwise comparison	LRT _{df=1}	p	AIC _C
Max. HC	0.021–0.049				
WL	0.001–0.004	Max. HC vs. WLR	27.4–36.4	$<.001$	Obs = 54.2–67.4 Cons = 82.5–100.9
WC	0.00003–0.00007	Max. HC vs. WC	121.1–125.5	$<.001$	Obs = –40.3 to –25.2 Cons = 78.8–97.9

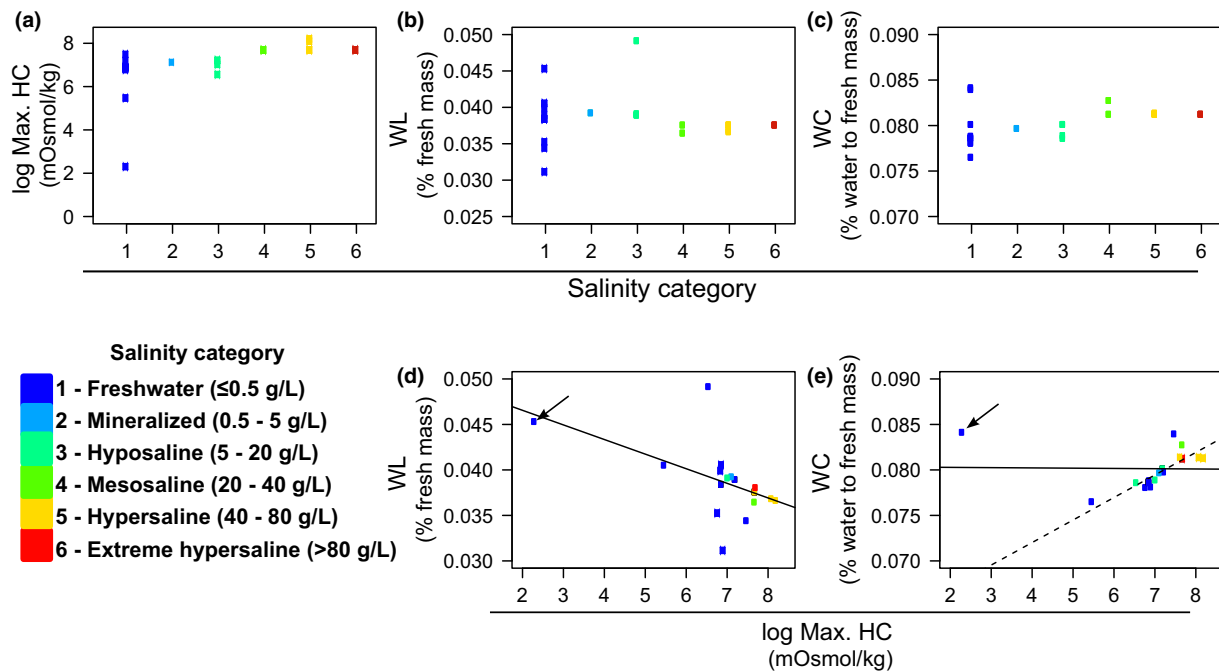


FIGURE 3 Relationships between habitat salinity, hypotonic capacity and desiccation traits. Regression lines are shown for significant relationships in PGLS (see Table S6). Dashed line for regressions excluding *Enochrus salomonis* (indicated by arrow). Max. HC, maximum hypotonic capacity; WL, water loss; WC, water content

an essential trait for the colonization of hyperosmotic media by aquatic insects, arose as a mechanism derived from those originally developed to deal with desiccation stress in this lineage, in agreement with our first hypothesis.

The ancestral reconstruction of water loss suggests that the most common recent ancestor of *Lumetus* had similar desiccation resistance to extant species of the subgenus. Water loss did not change abruptly through the evolutionary history of the lineage, but had 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), which show comparable water loss rates to those reported for the highly desiccation-resistant aquatic beetle *Pelodytes muticus* (Arlian & Staiger, 1979). The hypersaline *E. jesuarribasi* has much lower water loss rates and higher resistance to desiccation than hypersaline diving beetles studied to date (Pallarés et al., 2017), which seem to have more permeable cuticles than *Enochrus* species (Botella-Cruz et al., 2017). Our data suggest a 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 itself. Despite the lack of data on desiccation resistance of other hydrophilids, the unusually frequent transitions between terrestrial and aquatic environments within this family (Bernhard, Schmidt, Korte, Fritzsche, & Beutel, 2006; Short & Fikáček, 2013) would be in agreement with this hypothesis.

The ancestor of *Lumetus* was inferred to have lived in mineralized waters, and to have had moderate hyporegulation ability. In contrast to the low variation in water loss, hyporegulation ability underwent large and, in some cases, accelerated changes through 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 at a higher rate than habitat transitions 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 their hyporegulation ability.

Species living in the most saline conditions showed high hypotonic capacity, but also an increased desiccation resistance (i.e., lower water loss). In the case of species living in fresh to hyposaline waters, we found (i) some species with comparable or even higher desiccation resistance than their saline water relatives, but relatively low hypotonic capacity (e.g., *E. ochropterus*) and (ii) species which had both high desiccation resistance and hypotonic capacity. For example, *E. testaceus* and *E. politus* were able to hyporegulate at salinities well above those encountered by these beetles in nature. According to the ancestral reconstruction of habitat salinity, neither *E. testaceus* nor *E. politus* had saline ancestors, something that is only compatible with the first of our proposed hypotheses, that is, that hyporegulation ability was co-opted from desiccation resistance mechanisms. A lack of association between habitat salinity and osmoregulatory ability has also been reported in some crustaceans (e.g., Faria et al., 2017; McNamara & Faria, 2012). Grapsid and ocy-podid crabs present an example of how selection on mechanisms to reduce water loss under aerial desiccation (gill function in this case) indirectly has improved underwater osmoregulation ability, meaning desiccation resistance and osmoregulation capacities are positively associated (Faria et al., 2017; Takeda, Matsumasa, Kikuchi,

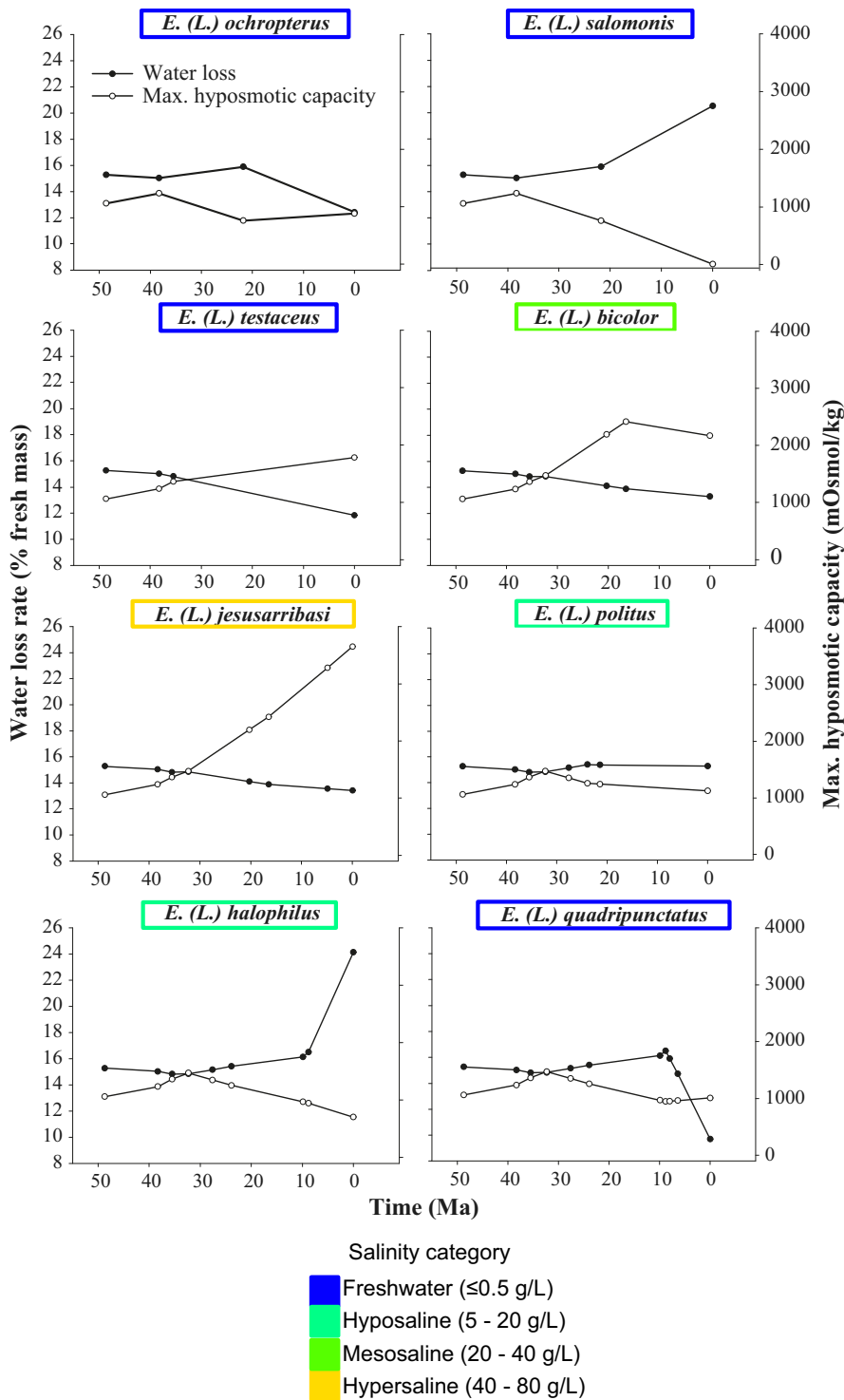


FIGURE 4 Values of water loss and maximum hyposmotic capacity through the full evolutionary path of the *Lumetis* species used in desiccation and osmoregulation experiments

Poovachiranon, & Murai, 1996). In the case of water beetles, selection on mechanisms such as those involved in ion transport, cell volume regulation or cuticle permeability for the control of water loss under desiccation might have resulted in enhanced hyporegulation ability.

Overall, our findings are consistent with an evolutionary sequence in which improved desiccation resistance in *Lumetis* 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 hyposmotic 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

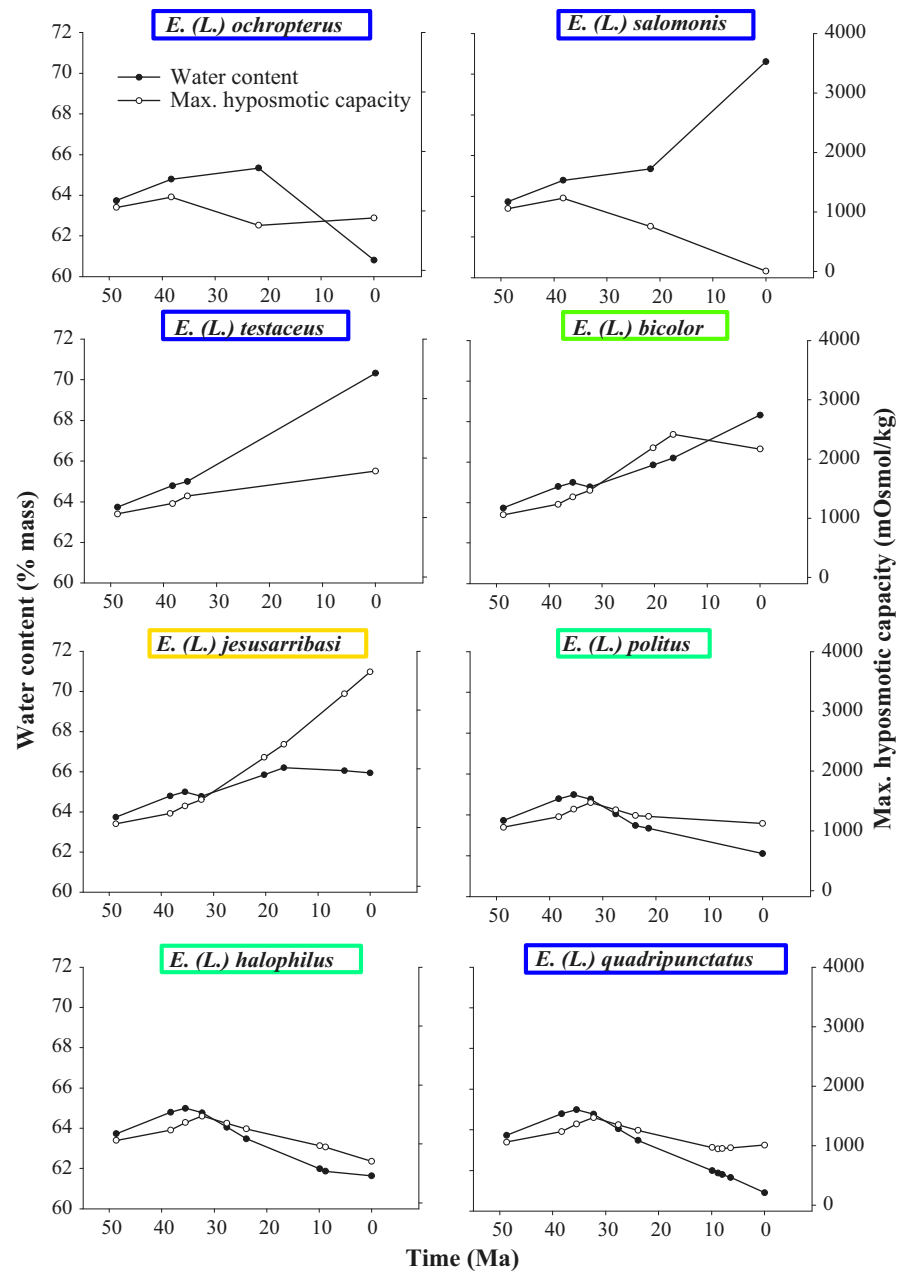


FIGURE 5 Values of water content and maximum osmotic capacity through the full evolutionary path of the *Lumetis* species used in desiccation and osmoregulation experiments

in hyporegulation (Bradley, 2009) is more likely to occur when such a mechanistic basis is already present (Barrett & Schluter, 2008; Roesti, Gavrillets, Hendry, Salzburger, & Berner, 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 (Finarelli & Goswami, 2013). This could underestimate the real interspecific variation of some traits in *Lumetis*. 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 hyposmotic 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., *Enochrus quadripunctatus* and *Enochrus falcarius*), for which no experimental data were available, may possess higher hyporegulation abilities than those inferred in our ancestral reconstructions. Such high

hyporegulation ability 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 hypothesized increase in desiccation resistance preceding any improvements in hyposmotic capacity. Rapid increases in hyposmotic capacity were associated with parallel weak decreases in water loss and increases in water content across the evolutionary path of the strongest hyporegulator species. Despite these parallel changes, a correlated evolution of both tolerances, constrained by identical genes and mechanisms (genetic correlation sensu Kellermann, Overgaard, Loeschcke, Kristensen, & Hoffmann, 2013;—i.e., our third hypothesis), is incompatible with the occurrence of species resistant to desiccation but with reduced hyporegulation ability, such as *E. ochropterus*. Nevertheless, further research identifying potential gene expression pathways related with either desiccation (e.g., Lopez-Martinez et al., 2009) or salinity stress (e.g., Uyhelji, Cheng, & Besansky, 2016), as well as those common to both stressors, would be needed to shed light on the degree of mechanistic overlap between desiccation and salinity tolerances.

Parallel increases in desiccation resistance and salinity tolerance could have been strengthened instead 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 ability in the *E. bicolor* group started to increase in parallel in the Late Eocene, a period of global aridification (Bosboom et al., 2014; Mosbrugger, Utescher, & Dilcher, 2005). Temporary habitats were presumably more abundant during such arid periods, which, together with an increase in the mineralization of the surface waters in some populations of these *Lumetus* species, could have posed a strong selective pressure on a further development of existing mechanisms to deal with saline stress and periodic exposure to desiccation. Other studies have proposed that global aridification events promoted diversification of several aquatic taxa (e.g., Dorn, Musilová, Platzer, Reichwald, & Cellerino, 2014; Pinceel et al., 2013). Aridification, by enhancing the linked tolerance of desiccation and salinity, could have also been a key driver in the diversification of *Lumetus*.

Euryhalinity is also an important source of evolutionary diversity (Brauner, Gonzales, & Wilson, 2013; Schultz & McCormick, 2012). 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, Weider, Colbourne, & Pfrender, 2012). In general, species of *Lumetus* (and other beetle genera) typical of hypersaline waters are almost absent from freshwater habitats, despite been able to hyper-regulate (Céspedes et al., 2013; Pallarés et al., 2015; Tones, 1977)—although *E. bicolor* is regularly found in low mineralized waters in northern localities of Europe. Such a situation also holds for saline Hemiptera (corixids, Tones & Hammer, 1975), coastal and estuarine decapods (Faria et al., 2017; McNamara & Faria, 2012) and fish (Schultz & McCormick, 2012). The

maintenance of hyper-regulation ability despite the apparent loss of its ecological role may reflect positive pleiotropies or functional correlations between hypo- and hyper-regulatory mechanisms (e.g., Smith, VanEkeris, Okech, Harvey, & Linser, 2008; Smith, Raymond, Valenti, Smith, & Linser, 2010), but may also be just due to the low cost of maintaining functional osmoregulatory responses outside conditions commonly encountered in nature (Divino et al., 2016).

The fundamental salinity tolerance niche of some fresh-hyposaline species was also found to be much broader than their realized niches (e.g., in *E. testaceus*), something which supports the view that hyporegulation arose as a co-opted mechanism. The osmoregulatory physiology of water beetles is still poorly explored, so it is not known whether euryhalinity is common in freshwater species of other genera, but at least two dytiscid species of the genus *Nebrioporus* typical of freshwater habitats are unable to osmoregulate at salinities above their isosmotic point (Pallarés et al., 2015). The absence of species of *Lumetus* which 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, Palmer, Hills, Torpy, & Kefford, 2017).

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 the linked evolution of stress resistance traits could have been key for developing tolerance to extreme salinities.

ACKNOWLEDGEMENTS

We thank Anabela Cardoso, Marie Palmer and Almudena Gutiérrez for technical support. We also thank all the collectors listed in Tables S1 and S2 for providing specimens, Pedro Abellán and Adrián Villastrigo for helping with phylogenetic analyses and three anonymous referees for useful comments to the manuscript. This work was funded by the I+D+i project CGL2013-48950-C2-1&2-P (AEI/FEDER, UE), a predoctoral grant and a scholarship grant from the University of Murcia to S. Pallarés and two postdoctoral grants from the Royal Society UK (Newton International Program) and from the Spanish Ministry of Economy and Competitiveness (Juan de la Cierva Formación Program) to P. Arribas.

DATA ACCESSIBILITY

All sequences generated have been deposited in the EMBL database (accession numbers shown in Table S1). Sequence alignments are available via Dryad at <https://doi.org/10.5061/dryad.2j3c8>, and all data obtained in desiccation and osmoregulation experiments can be found in the Supporting Information.

AUTHOR CONTRIBUTIONS

All authors conceived the study. I.R., D.T.B., P.A., J.V. and A.M. helped in field collection of specimens. S.P. performed experiments. S.P., I.R. and P.A. analysed data. S.P. wrote the manuscript. All authors reviewed the manuscript.

ORCID

Susana Pallarés  <http://orcid.org/0000-0001-8677-7475>

REFERENCES

- Adams, D. C. (2013). Comparing evolutionary rates for different phenotypic traits on a phylogeny using likelihood. *Systematic Biology*, 62, 181–192.
- Albers, M. A., & Bradley, T. J. (2011). On the evolution of saline tolerance in the larvae of mosquitoes in the genus *Ochlerotatus*. *Physiological and Biochemical Zoology*, 84, 258–267.
- Andújar, C., Serrano, J., & Gómez-Zurita, J. (2012). Winding up the molecular clock in the genus *Carabus* (Coleoptera: Carabidae): Assessment of methodological decisions on rate and node age estimation. *BMC Evolutionary Biology*, 12, 40.
- Arlian, L., & Staiger, T. (1979). Water balance in the semiaquatic beetle, *Peltodytes muticus*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 62A, 1041–1047.
- Arribas, P., Andújar, C., Abellán, P., Velasco, J., Millán, A., & Ribera, I. (2014). Tempo and mode of the multiple origins of salinity tolerance in a water beetle lineage. *Molecular Ecology*, 23, 360–373.
- Arribas, P., Andújar, C., Sánchez-Fernández, D., Abellán, P., & Millán, A. (2013). Integrative taxonomy and conservation of cryptic beetles in the Mediterranean region (Hydrophilidae). *Zoologica Scripta*, 42, 182–200.
- Arribas, P., Velasco, J., Abellán, P., Sánchez-Fernández, D., Andújar, C., Calosi, P., ... Bilton, D. T. (2012). Dispersal ability rather than ecological tolerance drives differences in range size between lentic and lotic water beetles (Coleoptera: Hydrophilidae). *Journal of Biogeography*, 39, 984–994.
- Barrett, R. D., & Schluter, D. (2008). Adaptation from standing genetic variation. *Trends in Ecology and Evolution*, 23, 38–44.
- Barrieu, F., Marty-Mazars, D., Thomas, D., Chaumont, F., Charbonnier, M., & Marty, F. (1999). Desiccation and osmotic stress increase the abundance of mRNA of the tonoplast aquaporin BobTIP26-1 in cauliflower cells. *Planta*, 209, 77–86.
- Bernhard, D., Schmidt, C., Korte, A., Fritzsche, G., & Beutel, R. G. (2006). From terrestrial to aquatic habitats and back again – Molecular insights into the evolution and phylogeny of Hydrophiloidea (Coleoptera) using multigene analyses. *Zoologica Scripta*, 35, 597–606.
- Beyenbach, K. W. (2016). The plasticity of extracellular fluid homeostasis in insects. *Journal of Experimental Biology*, 219, 2596–2607.
- Boettiger, C., Coop, G., & Ralph, P. (2012). Is your phylogeny informative? Measuring the power of comparative methods. *Evolution*, 66, 2240–2251.
- Bosboom, R. E., Abels, H. A., Hoorn, C., van den Berg, B. C. J., Guo, Z., & Dupont-Nivet, G. (2014). Aridification in continental Asia after the Middle Eocene Climatic Optimum (MECO). *Earth and Planetary Science Letters*, 389, 34–42.
- Botella-Cruz, M., Villastrigo, A., Pallarés, S., López-Gallego, E., Millán, A., & Velasco, J. (2017). Cuticle hydrocarbons in saline aquatic beetles. *PeerJ*, 5, e3562.
- Bradley, T. J. (2009). *Animal osmoregulation*. New York, NY: Oxford University Press.
- Bradley, T. J., Briscoe, A. D., Brady, S. G., Contreras, H. L., Danforth, B. N., Dudley, R., ... Yanoviak, S. P. (2009). Episodes in insect evolution. *Integrative and Comparative Biology*, 49, 590–606.
- Brauner, C. J., Gonzales, R. J., & Wilson, J. M. (2013). Extreme environments; hypersaline, alkaline and ion poor waters. In S. D. McCormick, A. P. Farrell, & C. J. Brauner (Eds.), *Fish physiology*, vol. 32, *Euryhaline Fishes* (pp. 433–474). New York, NY: Elsevier.
- Bruggeman, J., Heringa, J., & Brandt, B. W. (2009). PHYLOPARS: Estimation of missing parameter values using phylogeny. *Nucleic Acids Research*, 37, 179–184.
- Calosi, P., Ugolini, A., & Morritt, D. (2005). Physiological responses to hyposmotic stress in the supralittoral amphipod *Talitrus saltator* (Crustacea: Amphipoda). *Comparative Biochemistry and Physiology A*, 142, 267–275.
- Carbonell, J. A., Millán, A., & Velasco, J. (2012). Concordance between realised and fundamental niches in three Iberian *Sigara* species (Hemiptera: Corixidae) along a gradient of salinity and anionic composition. *Freshwater Biology*, 57, 2580–2590.
- Cayuela, E., Muñoz-Mayor, A., Vicente-Agulló, F., Moyano, E., García-Abellán, J. O., Estañ, M. T., & Bolarín, M. C. (2007). Drought pretreatment increases the salinity resistance of tomato plants. *Journal of Plant Nutrition and Soil Science*, 170, 479–484.
- 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.
- Charmantier, G., Charmantier-Daures, M., & Aiken, D. E. (1984). Variation des capacités osmoregulatrices des larves et postlarves de *Homarus americanus* Milnes-Edwards, 1837 (Crustacea, Decapoda). *Comptes Rendus de l'Académie des Sciences*, 299, 863–866.
- Cheng, C. H. C., & Chen, L. (1999). Evolution of an antifreeze glycoprotein. *Nature*, 401, 463–464.
- Cohen, E. (2012). Roles of aquaporins in osmoregulation, desiccation and cold hardiness in insects. *Entomology, Ornithology and Herpetology*, 51, 1–17.
- Cooper, N., Thomas, G. H., Venditti, C., Meade, A., & Freckleton, R. P. (2016). A cautionary note on the use of Ornstein Uhlenbeck models in macroevolutionary studies. *Biological Journal of the Linnean Society*, 118, 64–77.
- Divino, J. N., Monette, M. Y., McCormick, S. D., Yancey, P. H., Flannery, K. G., Bell, M. A., ... Schultz, E. T. (2016). Osmoregulatory physiology and rapid evolution of salinity tolerance in threespine stickleback recently introduced to fresh water. *Evolutionary Ecology Research*, 17, 179–201.
- Dorn, A., Musilová, Z., Platzer, M., Reichwald, K., & Cellerino, A. (2014). The strange case of East African annual fishes: Aridification correlates with diversification for a savannah aquatic group? *BMC Evolutionary Biology*, 14, 210.
- Dow, J. A. T., & Davies, S. A. (2006). The Malpighian tubule: Rapid insights from post-genomic biology. *Journal of Insect Physiology*, 52, 365–378.
- Dowse, R., Palmer, C. G., Hills, K., Torpy, F., & Kefford, B. J. (2017). The mayfly nymph *Austrophlebioides pusillus* Harker defies common osmoregulatory assumptions. *Royal Society Open Science*, 4, 160520.
- Drummond, A. J., Suchard, M. A., Xie, D., & Rambaut, A. (2012). Bayesian phylogenetics with BEAUTI and the BEAST 1.7. *Molecular Biology and Evolution*, 29, 1969–1973.
- Dunson, W. A., & Travis, J. (1991). The role of abiotic factors in community organization. *The American Naturalist*, 138, 1067–1091.
- Elnitsky, M. A., Benoit, J. B., Lopez-Martinez, G., Denlinger, D. L., & Lee, R.E. Jr (2009). Osmoregulation and salinity tolerance in the Antarctic midge, *Belgica antarctica*: Seawater exposure confers enhanced tolerance to freezing and dehydration. *Journal of Experimental Biology*, 212, 2864–2871.
- Evans, D. H. (2008). *Osmotic and ionic regulation: Cells and animals*. Boca Raton, FL: CRC Press.

- Faria, S. C., Augusto, A. S., & McNamara, J. C. (2011). Intra- and extracellular osmotic regulation in the hololimnetic *Caridea* and *Anomura*: A phylogenetic perspective on the conquest of fresh water by the decapod Crustacea. *Journal of Comparative Physiology B*, 181, 175–186.
- Faria, S. C., Provete, D. B., Thurman, C. L., & McNamara, J. C. (2017). Phylogenetic patterns and the adaptive evolution of osmoregulation in fiddler crabs (*Brachyura*, *Uca*). *PLoS ONE*, 12, e0171870.
- Finarelli, J. A., & Goswami, A. (2013). Potential pitfalls of reconstructing deep time evolutionary history with only extant data, a case study using the Canidae (Mammalia, Carnivora). *Evolution*, 67, 3678–3685.
- Freckleton, R. P., Harvey, P. H., & Pagel, M. (2002). Phylogenetic analysis and comparative data: A test and review of evidence. *The American Naturalist*, 160, 712–726.
- Gibbs, A. G., & Rajpurohit, S. (2010). Cuticular lipids and water balance. In G. J. Blomquist, & A. G. Bagnères (Eds.), *Insect hydrocarbons: Biology, biochemistry, and chemical ecology* (pp. 100–120). Cambridge: Cambridge University Press.
- Gómez-Mestre, I., & Tejedo, M. (2005). Adaptation or exaptation? An experimental test of hypotheses on the origin of salinity tolerance in *Bufo calamita*. *Journal of Evolutionary Biology*, 18, 847–855.
- Goolsby, E. W., Bruggeman, J., & Ane, C. (2017). RPHYLOPARS: Fast multivariate phylogenetic comparative methods for missing data and within-species variation. *Methods in Ecology and Evolution*, 8, 22–27.
- Griffith, M. B. (2017). Toxicological perspective on the osmoregulation and ionoregulation physiology of major ions by freshwater animals: Teleost fish, crustacea, aquatic insects, and mollusca. *Environmental Toxicology and Chemistry*, 36, 576–600.
- Hadley, N. F. (1994). *Water relations of terrestrial arthropods*. San Diego, CA: Academic Press.
- Heard, S. B., & Hauser, D. L. (1995). Key evolutionary innovations and their ecological mechanisms. *Historical Biology*, 10, 151–173.
- Herbst, D. B. (1999). Biogeography and physiological adaptations of the brine fly genus *Ephydra* (Diptera: Ephydriidae) in saline waters of the Great Basin. *Great Basin Naturalist*, 59, 127–135.
- Herbst, D. B. (2001). Gradients of salinity stress, environmental stability and water chemistry as a template for defining habitat types and physiological strategies in inland salt waters. *Hydrobiologia*, 466, 209–219.
- Herbst, D. B., Conte, F. P., & Brookes, V. J. (1988). Osmoregulation in an alkaline salt lake insect, *Ephydra* (Hydropyridae) hians Say (Diptera: Ephydriidae) in relation to water chemistry. *Journal of Insect Physiology*, 34, 903–909.
- Hossain, M. A., Mostofa, M. G., & Fujita, M. (2013). Cross protection by cold-shock to salinity and drought stress-induced oxidative stress in mustard (*Brassica campestris* L.) seedlings. *Molecular Plant Breeding*, 4, 50–70.
- Hunter, J. P. (1998). Key innovations and the ecology of macroevolution. *Trends in Ecology and Evolution*, 13, 31–36.
- Kaliontzopoulou, A., & Adams, D. C. (2016). Phylogenies, the comparative method, and the conflation of tempo and mode. *Systematic Biology*, 65, 1–15.
- Katoh, K., & Toh, H. (2008). Recent developments in the MAFFT multiple sequence alignment program. *Briefings in Bioinformatics*, 9, 286–298.
- Kellermann, V., Overgaard, J., Loeschcke, V., Kristensen, T. N., & Hoffmann, A. A. (2013). Trait associations across evolutionary time within a *Drosophila* phylogeny: Correlated selection or genetic constraint? *PLoS ONE*, 8, e72072.
- Landfear, R., Calcott, B., Ho, S. Y., & Guindon, S. (2012). PARTITION FINDER: Combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*, 29, 1695–1701.
- Larsen, E. H., Deaton, L. E., Onken, H., O'Donnell, M., Grosell, M., Dantzer, W. H., & Weihrauch, D. (2014). Osmoregulation and excretion. *Comprehensive Physiology*, 4, 405–573.
- Latta, L. C., Weider, L. J., Colbourne, J. K., & Pfrender, M. E. (2012). The evolution of salinity tolerance in *Daphnia*: A functional genomics approach. *Ecology Letters*, 15, 794–802.
- Lee, C. E., Kiergaard, M. G., Gelembiuk, W., Eads, B. D., & Posavi, M. (2011). Pumping ions: Rapid parallel evolution of ionic regulation following habitat invasions. *Evolution*, 65, 2229–2244.
- Lopez-Martinez, G., Benoit, J. B., Rinehart, J. P., Elnitsky, M. A., Lee, R. E. Jr., & Denlinger, D. L. (2009). Dehydration, rehydration, and overhydration alter patterns of gene expression in the Antarctic midge, *Belgica antarctica*. *Journal of Comparative Physiology B*, 179, 481–491.
- McNamara, J., & Faria, S. (2012). Evolution of osmoregulatory patterns and gill ion transport mechanisms in the decapod Crustacea: A review. *Journal of Comparative Physiology B*, 182, 997–1014.
- Miller, M. A., Pfeiffer, W., & Schwartz, T. (2010). Creating the CIPRES Science Gateway for inference of large phylogenetic trees. In *Proceedings of the Gateway Computing Environments Workshop (GCE)*, 14 November 2010 (pp. 1–8). New Orleans, LA.
- Moczek, A. P. (2008). On the origins of novelty in development and evolution. *BioEssays*, 30, 432–447.
- Mosbrugger, V., Utescher, T., & Dilcher, D. L. (2005). Cenozoic continental climatic evolution of Central Europe. *Proceedings of the National Academy of Sciences of the United States of America*, 102, 14964–14969.
- Pagel, M. (1999). Inferring the historical patterns of biological evolution. *Nature*, 401, 877–884.
- 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.
- 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*, 220, 1277–1286.
- 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.
- Papadopoulou, A., Anastasiou, I., & Vogler, A. P. (2010). Revisiting the insect mitochondrial molecular clock: The mid-Aegean trench calibration. *Molecular Biology and Evolution*, 27, 1659–1672.
- Penone, C., Davidson, A. D., Shoemaker, K. T., Di Marco, M., Rondinini, C., Brooks, T. M., ... Costa, G. C. (2014). Imputation of missing data in life-history datasets: Which approach performs the best? *Methods in Ecology and Evolution*, 5, 961–970.
- Pinceel, T., Brendonck, L., Larmuseau, M. H. D., Vanhove, M. P. M., Timms, B. V., & Vanschoenwinkel, B. (2013). Environmental change as a driver of diversification in temporary aquatic habitats: Does the genetic structure of extant fairy shrimp populations reflect historic aridification? *Freshwater Biology*, 58, 1556–1572.
- Pollux, B. J. A., Meredith, R. W., Springer, M. S., Garland, T., & Reznick, D. N. (2014). The evolution of the placenta drives a shift in sexual selection in live bearing fish. *Nature*, 513, 233–236.
- Revell, L. J. (2012). PHYTOOLS: An R package for phylogenetic comparative biology (and other things). *Methods in Ecology and Evolution*, 3, 217–223.
- Ribera, I., Fresneda, J., Bucur, R., Izquierdo, A., Vogler, A. P., Salgado, J. M., & Cieslak, A. (2010). Ancient origin of a Western Mediterranean radiation of subterranean beetles. *BMC Evolutionary Biology*, 10, 29.
- Roesti, M., Gavrillets, S., Hendry, A. P., Salzburger, W., & Berner, D. (2014). The genomic signature of parallel adaptation from shared genetic variation. *Molecular Ecology*, 23, 3944–3956.
- Schultz, E. T., & McCormick, S. D. (2012). Euryhalinity in an evolutionary context. *Fish Physiology*, 32, 477–533.
- Short, A. E. Z., & Fikáček, M. (2013). Molecular phylogeny, evolution and classification of the Hydrophilidae (Coleoptera). *Systematic Entomology*, 38, 723–752.

- Sinclair, B. J., Ferguson, L. V., Salehipour-shirazi, G., & MacMillan, H. A. (2013). Cross-tolerance and cross-talk in the cold: Relating low temperatures to desiccation and immune stress in insects. *Integrative and Comparative Biology*, 53, 545–556.
- Smith, K. E., Raymond, S. L., Valenti, M. L., Smith, P. J. S., & Linser, P. J. (2010). Physiological and pharmacological characterizations of the larval *Anopheles albimanus* rectum support a change in protein distribution and/or function in varying salinities. *Comparative Biochemistry and Physiology A*, 157, 55–62.
- Smith, K. E., VanEkeris, L. A., Okech, B. A., Harvey, W. R., & Linser, P. J. (2008). Larval anopheline mosquito recta exhibit a dramatic change in localization patterns of ion transport proteins in response to shifting salinity: A comparison between anopheline and culine larvae. *Journal of Experimental Biology*, 211, 3067–3076.
- Takeda, S., Matsumasa, M., Kikuchi, S., Poovachiranon, S., & Murai, M. (1996). Variation in the branchial formula of semiterrestrial crabs (Decapoda: Brachyura: Grapsidae and Ocypodidae) in relation to physiological adaptations to the environment. *Journal of Crustacean Biology*, 16, 472–486.
- Tobler, M., & Plath, M. (2011). Living in extreme habitats. In J. Evans, A. Pilastro, & I. Schlupp (Eds.), *Ecology and evolution of Poeciliid fishes* (pp. 120–127). Chicago, IL: University of Chicago Press.
- Todgham, A. E., & Stillman, J. H. (2013). Physiological responses to shifts in multiple environmental stressors: Relevance in a changing world. *Integrative and Comparative Biology*, 53, 539–544.
- Tones, P. I. (1977). Osmoregulation in adults and larvae of *Hygrotus salinarius* Wallis (Coleoptera, Dytiscidae). *Comparative Biochemistry and Physiology A*, 60, 247–250.
- Tones, P. I., & Hammer, U. T. (1975). Osmoregulation in *Trichocorixa verticalis interiores* Sailer (Hemiptera, Corixidae) – An inhabitant of Saskatchewan saline lakes, Canada. *Canadian Journal of Zoology*, 53, 1207–1212.
- Uyhelji, H. A., Cheng, C., & Besansky, N. J. (2016). Transcriptomic differences between euryhaline and stenohaline malaria vector sibling species in response to salinity stress. *Molecular Ecology*, 25, 2210–2225.

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Pallarés S, Arribas P, Bilton DT, Millán A, Velasco J, Ribera I. The chicken or the egg? Adaptation to desiccation and salinity tolerance in a lineage of water beetles. *Mol Ecol*. 2017;00:1–15. <https://doi.org/10.1111/mec.14334>

Supporting information

The chicken or the egg? Adaptation to desiccation and salinity tolerance in a lineage of water beetles

S. PALLARÉS¹, P. ARRIBAS², D. T. BILTON³, A. MILLÁN¹, J. VELASCO¹, I. RIBERA⁴

¹ Department of Ecology and Hydrology, University of Murcia, Facultad de Biología, Campus Espinardo, Murcia, 30100, Spain

² Island Ecology and Evolution Research Group, IPNA-CSIC, Avda. Astrofísico Francisco Sánchez, 3, San Cristóbal de La Laguna, 38206, Santa Cruz de Tenerife, Spain

³ Marine Biology and Ecology Research Centre, School of Biological and Marine Sciences, Plymouth University, Drake Circus, Plymouth, PL4 8AA, United Kingdom

⁴ Institute of Evolutionary Biology (CSIC-Universitat Pompeu Fabra), Passeig de la Barceloneta 37, Barcelona, 08003, Spain

Supporting information for this paper consists on seven tables (Tables S1-S7), five figures (Figures S1-S5) and supporting data (Data S1), all of them contained in this pdf.

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

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

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

ENObico_SPA_HUV_PINA_AB287	<i>Enochrus (Lumetus) bicolor</i>	Spain, Huelva, Marismas del Pinar	30-01-10	Andújar & Arribas Leg	IBE-AB287
ENObico_SPA_JAE_BRUJ_AB59	<i>Enochrus (Lumetus) bicolor</i>	Spain, Jaén, Brujuelo, Arroyo en las Salinas de Brujuelo	27-07-07	Millán et col. Leg	IBE-AB59
ENObico_SPA_MUR_REVE_AB58	<i>Enochrus (Lumetus) bicolor</i>	Spain, Murcia, Rambla del Reventón	18-09-07	Millán et col. Leg	IBE-AB58
ENObico_SPA_NAV_MEND_AB7	<i>Enochrus (Lumetus) bicolor</i>	Spain, Navarra, Barranco Salado de Mendavia	29-07-07	Millán et col. Leg	IBE-AB7
ENObico_SPA_NAV_YUGO_AB176	<i>Enochrus (Lumetus) bicolor</i>	Spain, Navarra, El Yugo, Bárdenas Blancas	21-07-04	Ribera & Cieslak Leg	IBE-AB176
ENObico_SPA_TAR_GERR_AB170	<i>Enochrus (Lumetus) bicolor</i>	Spain, Lérida, Gerry de la Sal, Arroyo en Gerry de la Sal	27-09-08	Abellán Leg	IBE-AB170
ENObico_SPA_ZAR_MAGA_SP28	<i>Enochrus (Lumetus) bicolor</i>	Spain, Magallón, Lagunas de Magallón	2014	Ribera & Cieslak Leg	IBE-SP28
ENObico_SPA_ZAR_SMAR_AB43	<i>Enochrus (Lumetus) bicolor</i>	Spain, Zaragoza, San Marcos, Chiprana pond	20-07-04	Ribera & Cieslak Leg	IBE-AB43
ENObiaz_MOR_JOR_MGHA_AB246	<i>Enochrus (Lumetus) blazquezae</i>	Morocco, Jorf El Melha, Sidi Kacem, Oued Mghassem	18-04-06	Millán et col. Leg	IBE-AB246
ENObiaz_MOR_TIS_TISA_AB247	<i>Enochrus (Lumetus) blazquezae</i>	Morocco, Tissa, Taounate, Salines de Tissa	19-04-06	Millán et col. Leg	IBE-AB247
ENOcoar_ENG_NOR_BROA_AB312	<i>Enochrus (Methydus) coarctatus</i>	England, Norfolk, The Broads, Catfield Fen 4	07-05-06	Ribera Leg	IBE-AB312
ENOcoar_ENG_NOR_HOWH2_AB37	<i>Enochrus (Methydus) coarctatus</i>	England, Norfolk, Lufham, How Hill Marsh 2	06-05-06	Ribera Leg	IBE-AB37
ENOcoar_IRE_CLA_RINE_AB305	<i>Enochrus (Methydus) coarctatus</i>	Ireland, Clare, Rinecaha, Rinecaha fen	22-05-10	Ribera Leg	IBE-AB305
ENOcoar_ITA_BRE_SANA_AB236	<i>Enochrus (Methydus) coarctatus</i>	Italy, Brescia, Santa Anna, Santa Anna pond	17-10-02	Ribera, Cieslak, Toledo & Mazzoldi Leg	IBE-AB236
ENOdif USA_CAL_AMAR_AN378	<i>Enochrus (Lumetus) diffusus</i>	California, Amargosa River in Tecopa	2008	Abellán Leg	IBE-AN378
ENOdif USA_CAL_BALD_AN372	<i>Enochrus (Lumetus) diffusus</i>	California, Baldwin Lake	2008	Abellán Leg	IBE-AN372
ENOdif USA_CAL_SODA_AB49	<i>Enochrus (Lumetus) diffusus</i>	United States, California, Baker, Soda lake	07-04-08	Abellán Leg	IBE-AB49
ENOfalc_ITA_SIC_SALS_AB224	<i>Enochrus (Lumetus) falcarius</i>	Italy, Sicily, Villadoro, Afluente mesosalino del Salso	27-07-09	Gutiérrez-Cánovas Leg	IBE-AB224
ENOfalc_ITA_SIC_TURV_AB223	<i>Enochrus (Lumetus) falcarius</i>	Italy, Sicily, Cianciana, Fiume Tùrvoli	26-07-09	Gutiérrez-Cánovas Leg	IBE-AB223
ENOfalc_ITA_SIC_VACC_AB23	<i>Enochrus (Lumetus) falcarius</i>	Italy, Sicily, Caltanissetta, Torrente Vaccarizzo, Castello	12-06-07	Abellán & Picazo Leg	IBE-AB23
ENOhalo_ENG_SHE_NHM3	<i>Enochrus (Lumetus) halophilus</i>	England, Sheppey Island	2016	Arribas & Andújar Leg	NHM-3
ENOhalo_MOR_AZR_AFEN_AB323	<i>Enochrus (Lumetus) halophilus</i>	Morocco, Azrou, Afenourir, Lac Afenourir	29-04-00	Ribera, Aguilar, Hernando & Millán Leg	IBE-AB323

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

		Salinas de Brujuelo			
ENOjesu_SPA_JAE_PORC_AB387	<i>Enochrus (Lumetus) jesuarribasi</i>	Spain, Jaén, Porcuna, Arroyo en las Salinas de Porcuna	28-07-98	Ribera Leg	IBE-AB387
ENOjesu_SPA_JAE_SILE_AB222	<i>Enochrus (Lumetus) jesuarribasi</i>	Spain, Jaén, Siles, Arroyo Salado en Siles	24-10-09	Andújar & Arribas Leg	IBE-AB222
ENOjesu_SPA_MUR_AMAR_AB457	<i>Enochrus (Lumetus) jesuarribasi</i>	Spain, Murcia, Rambla de Agua Amarga	27-11-10	Andújar & Arribas Leg	IBE-AB457
ENOjesu_SPA_MUR_CHIC_AB386	<i>Enochrus (Lumetus) jesuarribasi</i>	Spain, Murcia, Abanilla, Río Chicamo	21-09-07	Millán et col. Leg	IBE-AB386
ENOjesu_SPA_MUR_REVE_AB463	<i>Enochrus (Lumetus) jesuarribasi</i>	Spain, Murcia, Rambla del Reventón	27-11-10	Andújar & Arribas Leg	IBE-AB463
ENOjesu_SPA_MUR_RSAL_AB79	<i>Enochrus (Lumetus) jesuarribasi</i>	Spain, Murcia, Fortuna, Rambla Salada en las Salinas	21-09-07	Millán et col. Leg	IBE-AB79
ENOjesu_SPA_MUR_SANG_AB9	<i>Enochrus (Lumetus) jesuarribasi</i>	Spain, Murcia, Sangonera, Arroyo de las Salinas de Sangonera	22-01-08	Millán et col. Leg	IBE-AB9
ENOjesu_SPA_SEV_MONT_AB82	<i>Enochrus (Lumetus) jesuarribasi</i>	Spain, Sevilla, Montellano, Arroyo Montero	21-07-06	Millán et col. Leg	IBE-AB82
ENOjesu_SPA_SEV_PINT_AB393	<i>Enochrus (Lumetus) jesuarribasi</i>	Spain, Sevilla, Osuna, Arroyo El Pintado	27-07-98	Ribera Leg	IBE-AB393
ENOmela_ENG_SHE_NHM1	<i>Enochrus (Enochrus) melanocephalus</i>	England, Sheppey Island	2016	Arribas & Andújar Leg	NHM-1
ENOmela_ENG_SHE_NHM2	<i>Enochrus (Enochrus) melanocephalus</i>	England, Sheppey Island	2016	Arribas & Andújar Leg	NHM-2
ENOmores_POR_ALG_CABE_AB317	<i>Enochrus (Methydrus) morenae</i>	Portugal, Algarbe, Cabezo Gordo	01-05-09	Andújar, Arribas & Sánchez-Gea Leg	IBE-AB317
ENOmores_SPA_CAC_VID_AB62	<i>Enochrus (Methydrus) morenae</i>	Spain, Cáceres, Arroyo de la Vid, Arroyo de la Vid	14-05-05	Ribera Leg	IBE-AB62
ENOmores_SPA_CAC_VISE_AB318	<i>Enochrus (Methydrus) morenae</i>	Spain, Cáceres, Villarreal, PN Monfragüe, Villarreal y Serradilla	2009	Ribera & Abellán Leg	IBE-AB318
ENOnata_MOR_GHA_KHOU_AB308	<i>Enochrus (Methydrus) natalensis</i>	Morocco, Gharviya, Oued Khoulj	21-04-06	Millán et col. Leg	IBE-AB308
ENOnata_MOR_KENI_DICH_AB60	<i>Enochrus (Methydrus) natalensis</i>	Morocco, Kenitra, ditch Kenitra	04-04-02	Millán et col. Leg	IBE-AB60
ENOnata_MOR_MOU_DRAD_AB3	<i>Enochrus (Methydrus) natalensis</i>	Morocco, Moulay, Bousselahm, Oued Drader	12-04-07	Ribera, Aguilar, Hernando Leg	IBE-AB3
ENOnigr_ITA_SIC_UBRI_AB314	<i>Enochrus (Methydrus) nigrinus</i>	Italy, Sicily, Parco dei Nebrodi, Lago Ubrio Quattrocchi	12-06-07	Abellán & Picazo Leg	IBE-AB314
ENOnigr_SPA_MAD_PENA_AB44	<i>Enochrus (Methydrus) nigrinus</i>	Spain, Madrid, Rascafría, Laguna Grande, PN Peñalara	02-06-07	Ribera & Hidalgo Leg	IBE-AB44
ENOochr_BEL_LUX_REGN_RA995	<i>Enochrus (Lumetus) ochropterus</i>	Belgium, Luxembourg, nr Regné	31-07-12	Foster Leg	IBE-RA995
ENOochr_SWE_OLA_MOK_RA817	<i>Enochrus (Lumetus) ochropterus</i>	Sweden, Öland, Möckelmossen, ponds in limestone	22-05-11	Ribera Leg	IBE-RA817
ENOpoli_ITA_SIC_MAND_AB27	<i>Enochrus (Lumetus) politus</i>	Italy, Sicily, Nicosia, Villadoro, Torrente Mandre	12-06-07	Abellán & Picazo Leg	IBE-AB27

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

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

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

ENOquad_TUR_SIN_YESI_AB163	<i>Enochrus (Lumetus) quadripunctatus</i> cplx	Turkey, Sinop, Sarayduzu, Boyabat, Yesilyurt stream	27-04-06	Ribera, Cieslak, Aguilar, Hernando Leg	IBE-AB163
ENOquan_OMA_MUQ_LOTI_RA490	<i>Enochrus (Lumetus) quadrinotatus</i> cf	Oman, Wadi Muqshin	2011	Carranza Leg	IBE-RA490
ENOquan_OMA_MUQ_LOTI_RA553	<i>Enochrus (Lumetus) quadrinotatus</i> cf	Oman, Wadi Muqshin	2011	Carranza Leg	IBE-RA553
ENOrisi_MOR_AGUI_AB280	<i>Enochrus (Lumetus) risii</i>	Morocco, Oued El-Aguig	03-04-07	Millán et col. Leg	IBE-AB280
ENOrisi_MOR_AOUD_AB229	<i>Enochrus (Lumetus) risii</i>	Morocco, Oued Aoudrei	05-04-07	Millán et col. Leg	IBE-AB229
ENOrisi_MOR_KHN_KHAN_AB248	<i>Enochrus (Lumetus) risii</i>	Morocco, Khniffis, Oued Khanoi Naâm	04-04-07	Millán et col. Leg	IBE-AB248
ENOrisi_TUN_TOZ_MELA_AB40	<i>Enochrus (Lumetus) risii</i>	Tunisia, Tozeur, Gafsa, Oued El Melah	26-10-01	Ribera & Cieslak Leg	IBE-AB40
ENOsalo_SPA_ALB_PET_NHM8	<i>Enochrus (Lumetus) salomonis</i>	Spain, Albacete, Pétrola, Laguna de Pétrola	2016	Arribas & Andújar Leg	NHM-8
ENOsalo_SPA_ALB_PET_NHM9	<i>Enochrus (Lumetus) salomonis</i>	Spain, Albacete, Pétrola, Laguna de Pétrola	2016	Arribas & Andújar Leg	NHM-9
ENOsalo_SPA_NAV_BARD_AB240	<i>Enochrus (Lumetus) salomonis</i>	Spain, Navarra, Bárdenas Reales, Arroyo Salado en las Bárdenas Reales	18-10-09	Andújar & Arribas Leg	IBE-AB240
ENOsalo_SPA_ZAR_MAGA_SP27	<i>Enochrus (Lumetus) salomonis</i>	Spain, Zaragoza, Magallón, Lagunas de Magallón	2014	Ribera & Cieslak Leg	IBE-SP27
ENOsegm_FRA_MON_PCAM_AB288	<i>Enochrus (Lumetus) segmentinotatus</i>	France, Montpellier, Mauguio, Petit Camargua. Etang d'Or	11-10-09	Andújar & Arribas Leg	IBE-AB288
ENOsegm_FRA_SIG_PALM_AB289	<i>Enochrus (Lumetus) segmentinotatus</i>	France, Sigean, Salin de Lapalme	16-11-09	Andújar & Arribas Leg	IBE-AB289
ENOsegm_MOR_BRI_AZIL_AB274	<i>Enochrus (Lumetus) segmentinotatus</i>	Morocco, Brieche, Azilah, Salines Azilah	21-04-06	Millán et col. Leg	IBE-AB274
ENOsegm_SPA_BAL_POLL_AB162	<i>Enochrus (Lumetus) segmentinotatus</i>	Spain, Baleares, Mallorca, Port de Pollensa pozas	11-11-00	Ribera & Cieslak Leg	IBE-AB162
ENOsegm_SPA_CAD_SMAR_AB231	<i>Enochrus (Lumetus) segmentinotatus</i>	Spain, Cádiz, Puerto de Santa María, Salinas de Santa María	30-01-10	Andújar & Arribas Leg	IBE-AB231
ENOsP_ARG_BAI_LORO_AB326	<i>Enochrus (Hugoscottia) sp</i>	Argentina, Buenos Aires, Sierra de la Ventana, Arroyo el Loro	14-12-00	Villalobos Leg	IBE-AB326
ENOsP_CAN_ALB_WATE2_AB87	<i>Enochrus (Methydus) sp</i>	Canada, Alberta, Waterton lakes park limit	28-06-00	Ribera & Cieslak Leg	IBE-AB87
ENOsP_CHI_CHI_CUCA_AB88	<i>Enochrus (Hugoscottia) sp</i>	Chile, Chiloé, Chiloé, Laguna de Cucao	03-11-99	Ribera & Guerrero Leg	IBE-AB88
ENOsP_CHI_CHI_TRIN_AB89	<i>Enochrus (Hugoscottia) sp</i>	Chile, Chiloé, Chiloé, Arroyo en Quellón Trincao	05-11-99	Ribera & Guerrero Leg	IBE-AB89
ENOsP_SAF_CAP_FRAN_AB310	<i>Enochrus (Methydus) sp</i>	South Africa, Cape, Franschhoek, Franschhoek river	26-03-01	Ribera & Cieslak Leg	IBE-AB310
ENOsP_SAF_CAP_HOUT_AB85	<i>Enochrus (Enochrus) sp</i>	South Africa, Cape, Hout Bay, Hout river	19-03-01	Ribera & Cieslak Leg	IBE-AB85
ENOsP_SAF_CAPE_HARO_AB84	<i>Enochrus (Methydus) sp</i>	South Africa, Cape, Pringle Bay, Harold Porter pond	21-03-01	Ribera & Cieslak Leg	IBE-AB84
ENOsP_USA_CAL_COAL_AB55	<i>Enochrus (Methydus) sp</i>	United States, California, Coal Oil Point Reserve	26-03-08	Abellán Leg	IBE-AB55

ENOfest_AUS_VOR_RHEI_AB306	<i>Enochrus (Lumetus) testaceus</i>	Austria, Voralberg, Rheintal, Bodensee, Hard Rheinvorland	02-07-01	Ribera Leg	IBE-AB306
ENOfest_AZE_QOB_SP36	<i>Enochrus (Lumetus) testaceus</i>	Azerbaijan, Qobustan, ponds in beach	2014	Ribera & Rudoy	IBE-SP36
ENOfest_ENG_NOR_BROA_AB36	<i>Enochrus (Lumetus) testaceus</i>	England, Norfolk, The Broads, Catfield Fen 4	07-05-06	Ribera Leg	IBE-AB36
ENOfest_ENG_SHE_NHM7	<i>Enochrus (Lumetus) testaceus</i>	England, Sheppey Island	2016	Arribas & Andújar Leg	NHM-7
ENOfest_IRE_CLA_RINE_AB304	<i>Enochrus (Lumetus) testaceus</i>	Ireland, Clare, Rinecaha, Rinecaha fen	22-05-10	Ribera Leg	IBE-AB304
ENOfest_POL_ZAC_SP42	<i>Enochrus (Lumetus) testaceus</i>	Poland, Zachodniopomorsky, Dygowo, garden pond	16-08-04	Ribera Leg	IBE-SP42
ENOfest_SLO_HAM_SP21	<i>Enochrus (Lumetus) testaceus</i>	Slovakia, Hámske trstie, ditch near Čičov	07-06-09	Ribera Leg	IBE-SP21
ENOfest_SPA_GER_CAMP_AB45	<i>Enochrus (Lumetus) testaceus</i>	Spain, Gerona, Capmany, Estany inferior	09-05-98	Ribera & Foster Leg	IBE-AB45
ENOfura_CYP_ORO_AN458	<i>Enochrus (Lumetus) turanicus cf</i>	Cyprus, Oroklini, saline wetland	25-04-16	Millán, Ribera, Velasco & Villastrigo Leg	IBE-AN458
ENOfura_GRE_ARK_MOUS_SP4	<i>Enochrus (Lumetus) turanicus cf</i>	Greece, Arkadia, Astros, lake Moustou and nearby wetland	2013	Ribera & Cieslak Leg	IBE-SP4
ENOfura_GRE_ATT_ARTE_SP6	<i>Enochrus (Lumetus) turanicus cf</i>	Greece, Attika, Artemida, ditch in beach next to wetland	2013	Ribera & Cieslak Leg	IBE-SP6
ENOfura_ISR_ATL_COAS_RA194	<i>Enochrus (Lumetus) turanicus cf</i>	Israel, Atlit	29-07-10	Rudoy Leg	IBE-RA194
ENOfura_TUR_BAL_KUCU_SP15	<i>Enochrus (Lumetus) turanicus cf</i>	Turkey, Balikesir prov.. Küçükköy, salty marsh	22-07-14	Ribera & Cieslak	IBE-SP15
ENOfura_TUR_BAL_SARI_SP16	<i>Enochrus (Lumetus) turanicus cf</i>	Turkey, Balikesir prov., Sarimsakli, salty ditch	23-07-14	Ribera & Cieslak	IBE-SP16
ENOfura_TUR_CAN_SP14	<i>Enochrus (Lumetus) turanicus cf</i>	Turkey, Çanakkale prov., Dalyan, salty stream	21-07-14	Ribera & Cieslak	IBE-SP14
ENOfura_TUR_IZM_GEDI_SP17	<i>Enochrus (Lumetus) turanicus cf</i>	Turkey, Izmir prov., Gediz Nehri Delta, saline pond	24-07-14	Ribera & Cieslak	IBE-SP17
ENOfura_TUR_IZM_KUCU_SP19	<i>Enochrus (Lumetus) turanicus cf</i>	Turkey, Izmir prov., Selçuk, delta Küçük menderes	25-07-14	Ribera & Cieslak	IBE-SP19
HEL_MOR_AZR_AFEN_SP40	<i>Helochares sp</i>	Morocco, Azrou, Lac Afenourir			IBE-SP40
HELLivi_SPA_ZAR_MAGD_AB83	<i>Helochares lividus</i>	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	<i>Hydrobius convexus</i>	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	Conductivity (mS cm ⁻¹)	Collector(s)
<i>E. (M.) coarctatus</i> (Gredler 1863)	Carrick ponds, Kirkcudbright, Scotland, U.K.	0.5	D.T. Bilton & G.N. Foster; G. Robinson & S. Routledge
<i>E. (L.) salomonis</i> (J. Sahlberg, 1900)	Pétrola ponds, Pétrola, Albacete, Spain	2.6	A. Millán, J. Velasco & S. Pallarés
<i>E. (L.) ochropterus</i> (Marshall 1802)	Carrick ponds, Kirkcudbright, Scotland, U.K.	0.5	D.T. Bilton & G.N. Foster; G. Robinson & S. Routledge
<i>E. (L.) politus</i> (Küster, 1849)	Chícamo stream, Abanilla, Murcia, Spain	14	A. Millán, J. Velasco & S. Pallarés
<i>E. (L.) quadripunctatus</i> (Herbst, 1797)	Drakeland Corner, Plymouth (Devon), UK	0.5	D.T. Bilton
	Knockewart moss, Ayrshire, Scotland, UK.	0.5	G.N. Foster
<i>E. (L.) testaceus</i> (Fabricius, 1801)	Carrick ponds, Kirkcudbright, Scotland, U.K.	0.5	D.T. Bilton & G.N. Foster; G. Robinson & S. Routledge
<i>E. (L.) halophilus</i> (Bedel, 1878)	Pétrola ponds, Pétrola, Albacete, Spain	2.6	A. Millán, J. Velasco & S. Pallarés
<i>E. (L.) bicolor</i> (Fabricius, 1792)	Mojón Blanco pond, Albacete, Spain	39	A. Millán, J. Velasco & S. Pallarés
<i>E. (L.) jesuarribasi</i> Arribas y Millán, 2013	Rambla Salada stream, Fortuna, Murcia, Spain	58	A. Millán, J. Velasco & S. Pallarés

Table S3. Primers used for PCR and sequencing.

Marker	Primer		Sequence (5'–3')	Reference
<i>cox1-A</i>	Jerry	F	CAACATTTATTTTGATTTTGG	Simon <i>et al.</i> (1994)
	Pat	R	TCCAATGCACTAATCTGCCATATTA	
<i>cyt b</i>	CB3	F	GAGGAGCAACTGTAATTACTAA	Barracough <i>et al.</i> (1999)
	CB4	R	AAAAGAAA(A/G)TATCATTGAGGTTGAAT	
<i>rrnL+trnL+nd1</i>	16SaR	F	CGCCTGTTTATCAAAAACAT	Simon <i>et al.</i> (1994)
	ND1A	R	GGTCCCTTACGAATTTGAATATATCCT	
<i>LSU</i>	Ka	F	ACACGGACCAAGGAGTCTAGCATG	Monaghan <i>et al.</i> (2007)
	Kb	R	CGTCCTGCTGTCTTAAGTTAC	
<i>cox1-B</i>	lco1490	F	GGTCAACAAATCATAAAGATATTGG	Folmer <i>et al.</i> (1994)
	hco2198	R	TAAACTTCAGGGTGACCAAAAAATCA	
<i>ITS2</i>	5.8sF	F	GTGAATTCTGTGAACTGCAGGACACATGAAC	Porter & Collins (1991)
	28sR	R	ATGCTTAAATTTAGGGGGTA	

F, forward; R, reverse

REFERENCES

- Barracough TG, Hogan JE, Vogler AP (1999) Testing whether ecological factors promote cladogenesis in a group of tiger beetles (Coleoptera: Cicindelidae). *Proceedings of the Royal Society of London Series B*, **266**, 1061-1067.
- Folmer O, Black M, Hoeh W *et al.* (1994) DNA primers for amplification of mitochondrial cytochrome oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology*, **3**, 294-299.
- Monaghan MT, Inward DJG, Hunt T *et al.* (2007) A molecular phylogenetic analysis of the Scarabaeinae (dung beetles). *Molecular Phylogenetics and Evolution*, **45**, 674-692.
- Porter CH, Collins FH (1991) Species-diagnostic differences in a ribosomal DNA internal transcribed spacer from the sibling species *Anopheles freeborni* and *Anopheles hermsi* (Diptera: Culicidae). *The American Journal of Tropical medicine and Hygiene*, **45**, 271-279.
- Ribera I, Fresneda J, Bucur R *et al.* (2010) Ancient origin of a Western Mediterranean radiation of subterranean beetles. *BMC Evolutionary Biology*, **10**, 29.
- Simon C, Frati F, Beckenbach A *et al.* (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene-sequences and a compilation of conserved polymerase chain-reaction primers. *Annals of the Entomological Society of America*, **87**, 651-701.

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, N) for each salinity treatment is provided; the lethal limit of each species (treatment with mortality $\geq 50\%$ 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.

	<i>E. (M.) coarctatus</i>			<i>E. (L.) salomonis</i>			<i>E. (L.) ochropterus</i>			<i>E. (L.) politus</i>			<i>E. (L.) quadripunctatus</i>			<i>E. (L.) testaceus</i>			<i>E. (L.) halophilus</i>			<i>E. (L.) bicolor</i>			<i>E. (L.) jesusarribasi</i>			
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. Information on individual genes from the aligned sequence data and estimated substitution rates for each partition from the phylogenetic analyses.

Sequence	n	Length	Variable sites	Subst. Rate (95% HPD interval) (subst/s/Ma)
mtDNA				
Protein coding				
cox1-A	216	787	350	0.0063 (0.0045 - 0.0083)
cox1-B	65	660	247	
cyt b	124	360	157	
Ribosomal				
<i>rrnL</i> + <i>trnL</i> +nd1	132	871	349	0.0031 (0.0022 - 0.0042)
nuDNA				
Ribosomal				
LSU	124	605	43	0.0034 (0.0019 - 0.0051)
ITS2	70	694	326	

Table S6. Branch measurements for the analyses of rates of phenotypic evolution.

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	initial	final	WLR	WC	HYPO	MAX OC
1	26	<i>E. coarctatus</i>	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	<i>E. ochropterus</i>	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	<i>E. salomonis</i>	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	<i>E. testaceus</i>	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	<i>E. bicolor</i>	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	<i>E. blazquezae</i>	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	<i>E. jesuarrribasi</i>	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	<i>E. quadrinotatus</i>	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	<i>E. segmentinotatus</i>	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	<i>E. falcarius</i>	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	<i>E. risi</i>	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	<i>E. turanicus</i>	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	<i>E. hamifer</i>	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	<i>E. hamiltoni</i>	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	<i>E. diffusus</i>	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	<i>E. politus</i>	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	<i>E. ater</i>	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	<i>E. quadripunctatus cplx. A</i>	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	<i>E. halophilus</i>	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	<i>E. quadripunctatus cplx. B</i>	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	<i>E. quadripunctatus cplx. C</i>	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	<i>E. quadripunctatus cplx. D</i>	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	<i>E. quadripunctatus cplx. E</i>	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	<i>E. quadripunctatus cplx. F</i>	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 S7. Correlation between traits in PGLS analyses. HC: hyposmotic capacity.

		Range R ²		
		Max HC	Water loss rate	Water content
Habitat salinity		-0.027 – 0.0001	-0.034 – -0.034	-0.045 – -0.040
Max HC	including <i>E. salomonis</i>		0.263 – 0.263 (100)	0.051 – 0.259 (58)
	excluding <i>E. salomonis</i>		-0.045 – -0.045	0.334 – 0.434 (100)

The range of adjusted R² for 1000 resampled post-burnin trees is presented. Significant correlations are highlighted in bold and the percentage of trees showing significant relationships (P<0.05) is indicated in parentheses.

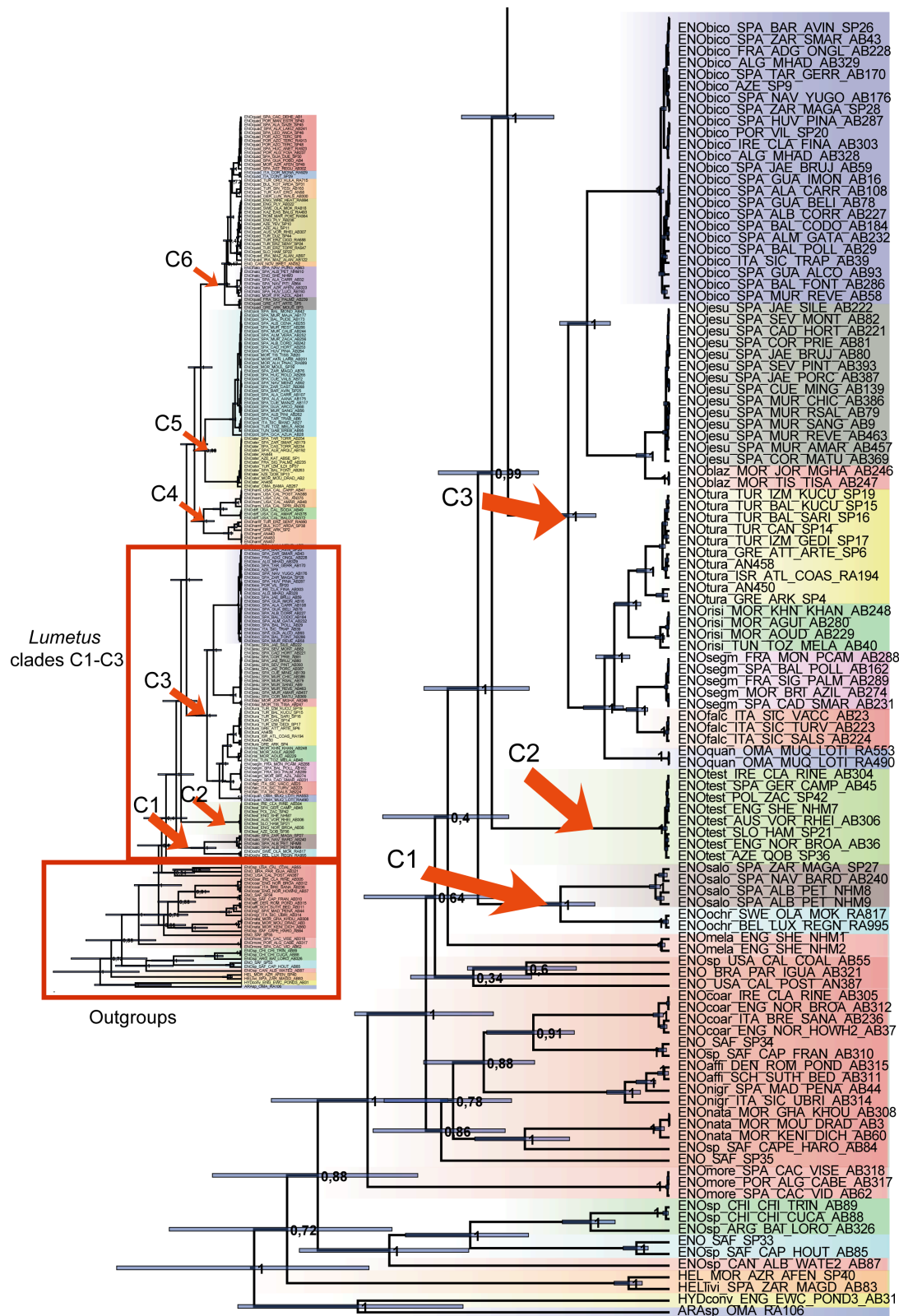


Figure S1. Phylogeny of *Lumetus*. Node numbers are posterior probabilities, bars on nodes are 95% confidence intervals for node ages. Letters: main clades as referred in the text. See Table S1 for details on the terminals.

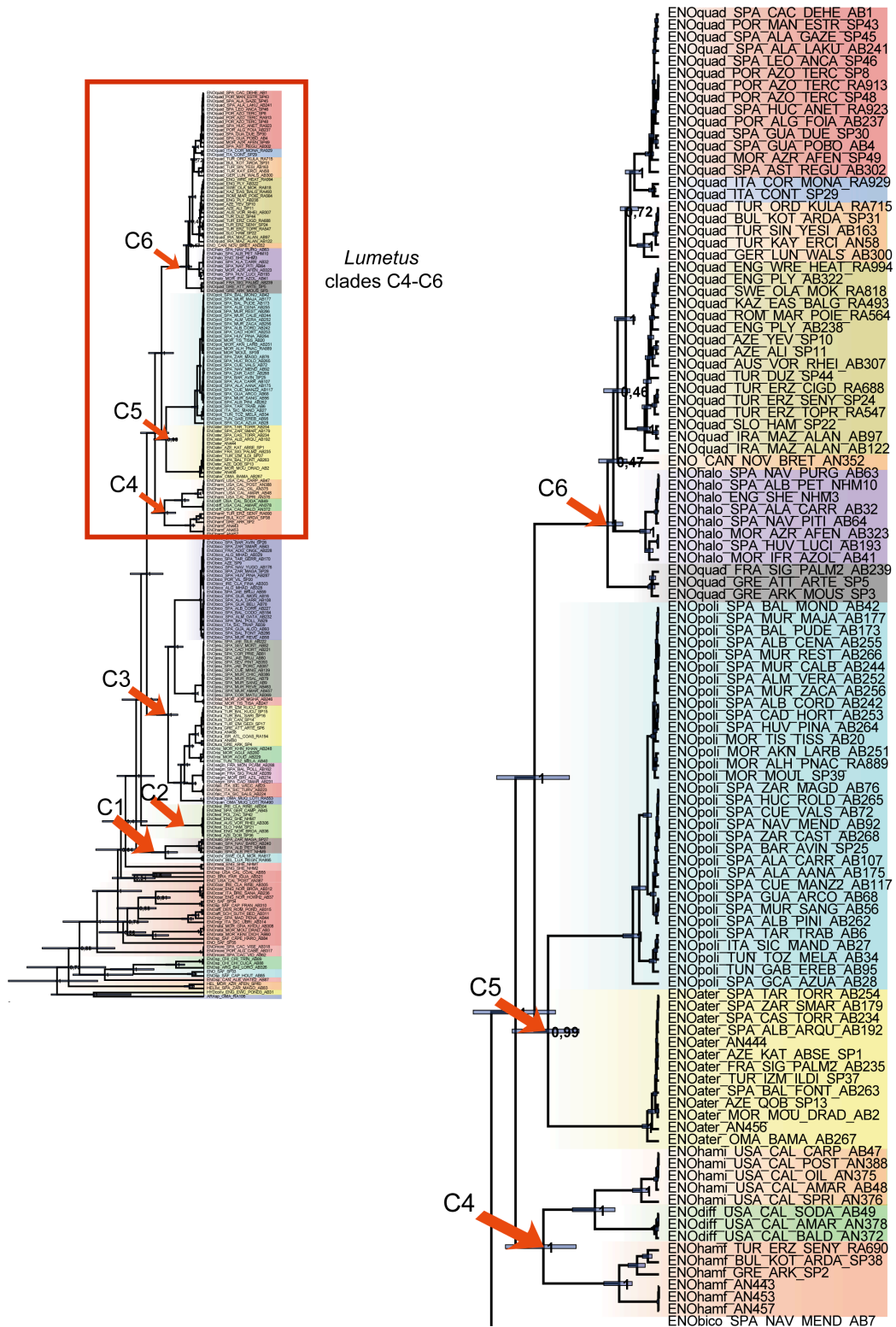


Figure S1. Continued

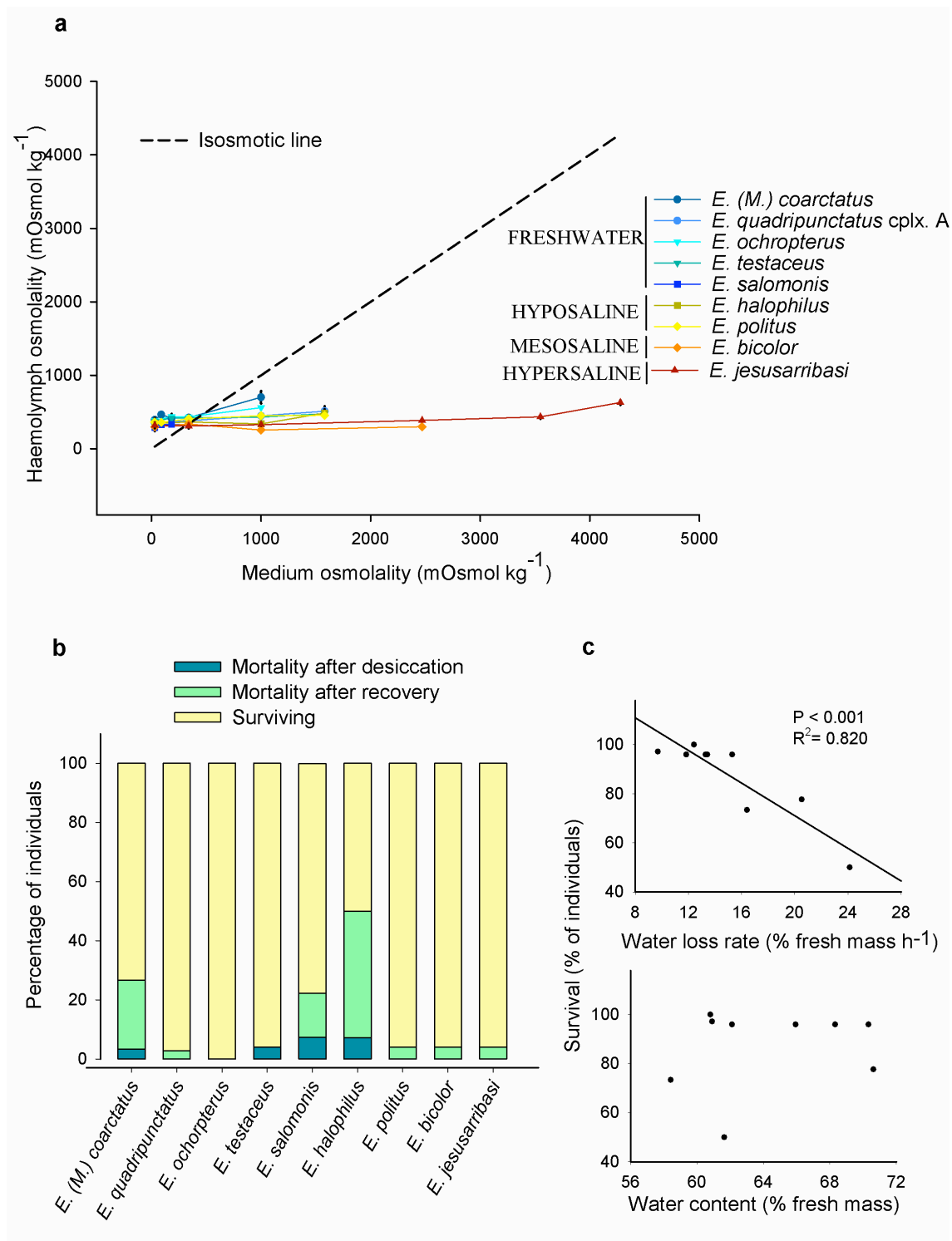


Figure S2. Summary of results of a) osmoregulation experiments; osmotic concentration of the salinity treatments (**medium osmolality**) vs. specimens' haemolymph and b-c) desiccation resistance experiments; b) mortality, recovery and survival percentages; c) relationships between survival to desiccation and water loss rate or water content.

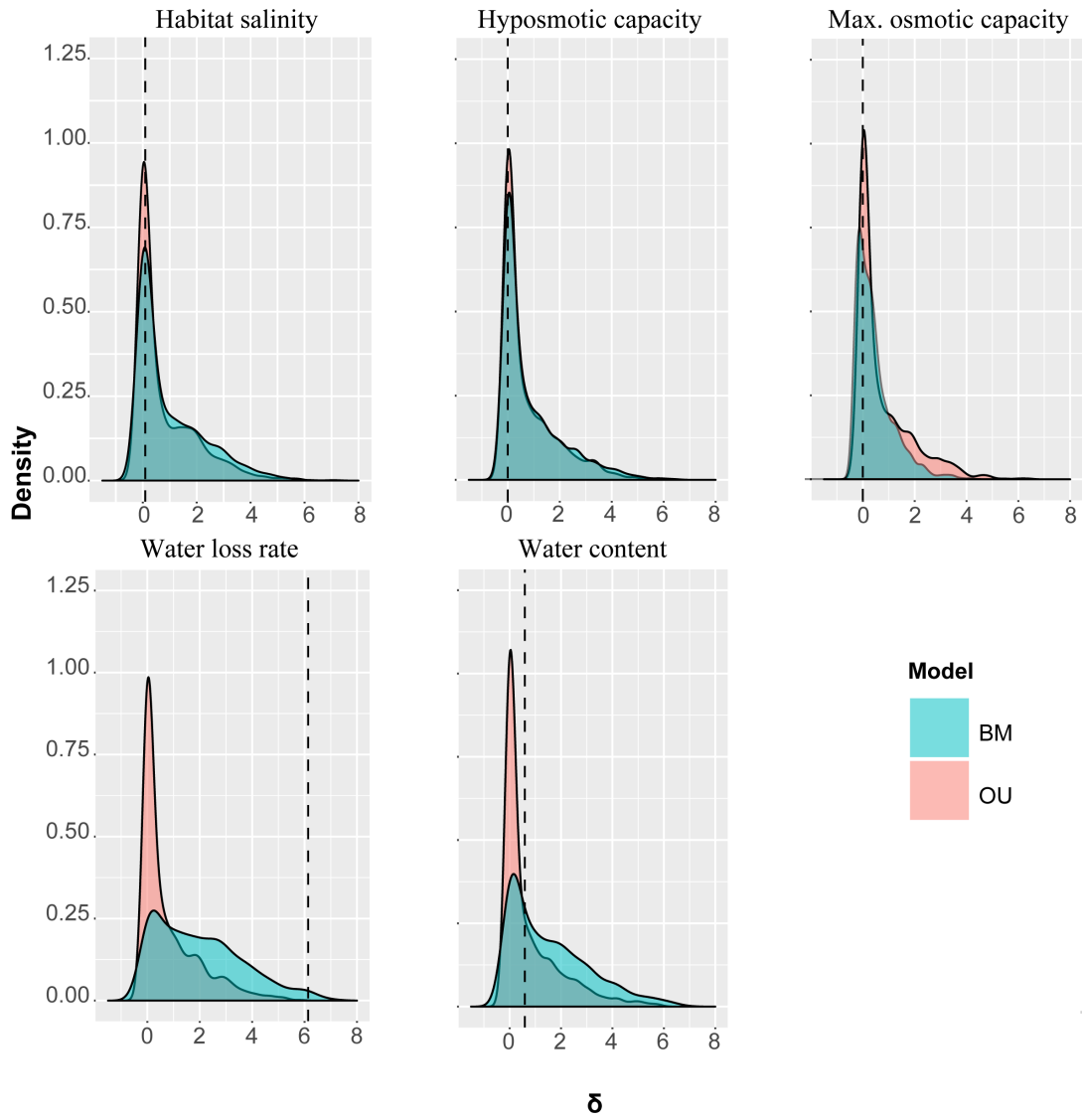


Figure S3. Distributions of the likelihood ratio statistic δ for model comparisons with Monte-Carlo simulations (n=1000 replicates). The dashed vertical lines indicate the observed value of δ when the models are fit to the *Lumetus* dataset. BM: Brownian Motion; OU: Ornstein-Uhlenbeck.

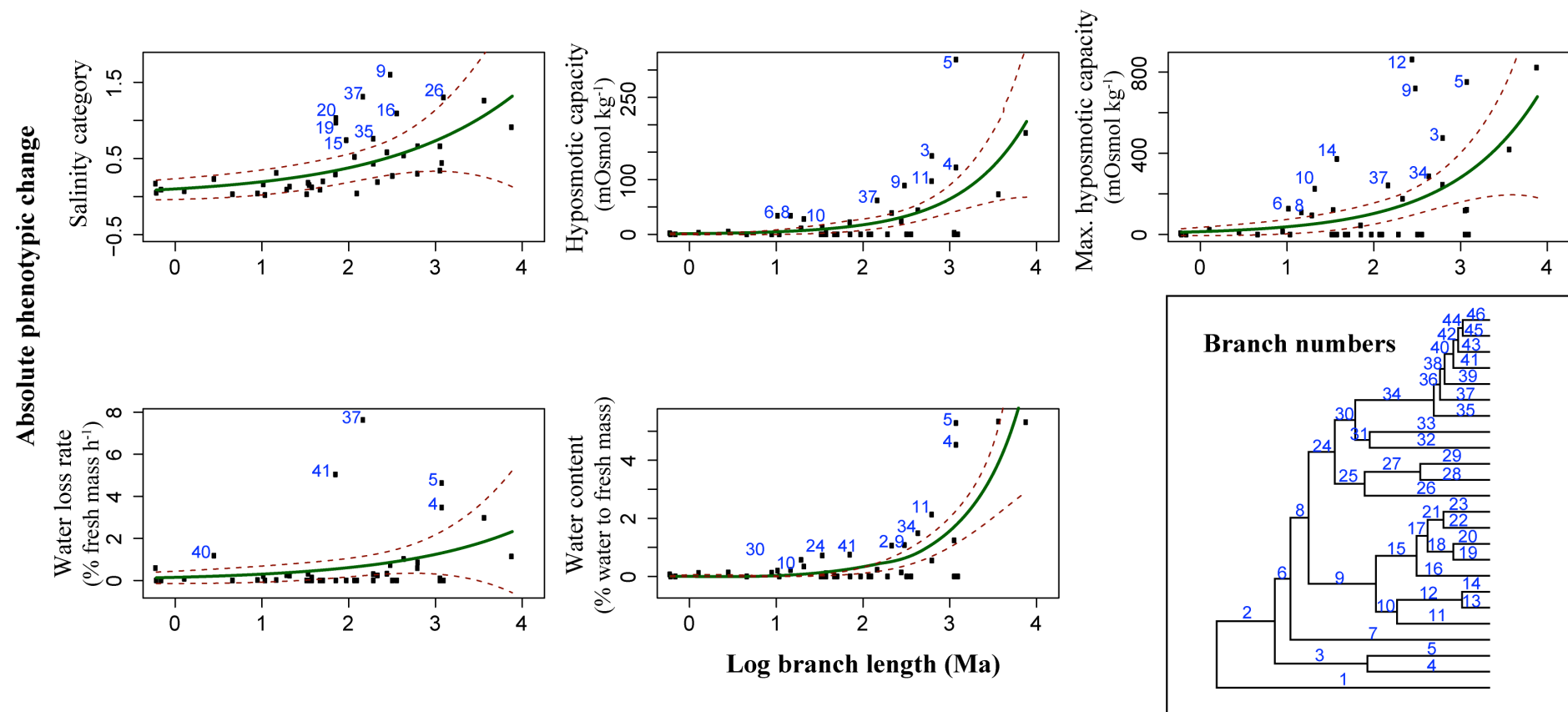


Figure S4. Plot of the branch length with the absolute phenotypic change of each trait. Green line: regression line; red dashed lines: 99% confidence intervals; numbers: branch numbers (only indicated for those branches with accelerated rates of evolution).

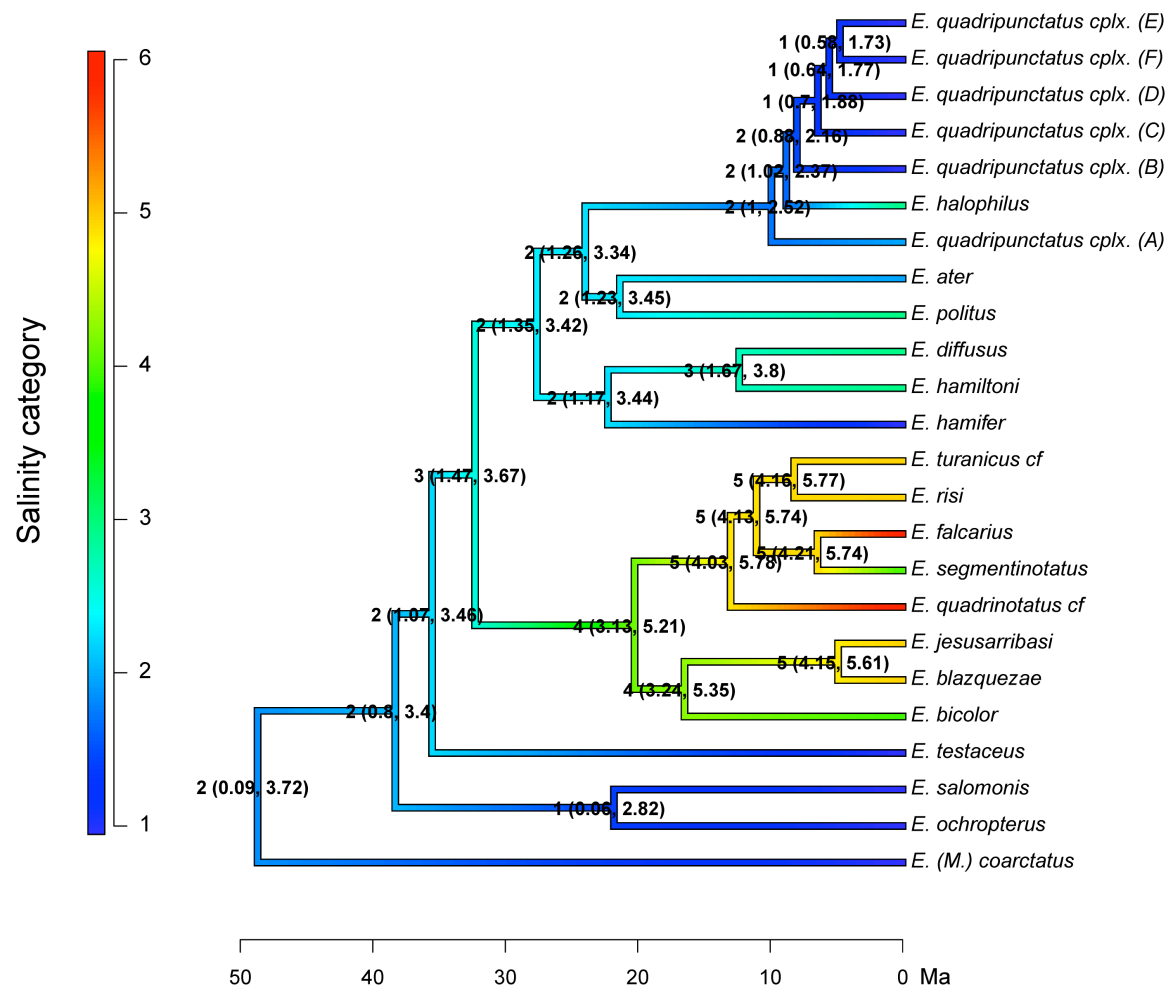


Figure S5. Ancestral reconstruction of desiccation and osmoregulation traits. The warmer (red) colours indicate higher resistance to desiccation or salinity than cooler (blue) colours. Mean reconstructed values and 95% confidence intervals are indicated on nodes.

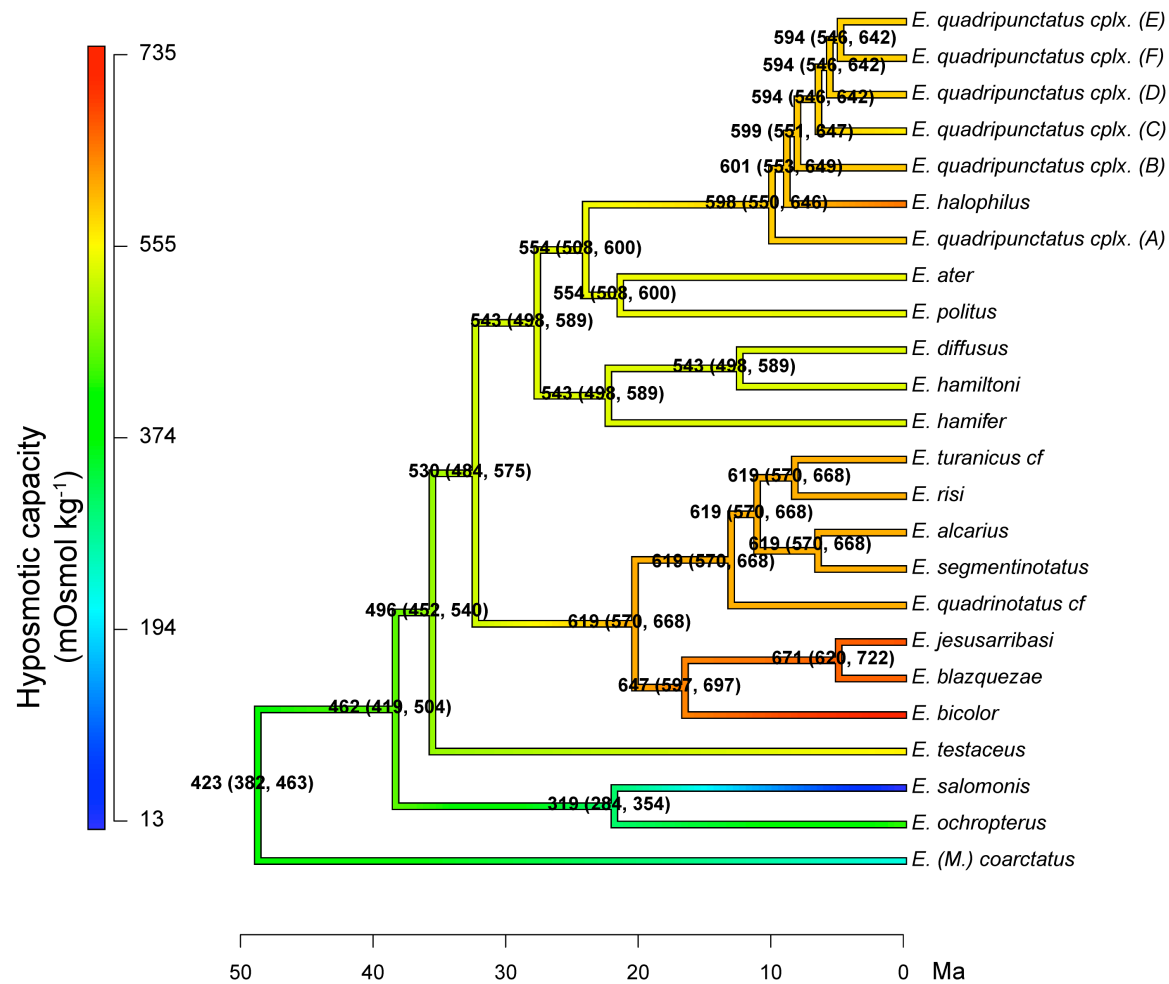


Figure S5. Continued.

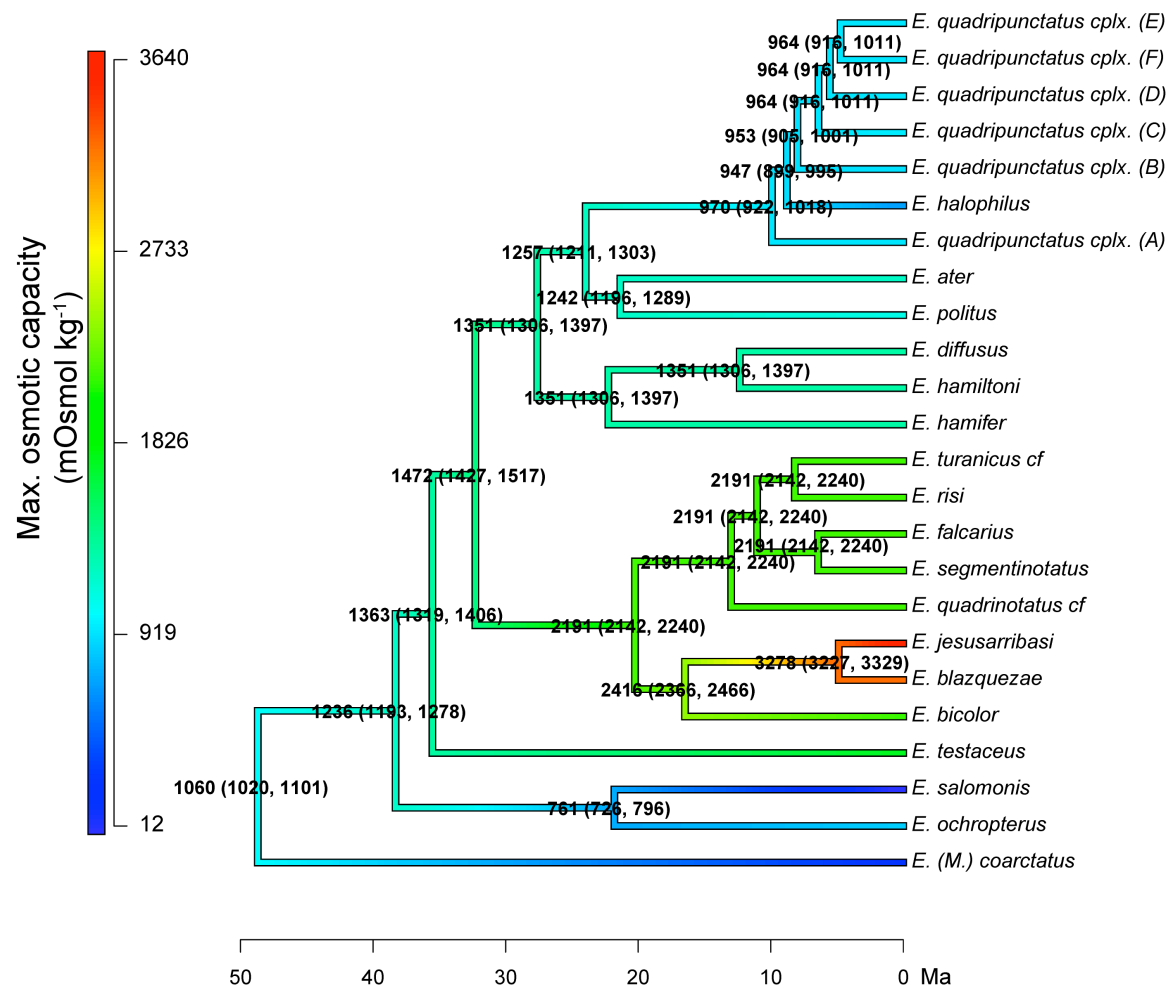


Figure S5. Continued.

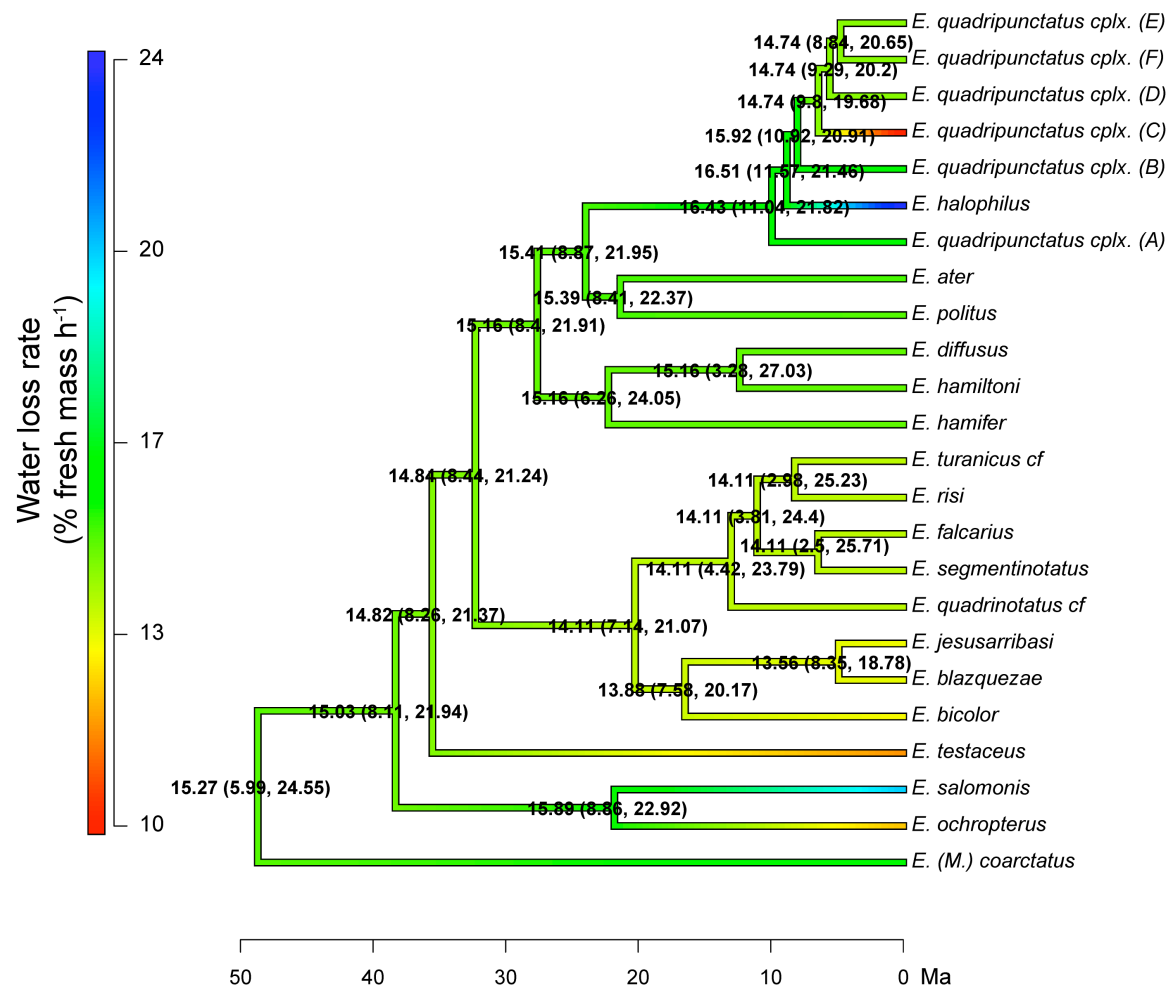


Figure S5. Continued.

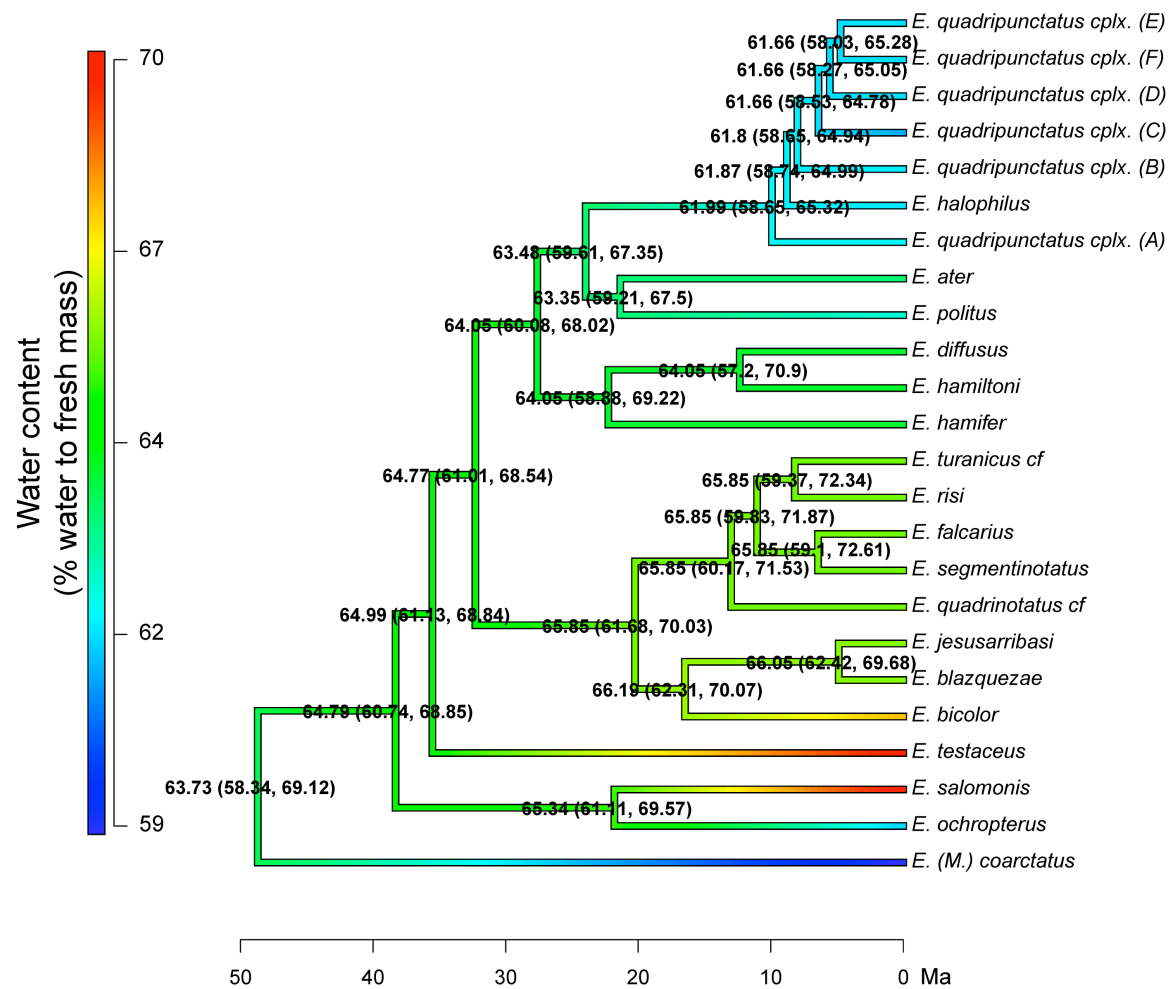


Figure S5. Continued.

Data S1. Details of osmoregulation and desiccation experiments

Osmoregulation experiments.

After field collection, individuals of each species were maintained for one week in aerated tanks placed in an environmental chamber (SANYO MLR-351, Sanyo Electric Co., Ltd., Moriguchi City, Osaka, Japan) at 20°C and 12:12 L:D cycle, with water from collection sites (Table S2). Specimens were fed during this period with macrophytes also collected in the source localities. Waters of different conductivity were prepared by dissolving an appropriate quantity of marine salt (Ocean Fish, Prodac, Cittadella, Padua, Italy) in distilled water. Specimens were transferred to individual plastic containers with 40 ml of the specific salinity solutions and exposed to the salinity treatments for 48 h (see the specific sample sizes and salinities in Table S4). Between 5-10 individuals of each species were kept at their original salinities as a control. Containers were held in the environmental chamber at constant temperature (20°C) and 12:12 L:D cycle. Food was not supplied during the exposure period.

For haemolymph extraction, specimens were gently 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 was 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 stored in the freezer until osmolality measurements.

Desiccation experiments

Specimens were maintained in the laboratory prior to experiments under the same conditions than in osmoregulation experiments. As previous salinity conditions have shown to influence desiccation resistance (Pallarés *et al.* 2017), specimens were kept 48 h before desiccation experiments in a dilute medium (ca. 0.1 mS cm⁻¹) at 20°C and 12:12 light:day cycle, without access to food. Groups of individuals (see specific sample size in Table S4) 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°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. Groups of 10 individuals per species were used as a control under no desiccation stress. These were kept in glass vials placed in a closed tank with deionized water in the base, producing a relative humidity ≥90%. After 6 h, specimens from control and test groups were re-weighed and allowed to recover for 24 h at 20±1°C in individual containers with 40 mL of the dilute solution. Mortality was monitored after desiccation exposure and after the recovery period. Specimens were then dried at 50°C for 48 h and re-weighed for estimation of the dry mass.