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

S. PALLARÉS[1], P. ARRIBAS[2], D. T. BILTON[3], A. MILLÁN[1], J. VELASCO[1], I. RIBERA[4]

[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

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Corresponding author:

S. Pallarés

susana.pallares@um.es

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

Tel. +34 868 88 49 77

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ABSTRACT

Transitions from fresh to saline habitats are restricted to a handful of insect lineages, since 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 colonisation 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.

INTRODUCTION

How organisms acquire novel traits or undergo adaptive trait divergence are central questions in evolutionary ecology, as these processes facilitate niche shifts and the colonisation of novel environments (Heard & Hauser 1995; Hunter 1998; Moczek 2008). In the aquatic realm, the evolution of hydric and osmotic regulation mechanisms was a key innovation allowing transitions from marine to freshwater habitats in some animal groups like fishes or crustaceans (e.g. Faria *et al*. 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. Bradley *et al*. 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 *et al.* 2012 for water bugs; or Herbst 1999 for flies). The physiological and evolutionary processes that may facilitate the colonisation of extreme habitats such as saline waters remain poorly understood, however, and require the study of relevant organismal traits within a phylogenetic context (Cheng & Chen 1999; Tobler *et al*. 2011).

In insects, the main osmoregulatory adaptations are a highly impermeable cuticle and a rectum capable of producing hyperosmotic excreta. These are ancestral characters, found in virtually all insect lineages and are clearly essential to their success on land, where desiccation 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 adaption, only present in a few insect orders (Bradley *et al*. 2009). In general,

insect species that show tolerance to salinities above that of seawater are efficient hyporegulators, i.e. 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. Tones & Hammer 1975; Herbst *et al*. 1988; Pallarés *et al*. 2015). 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 (Evans 2008; Bradley 2009; Cohen 2012). 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 *et al.* 2013) and to a lesser extent in animal taxa (Gómez-Mestre & Tejedo 2005; Faria *et al.* 2017). 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. Firstly, beetle adults (Pallarés *et al*. 2017) and dipteran larvae (Elnitsky *et al*. 2009) sequentially exposed to salinity and desiccation showed cross-

tolerance responses (Sinclair *et al*. 2013; Todgham & Stillman 2013), suggesting a mechanistic link between the response to both stressors. Secondly, a recent study reconstructing the colonisation of saline waters by *Enochrus* water beetles (Hydrophilidae) suggested that salinity tolerance arose during periods of global aridification, when multiple independent transitions from fresh to saline waters apparently occurred (Arribas *et al*. 2014). These authors also found a positive correlation between the 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 *et al.* (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 colonisation 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 *et al*. 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 of 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 *et al*. 2013). Mismatches between realised and fundamental niches may result when physiological tolerance evolved as a result of prior exposure to different stressors, since 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 colonisation 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*.

MATERIAL AND METHODS

Taxon sampling

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

Phylogeny of *Lumetus*

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

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

174 Bayesian phylogenetic analyses on the concatenated DNA matrix were implemented
175 in BEAST 1.8.0 (Drummond *et al*. 2012) and run in the CIPRES Science Gateway
176 (Miller *et al.* 2010). The concatenated data set was divided into 3 partitions: the three
177 protein-coding genes, the mitochondrial ribosomal gene and the two nuclear sequences.
178 Analyses were conducted by applying a GTR + I + G substitution model for each
179 partition, which was the best fitting model previously estimated with Partition Finder
180 (Lanfear *et al*. 2012). We applied a Yule speciation tree prior. To calibrate the tree, we
181 used as a prior for the age of *Lumetus* (time to most recent common ancestor, tMRCA)
182 the age distribution of this node obtained by Arribas *et al.* (2014) – i.e. ≈45 Ma (Gamma
183 distribution shape: 56.84, scale: 0.74). An uncorrelated lognormal clock was applied for
184 the nuclear partition, with an uniform prior distribution for the rate of substitutions set
185 between 0.0001 – 0.01 substitutions per site per time unit (subs/s/Ma) and an initial
186 value of 0.001, together with a strict clock for each of the mitochondrial partitions with
187 an uniform prior distribution for the rate with 0.01 (0.001 – 0.1) subst/s/Ma. The ranges
188 set as priors for the substitution rates cover the range of rates usually reported for
189 Coleoptera, which are faster for the mitochondrial than for the nuclear genes used in this
190 study (e.g. Papadopoulou *et al*. 2010; Ribera *et al.* 2010; Andújar *et al*. 2012).

191 We set two independent runs of 100 million MCMC steps each, sampling one tree
192 every 10,000 generations. LogCombiner (Drummond *et al*. 2012) was used to combine

trees from both runs and to obtain 1,000 randomly resampled postburnin 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).

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, 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 h 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^{-1}) and a hyperosmotic one (35 g L^{-1}) to obtain comparable osmolality measurements. For each species, the treatment in which mortality exceeded 50% of the tested individuals was considered as the upper lethal limit (e.g. Faria *et al*. 2017) (Table S4). From each treatment, we obtained haemolymph samples from a minimum of three of the exposed individuals (Table S4), as pilot trails showed low intraspecific variation within salinity treatments. Osmolality of the haemolymph and the saline media were measured using a calibrated nanolitre

osmometer (Otago Osmometers, Dunedin, New Zealand). For each treatment, we estimated the hyper- or hyposmotic capacity, i.e. 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 (Charmantier *et al*. 1984; Calosi *et al*. 2005). The hyposmotic capacity at 35 g L^{-1} (hyposmotic capacity hereafter) and the maximum hyposmotic capacity (i.e. that measured at the highest salinity tolerated by each species) showed the highest variation 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±5 % RH (relative humidity), 20±1ºC for 6 h. 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 h 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 supplementary material (Data S1).

Habitat transitions, evolution of desiccation resistance and osmoregulatory capacity

Ancestral trait reconstruction. We tested different models of trait evolution (Brownian motion – BM and Ornstein-Uhlenbeck – OU) (Kaliontzopoulou *et al*. 2016) to reconstruct ancestral values of habitat salinity (considered as a semi-continuous variable), hyposmotic capacity and desiccation resistance traits. Intraspecific variation, missing observations and small tree size can profoundly affect the performance of such models (Boettiger *et al*. 2012; Cooper *et al*. 2016). To account for this, we used a Monte-Carlo based approach to assess the 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 *et al*. 2009; Goolsby *et al*. 2016), which uses a maximum likelihood-based method to estimate trait covariance across (phylogenetic covariance) and within species (phenotypic covariance) for datasets with missing data and multiple within-species observations (e.g. Pollux *et al*. 2014). This method provides predicted trait values and variances for ancestral nodes and unmeasured extant species (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 inter-individual variation and measurement error (Bruggeman *et al*. 2009).

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 ($R^2 = 0.37$, $P < 0.001$).

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

289 *Relationships between traits*. Phylogenetic generalized least squares (PGLS) were
290 applied, using the R function *pgls* (caper), to explore the relationships between i) habitat
291 salinity and hyposmotic capacity, ii) habitat salinity and desiccation resistance, iii)
292 desiccation resistance and hyposmotic capacity. Proportional data (% water content and
293 % water loss) were arcsine transformed and hyposmotic capacity was log-transformed
294 prior to analyses to improve fit to a normal distribution. Again, for simplicity, only the
295 maximum hyposmotic capacity was used for these analyses (see above). We also traced
296 the relative order of appearance of changes in desiccation resistance and maximum
297 hyposmotic capacity across the entire tree (i.e. from root to the tip) for species for which
298 data were obtained experimentally by plotting the reconstructed value of the variable at
299 each of the nodes against the time of the node.

300 ### Topological uncertainty

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

304 ## RESULTS

305 ### Phylogeny of *Lumetus*

306 We obtained a well-resolved phylogeny of the subgenus *Lumetus,* with strong support
307 for most of the main nodes except for some internal nodes in the *E. quadripunctatus*
308 group (Figs 1 and S1). The first splits separated *E. ochropterus* and *E. salomonis* from
309 the rest of the *Lumetus* species at 38 (28–49 95% confidence interval, c.i.) Ma (clade
310 C1) and the lineage containing only *E. testaceus* at 36 (26–46 c.i.) Ma (clade C2).
311 Within the remaining *Lumetus* species, the next split, at 32 (23–42 c.i.) Ma, separated a

clade of saline species (the *E. bicolor* group, clade C3) from one including three subclades of Nearctic and Palaearctic 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 6 recently diverged lineages (the *E. quadripunctatus* complex) with well characterised geographical distributions. These included (A) a coastal Mediterranean clade; (B) another containing a single specimen from Canada; two Eurasian clades, one (C) widely distributed and another (D) restricted to Bulgaria and Turkey; (E) a clade apparently restricted to Italy; and (F) an Ibero-Moroccan clade. Sequence length, number of variable sites and the estimated substitution rates for each partition are provided in Table S5.

Hyporegulation ability and desiccation resistance

All species were hyperegulators at salinities below the isosmotic point. Under hyperosmotic conditions, all the species showed hyporegulation ability within specific salinity ranges, except for one freshwater species, *E. salomonis*, which did not survive exposure to hyperosmotic conditions (> 35 g L^{-1}) (Fig. S2a, Table S4). In desiccation experiments, *E. halophilus* was the least desiccation resistant species (highest mortality and lowest recovery capacity), followed by *E. coarctatus* and *E. salomonis*, all living in fresh-mineralized waters. Amongst the remaining species, most exposed specimens survived, and were able to recover after desiccation (Fig. S2b). No significant mortality was observed in control (non-desiccated) individuals. Survival under desiccation was highly correlated with water loss but not with water content (Fig. S2c).

Habitat transitions, evolution of desiccation resistance and hyporegulation ability

336 *Ancestral traits reconstruction and rates of evolution.* For all traits studied, the
337 distributions of δ under BM and OU models showed a high degree of overlap,
338 indicating limited power to distinguish between evolutionary models (Fig. S3).
339 Ancestral state reconstruction was therefore made assuming the simplest model. i.e.
340 BM. All measures of absolute phenotypic change (shown in Table S6) were
341 significantly related to branch length ($P < 0.05$), except for water loss ($P = 0.07$).
342 Accelerated rates of phenotypic evolution of all traits were identified in several
343 branches across the tree (Figs 2 and S4).

344 The ancestor of *Lumetus* was inferred to be a species which lived in mineralized
345 waters (Figs 2a and S5) with some degree of hyposmotic capacity (423 mOsmol kg^{-1} at
346 35 g L^{-1}, Figs 2b and S5), but within a limited salinity range (maximum estimated
347 hyposmotic capacity of 1,000 mOsmol kg^{-1}, Figs 2c and S5). A rapid, direct transition
348 to mesosaline waters took place at the origin of the *E. bicolor* group, as well as other
349 independent transitions to hyposaline waters (e.g. at the origin of *E. diffusus*-*E.*
350 *hamiltoni* or *E. politus*) and accelerated reversions to freshwater habitats in the
351 Nearctic-Palaearctic clades (Fig. 2a). In the *E. bicolor* group, transitions to meso and
352 hypersaline waters were preceded by rapid increases in hyposmotic capacity, whilst a
353 shift to freshwater habitats in *E. salomonis* was associated with the loss of
354 hyporegulation ability.

355 The reconstructed ancestral values of water loss and water content varied little across
356 *Lumetus* (13.6 – 16.5 % of fresh mass and 61.7 – 66.2 % of water to fresh mass,
357 respectively, Fig. S5). Water loss progressively decreased after the split of *E. testaceus*
358 and within the *E. bicolor* group, alongside occupation of meso- and hypersaline waters.
359 In the clades occupying fresh to hyposaline waters, desiccation rates remained almost
360 constant, although some accelerated changes were identified within these, mostly on

361 terminal branches (Fig. 2d). Water content showed accelerated increases on several
362 branches, in some cases coinciding with rapid increases in hyposmotic capacity and
363 transition to saline waters (*E. bicolor* group) and also accelerated and significant
364 decreases in the *E. quadripunctatus* group (Fig. 2e).

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

369 *Phylogenetic signal.* For all traits, except for water loss, estimates of Pagel's λ were
370 close to 1 in all the resampled trees (although for habitat salinity λ was < 1 in 14% of
371 trees) and significantly better than those obtained when the phylogenetic structure was
372 erased (λ = 0), indicating a significant phylogenetic signal (Table 2). For hyposmotic
373 capacity and water content, estimated λs were also better than those from a model in
374 which the distribution of trait values across the phylogeny was as expected under BM
375 (i.e. λ = 1) in all resampled trees. Water loss was the only trait consistently showing no
376 phylogenetic signal in all the analyzed trees (Table 2).

377 *Relationships between traits.* In PGLS analyses (Table S7) habitat salinity showed no
378 significant relationships either with maximum hyposmotic capacity or desiccation traits
379 (Fig. 3a-c) in any of the analysed trees. Variability in maximum hyposmotic capacity
380 and desiccation traits was higher amongst freshwater species than saline ones (i.e.
381 mineralized-hypersaline taxa). In saline species, hyposmotic capacity and desiccation
382 resistance tended to increase with habitat salinity (Fig. 3a-c).

383 Maximum hyposmotic capacity was negatively related to water loss in 100% of the
384 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 analyzed trees (Table S7, Fig. 3d-e).

When the relative order of appearance of changes in desiccation resistance and maximum hyposmotic capacity was traced across individual branches of the phylogeny (Figs 4 and 5), increases in hypoosmotic capacity were not clearly preceded by increases in desiccation resistance nor *vice versa.* Among the species with the highest hyporegulation ability (*E. testaceus, E. bicolor* and *E. jesusarribasi*), 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 dessication resistance. On the contrary, increases in desiccation resistance were not always associated with an increase in hyposmotic capacity, as in e.g. *E. ochopterus* and *E. quadripunctatus* in Fig. 4, or *E. salomonis* in Fig. 5.

DISCUSSION

The reconstruction of habitat transitions, desiccation and osmoregulatory traits in *Lumetus* species suggest that hyporegulation ability, an essential trait for the colonisation of hyperosmotic media by aquatic insects, arose as a mechanism derived from those originally developed to deal with desiccation stress in this lineage, 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 *Peltodytes muticus* (Arlian & Staiger 1979). The hypersaline *Enochrus jesusarribasi* 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 *et al*. 2006; Short & Fikacek 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 trough the evolutionary history of *Lumetus*, most of these being associated with habitat transitions across the salinity gradient. Arribas *et al.* (2014) found that transitions to saline habitats in the *E. bicolor* group occurred 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 the their hyporegulation ability.

433 Species living in the most saline conditions showed high hyposmotic capacity, but
434 also an increased desiccation resistance (i.e. lower water loss). In the case of species
435 living in fresh to hyposaline waters, we found i) some species with comparable or even
436 higher desiccation resistance than their saline water relatives, but relatively low
437 hyposmotic capacity (e.g. *E. ochropterus*) and ii) species which had both high
438 desiccation resistance and hyposmotic capacity. For example, *E. testaceus* and *E.*
439 *politus* were able to hyporegulate at salinities well above those encountered by these
440 beetles in nature. According to the ancestral reconstruction of habitat salinity, neither *E.*
441 *testaceus* nor *E. politus* had saline ancestors, something that is only compatible with the
442 first of our proposed hypotheses, i.e. that hyporegulation ability was co-opted from
443 desiccation resistance mechanisms. A lack of association between habitat salinity and
444 osmoregulatory ability has also been reported in some crustaceans (e.g. McNamara &
445 Faria 2012; Faria *et al*. 2017). Grapsid and ocypodid crabs present an example of how
446 selection on mechanisms to reduce water loss under aerial desiccation (gill function in
447 this case) indirectly has improved underwater osmoregulation ability, meaning
448 desiccation resistance and osmoregulation capacities are positively associated (Takeda
449 *et al*. 1996; Faria *et al.* 2017). In the case of water beetles, selection on mechanisms
450 such as those involved in ion transport, cell volume regulation or cuticle permeability
451 for the control of water loss under desiccation might have resulted in enhanced
452 hyporegulation ability.

453 Overall, our findings are consistent with an evolutionary sequence in which
454 improved desiccation resistance in *Lumetus* provided the physiological basis for the
455 development of efficient hyporegulation mechanisms, which in some cases allowed
456 them to colonize and diversify in the meso- and hypersaline habitats. The accelerated
457 increases of hyposmotic capacity in some parts of the phylogeny are consistent with the

458 hypothesis that such capacity is based on a derived mechanism (i.e. in agreement with
459 our first hypothesis). Accelerated evolution of complex mechanisms such as those
460 involved in hyporegulation (Bradley 2009) are more likely to occur when such a
461 mechanistic basis is already present (Barrett & Schluter 2008; Roesti *et al*. 2014).

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

475 Due to the high ancestral tolerance to desiccation in the subgenus *Lumetus* it was not
476 possible to reconstruct the hypothesised increase in desiccation resistance preceding any
477 improvements in hyposmotic capacity. Rapid increases in hyposmotic capacity were
478 associated with parallel weak decreases in water loss and increases in water content
479 across the evolutionary path of the strongest hyporegulator species. Despite these
480 parallel changes, a correlated evolution of both tolerances, constrained by identical
481 genes and mechanisms (genetic correlation *sensu* Kellermann *et al*. 2013 - i.e. our third
482 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. López-Martínez *et al.* 2009) or salinity stress (e.g. Uyhelhi *et al*. 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 (Mosbrugger *et al*. 2005; Bosboom *et al*. 2014). Temporary habitats were pressumably 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. Pinceel *et al*. 2013; Dorn *et al*. 2014). Aridification, by enhancing the linked tolerance of desiccation and salinity, could have also been a key driver in the diversification of *Lumetus*.

Euryhalinity is also an important source of evolutionary diversity (Schultz & McCormick 2012; Brauner *et al*. 2013). However, the process of adaption to saline inland waters seems to be a unidirectional path, likely reflecting trade-offs between competitive ability and tolerance to osmotic stress (Dunson & Travis 1991; Herbst 2001; Latta *et al*. 2012). In general, species of *Lumetus* (and other beetle genera) typical of hypersaline waters are almost absent from freshwater habitats, despite been able to hyperregulate (Tones 1977; Céspedes *et al.* 2013; Pallarés *et al*. 2015) – although *E.*

508 *bicolor* is regularly found in low mineralised waters in northern localities of Europe.
509 Such a situation also holds for saline Hemiptera (corixids, Tones & Hammer 1975),
510 coastal and estuarine decapods (McNamara & Faria 2012; Faria *et al*. 2017) and fish
511 (Schultz & McCormick 2012). The maintenance of hyperegulation ability despite the
512 apparent loss of its ecological role may reflect positive pleiotropies or functional
513 correlations between hypo- and hyperregulatory mechanisms (e.g. Smith *et al*. 2008,
514 2010), but may also be just due to the low cost of maintaining functional
515 osmoregulatory responses outside conditions commonly encountered in nature (Divino
516 *et al*. 2016).

517 The fundamental salinity tolerance niche of some fresh-hyposaline species was also
518 found to be much broader than their realized niches (e.g. in *E. testaceus*), something
519 which supports the view that hyporegulation arose as a co-opted mechanism. The
520 osmoregulatory physiology of water beetles is still poorly explored, so it is not known if
521 euryhalinity is common in freshwater species of other genera, but at least two dytiscid
522 species of the genus *Nebrioporus* typical of freshwater habitats are unable to
523 osmoregulate at salinities above their isosmotic point (Pallarés *et al*. 2015). The absence
524 of species of *Lumetus* which able to osmoregulate in saline habitats may be due to
525 multiple factors, amongst them biological interactions, ecological requirements of
526 juvenile stages, or physiological traits other than osmoregulation (e.g. Dowse *et al*.
527 2017).

528 Our results demonstrate how a combination of ecological, experimental and
529 phylogenetic data can offer powerful insights into the origin and evolution of traits
530 underlying ecological transitions and the diversification of lineages into previously
531 unavailable areas of niche space. Further research is still needed to understand why only
532 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.

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DATA ACCESSIBILITY

All sequences generated have been deposited in the EMBL database (ascension numbers shown in Table S1). Sequence alingments are available via Dryad (doi:10.5061/dryad.2j3c8 at http://dx.doi.org/10.5061/dryad.2j3c8) and all data obtained in desiccation and osmoregulation experiments can be found in the supporting information.

AUTHOR CONTRIBUTIONS

788 Conceived the study: all authors
789 Field colection of specimens: I.R, D.T.B, P.A, J.V, A.M
790 Performed experiments: S.P
791 Analyzed data: S.P, I.R, P.A
792 Wrote the manuscript: S.P
793 Reviewed the manuscript: all authors
794

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

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

801

802

803 **Table 2**. Ranges of the estimated Pagel's λ (for the randomized sample of 1,000 post-burnin
804 trees) and P-values for the likelihood ratio test comparing estimated λ with a model assuming λ
805 = 0 or λ = 1 (for the consensus tree).

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

806

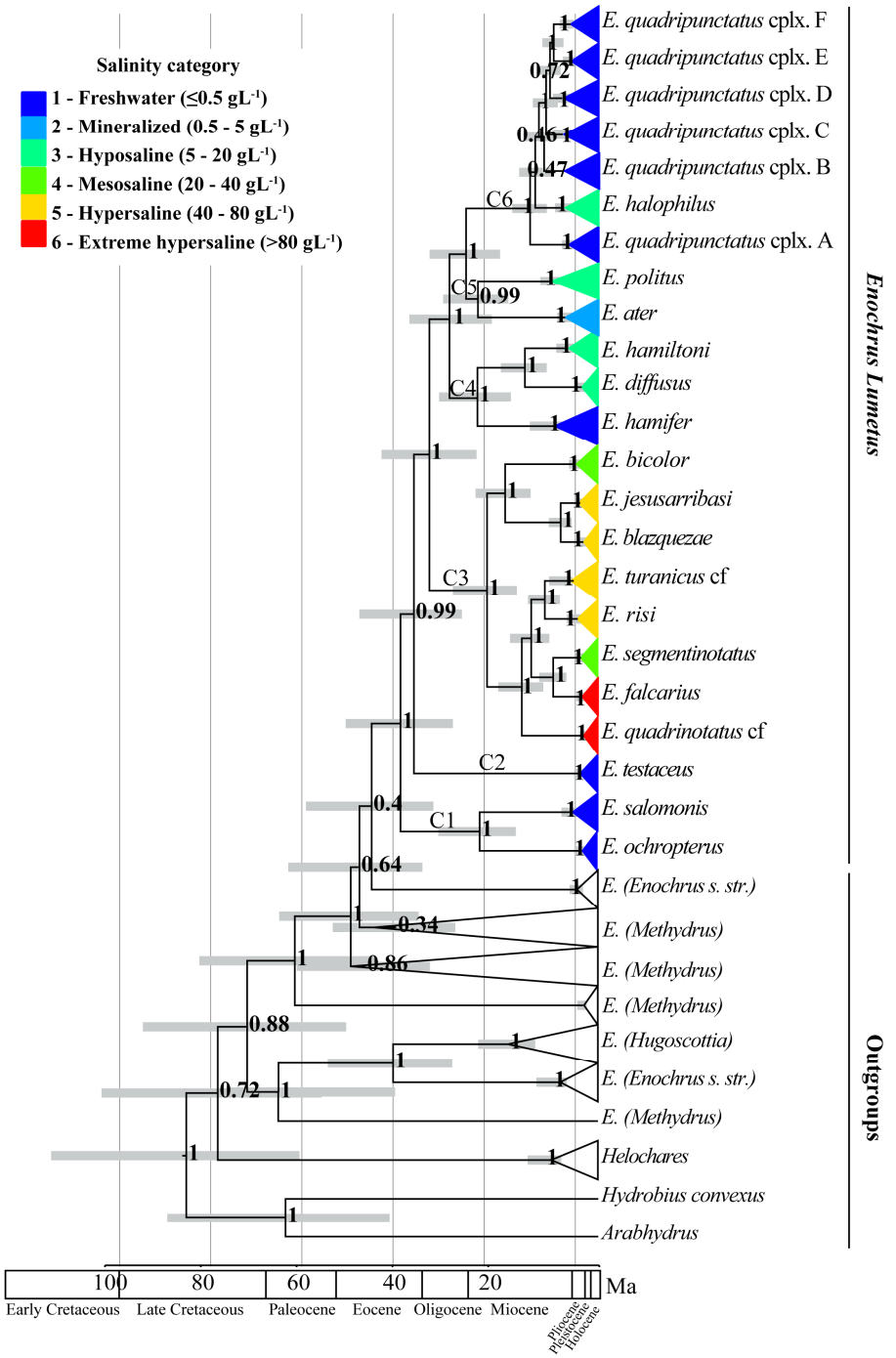

Figure 1. Dated phylogeny of *Lumetus*. Node numbers: posterior probabilities; bars on nodes: 95% confidence intervals for node ages; letters: main clades as referred to in the text. Terminals are collapsed to reflect species-level relationships (see Fig. S1 and Table S1 for details on terminals).

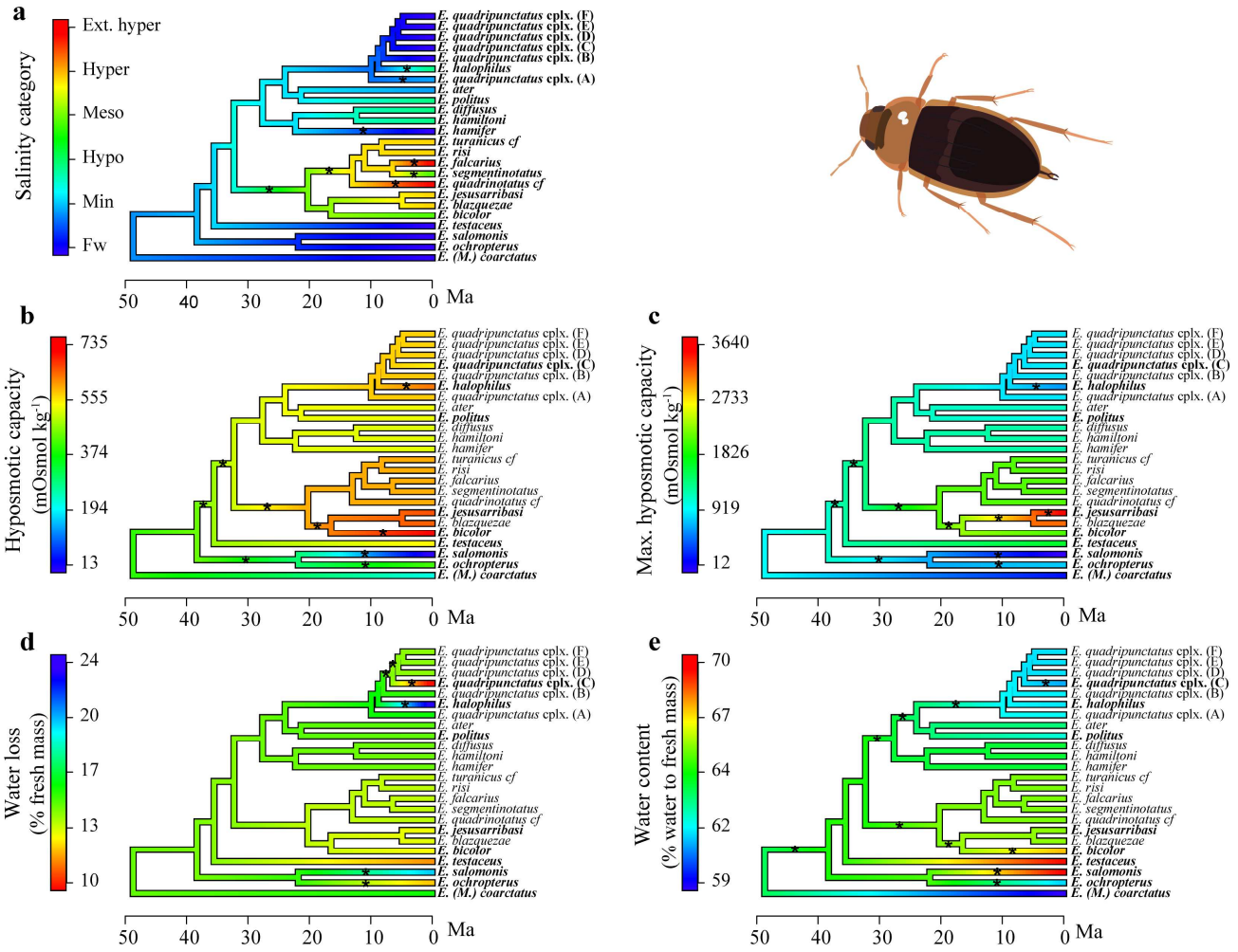

Figure 2. Ancestral reconstruction of desiccation and osmoregulation traits. The warmer (red) colours indicate higher resistance to desiccation or salinity than cooler (blue) colours. Branches where significantly accelerated increases or decreases in the rate of phenotypic change were identified (see 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.

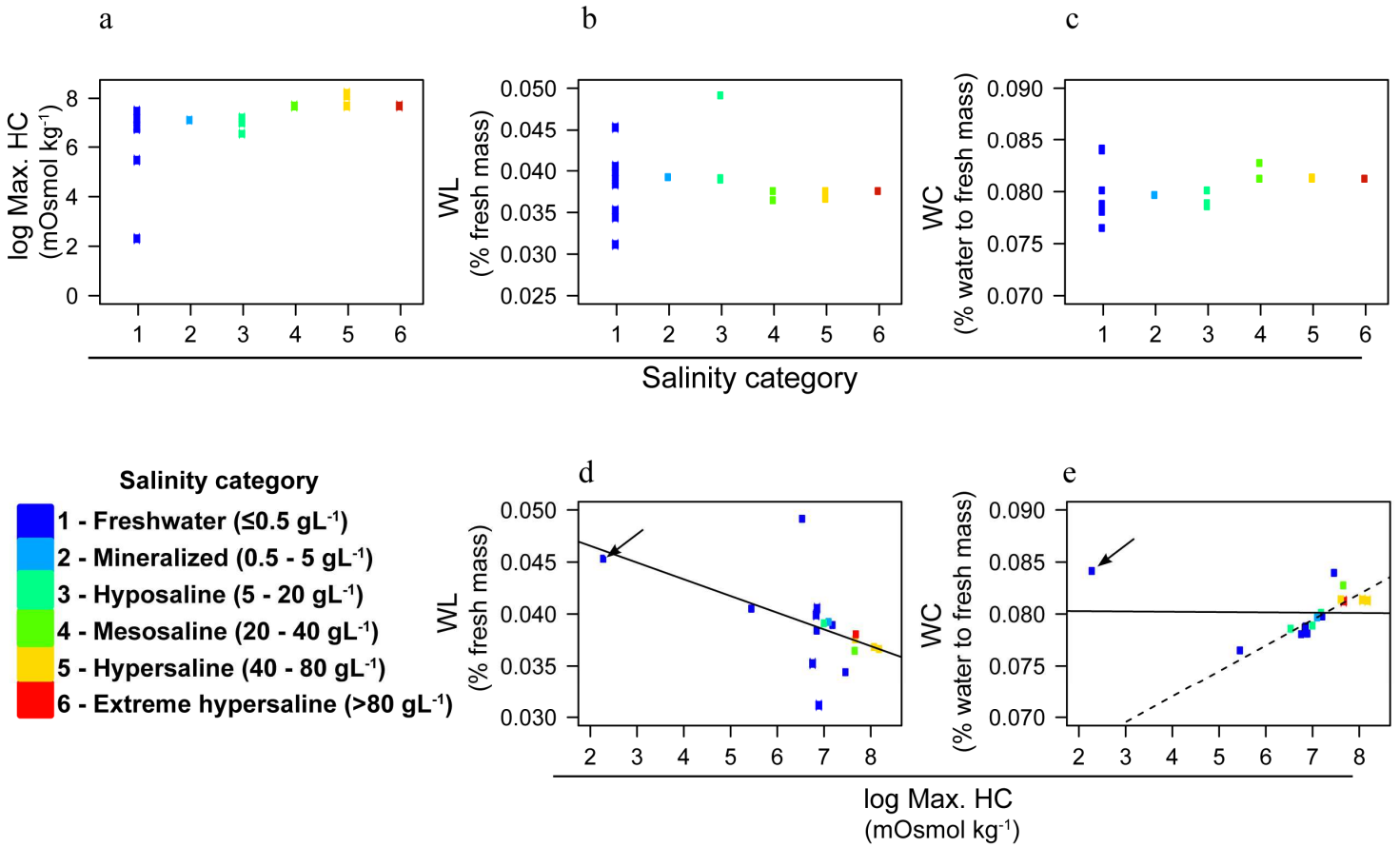

Figure 3. Relationships between habitat salinity, hyposmotic capacity and desiccation traits. Regression lines are shown for significant relationships in PGLS (see Table S6). Dashed line for regressions exluding *E. salomonis* (indicated by arrow). Max. HC: maximum hyposmotic capacity, WL: water loss,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

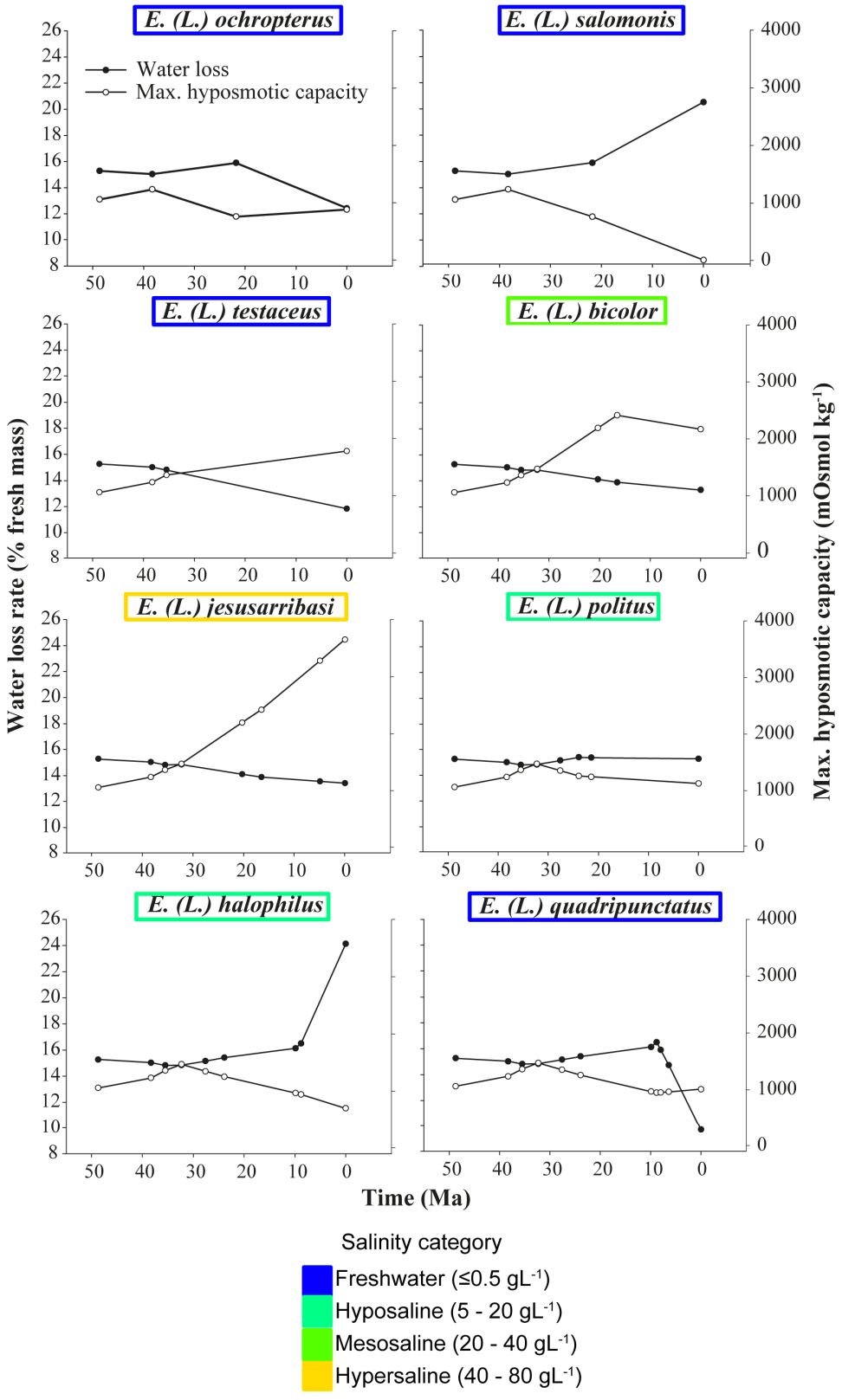

Figure 4. Values of water loss and maximum hyposmotic capacity through the full evolutionary path of the *Lumetus* species used in desiccation and osmoregulation experiments.

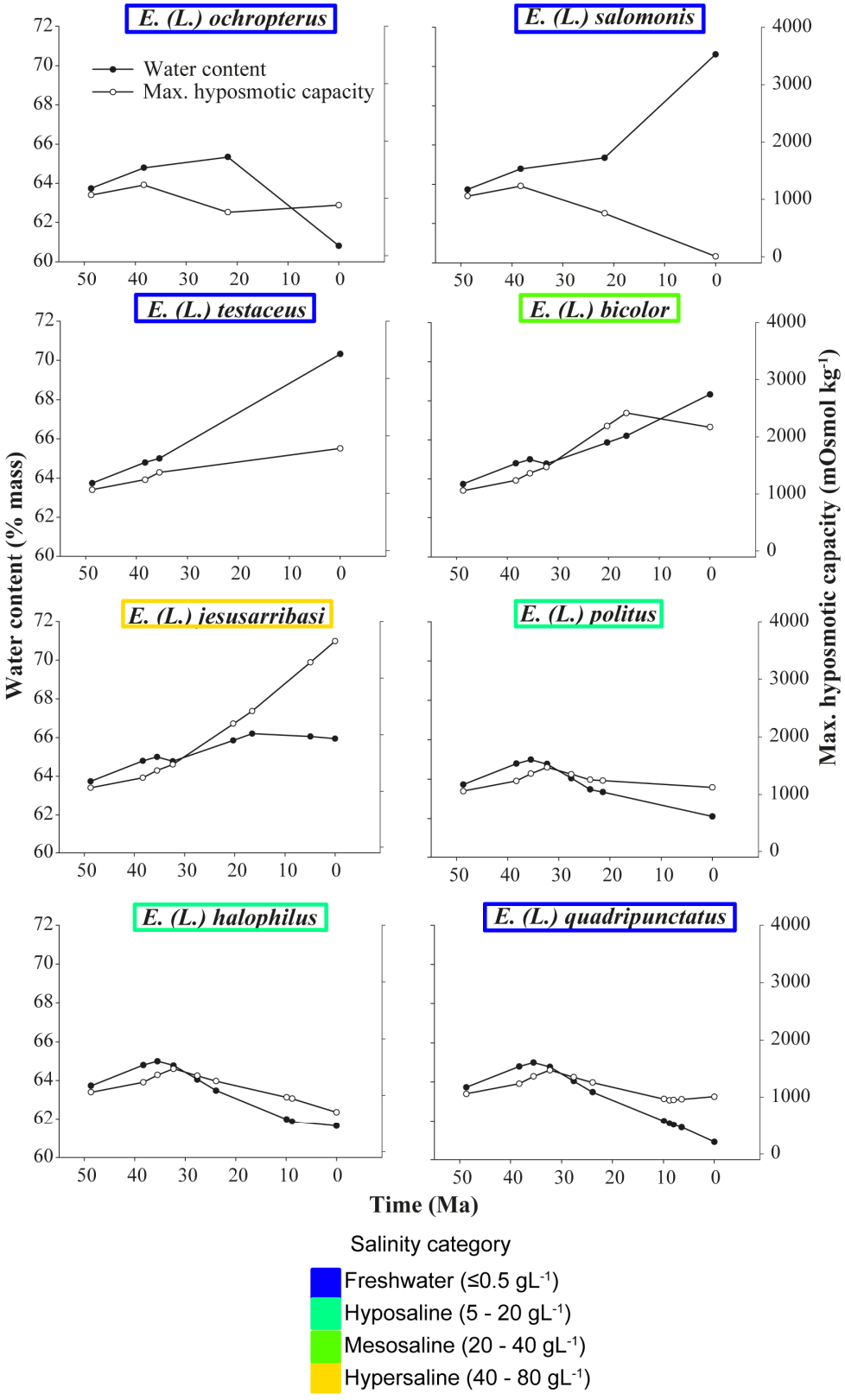

Figure 5. Values of water content and maximum osmotic capacity trough the full evolutionary path of the *Lumetus* species used in desiccation and osmoregulation experiments.

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 tress.

trait	σ^2	pairwise comparison	$LRT_{df=1}$	P	AICc
Max. HC	0.021 – 0.049				
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Table 2. Ranges of the estimated Pagel's λ (for the randomized sample of 1,000 post-burnin trees) and P-values for the likelihood ratio test comparing estimated λ with a model assuming λ = 0 or λ = 1 (for the consensus tree).

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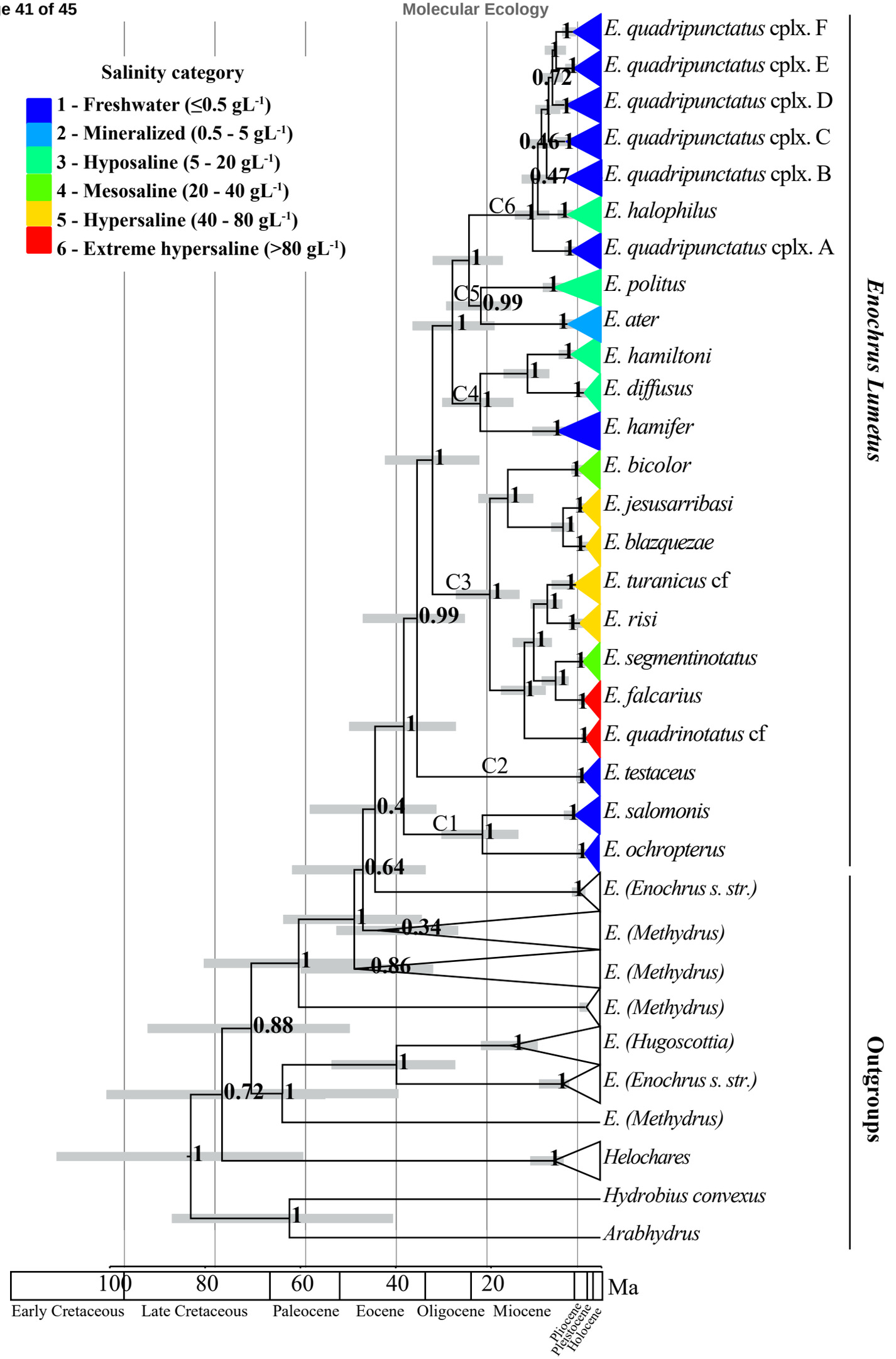
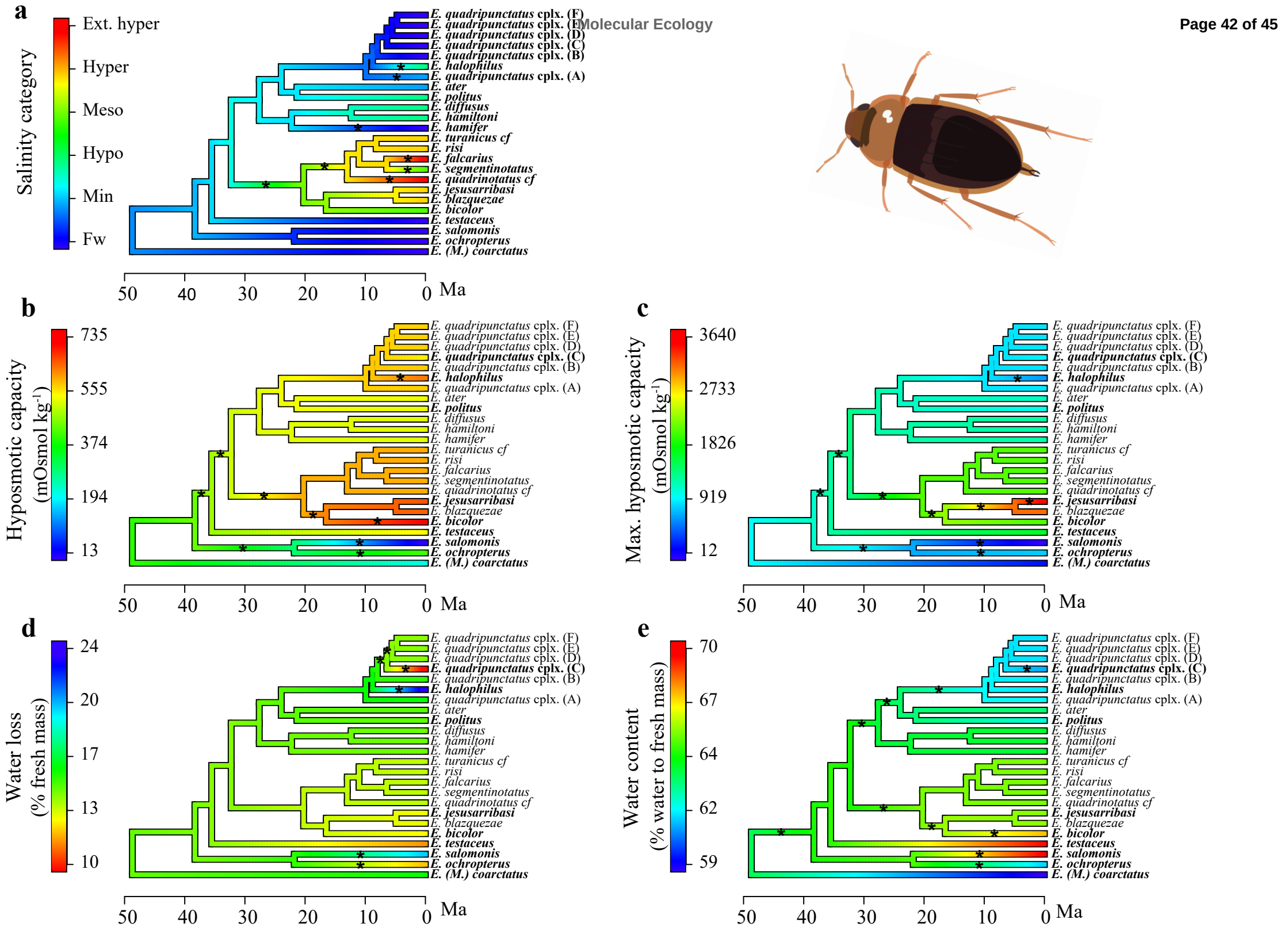
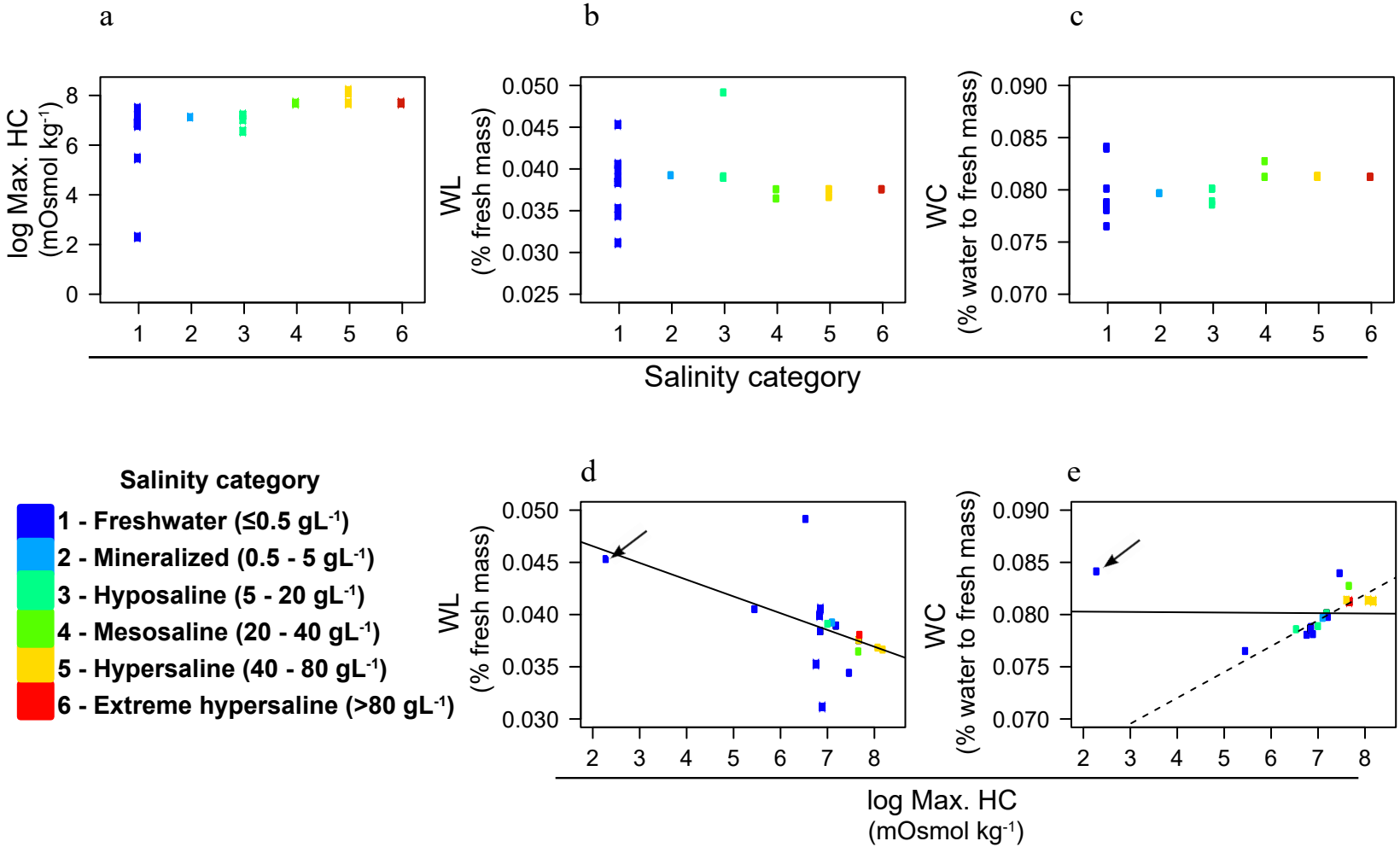
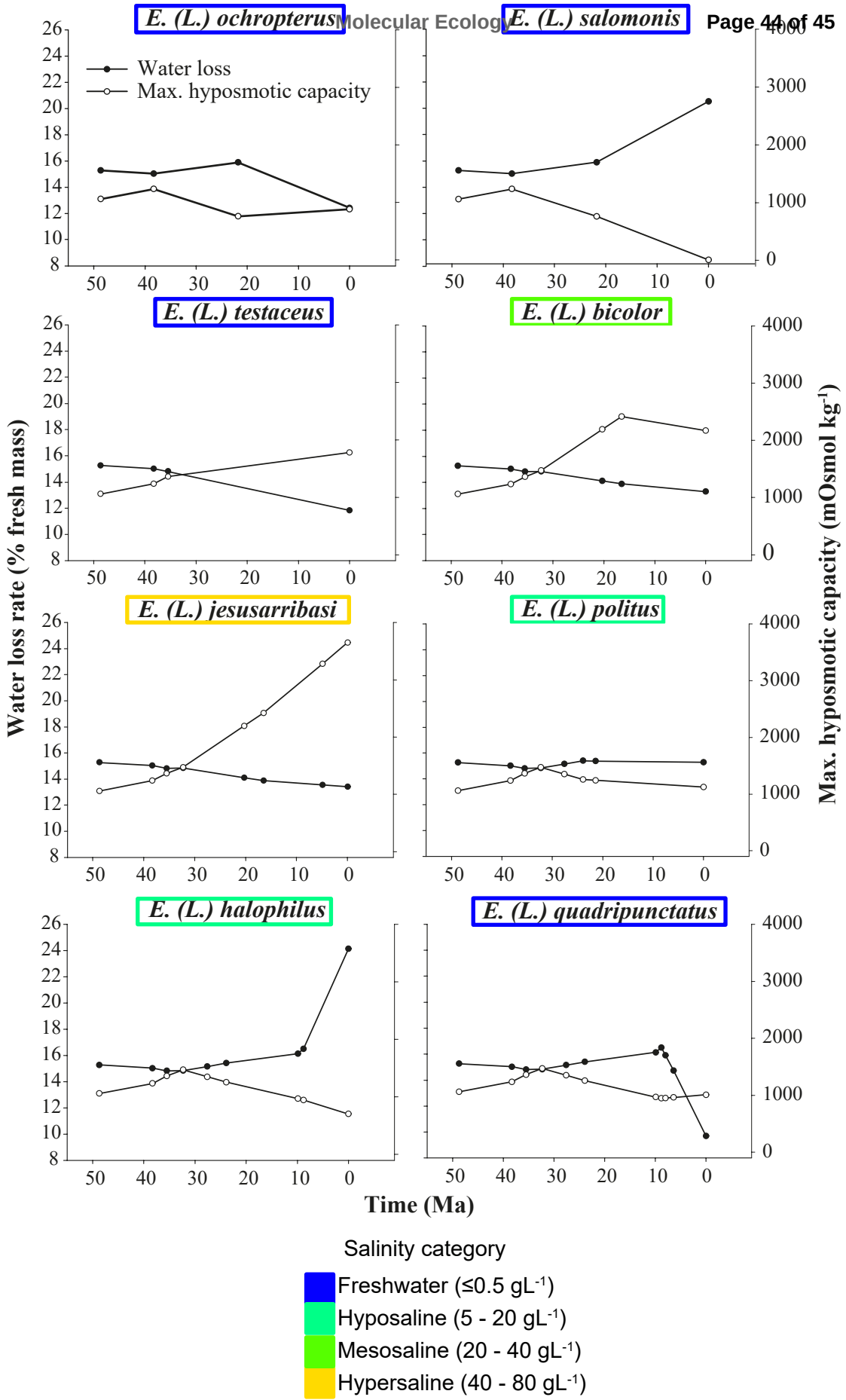
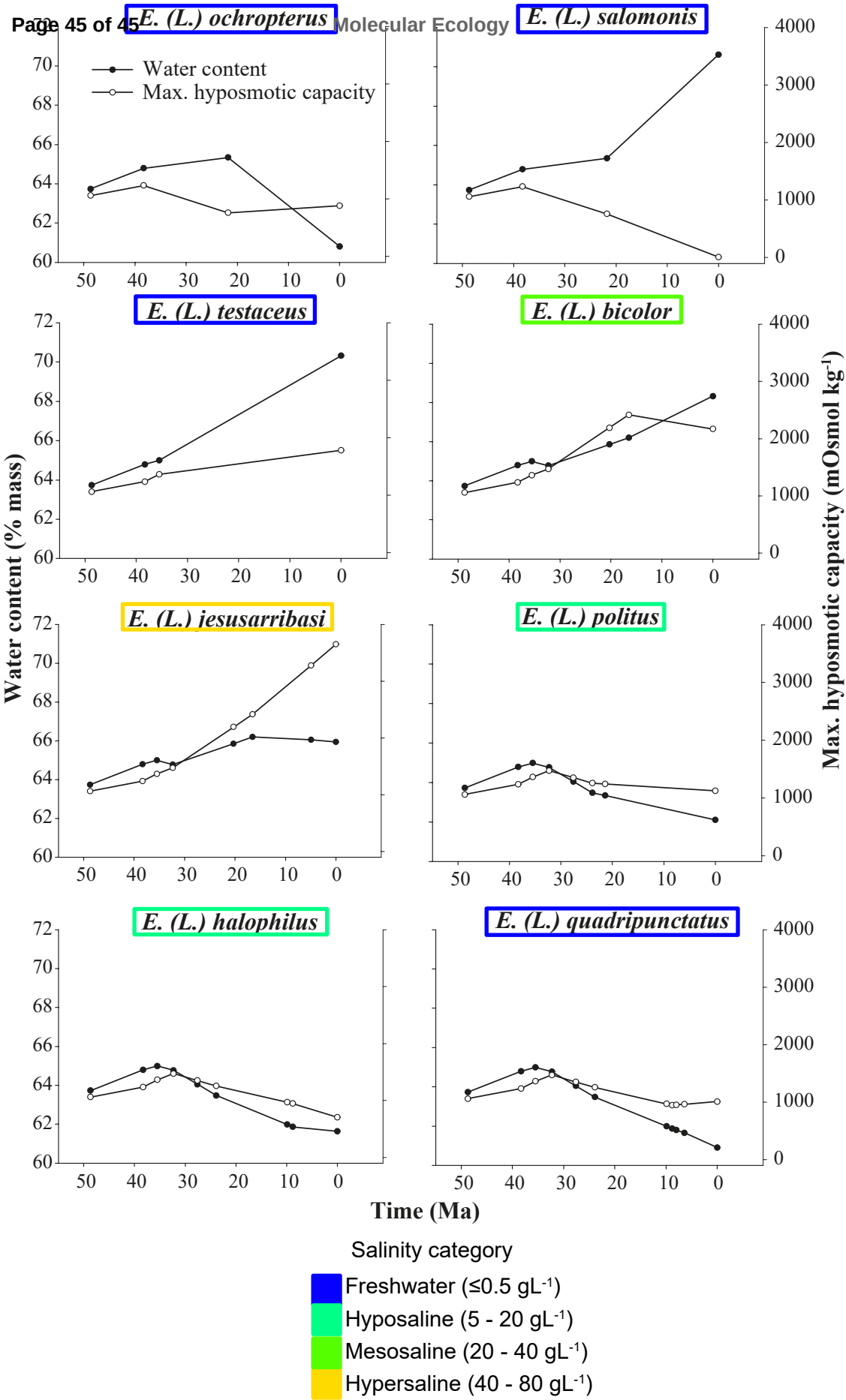
Max. hyposmotic capacity	1.04 – 1.13	< 0.001	0.051
Water loss	< 0.001	1	< 0.001
Water content	1.07 – 1.14	< 0.001	< 0.001


Salinity category
1 - Freshwater (≤0.5 gL-1)
2 - Mineralized (0.5 - 5 gL-1)
3 - Hyposaline (5 - 20 gL-1)
4 - Mesosaline (20 - 40 gL-1)
5 - Hypersaline (40 - 80 gL-1)
6 - Extreme hypersaline (>80 gL-1)
E. quadripunctatus cplx. F
E. quadripunctatus cplx. E
E. quadripunctatus cplx. D
E. quadripunctatus cplx. C
E. quadripunctatus cplx. B
E. halophilus
E. quadripunctatus cplx. A
E. politus
E. ater
E. hamiltoni
E. diffusus
E. hamifer
E. bicolor
E. jesusarribasi
E. blazquezae
E. turanicus cf
E. risi
E. segmentinotatus
E. falcarius
E. quadrinotatus cf
E. testaceus
E. salomonis
E. ochropterus
E. (Enochrus s. str.)
E. (Methydrus)
E. (Methydrus)
E. (Methydrus)
E. (Hugoscottia)
E. (Enochrus s. str.)
E. (Methydrus)
Helochares
Hydrobius convexus
Arabhydrus
C1
C2
C3
C4
C5
C6
0.72
0.46
0.47
0.99
0.99
0.4
0.64
0.34
0.86
0.88
0.72
Enochrus Lumetus
Outgroups
100
80
60
40
20
Ma
Early Cretaceous
Late Cretaceous
Paleocene
Eocene
Oligocene
Miocene
Pliocene
Pleistocene
Holocene


a
Salinity category
Ext. hyper
Hyper
Meso
Hypo
Min
Fw
E. quadripunctatus cplx. (F)
E. quadripunctatus cplx. (E)
E. quadripunctatus cplx. (D)
E. quadripunctatus cplx. (C)
E. quadripunctatus cplx. (B)
E. halophilus
E. quadripunctatus cplx. (A)
E. ater
E. politus
E. diffusus
E. hamiltoni
E. hamifer
E. turanicus cf
E. risi
E. falcarius
E. segmentinotatus
E. quadrinotatus cf
E. jesusarribasi
E. blazquezae
E. bicolor
E. testaceus
E. salomonis
E. ochropterus
E. (M.) coarctatus
50 40 30 20 10 0 Ma
b
Hyposmotic capacity (mOsmol kg-1)
735
555
374
194
13
c
Max. hyposmotic capacity (mOsmol kg-1)
3640
2733
1826
919
12
d
Water loss (% fresh mass)
24
20
17
13
10
e
Water content (% water to fresh mass)
70
67
64
62
59


a
b
c
log Max. HC (mOsmol kg-1)
WL (% fresh mass)
WC (% water to fresh mass)
Salinity category
Salinity category
1 - Freshwater (≤0.5 gL-1)
2 - Mineralized (0.5 - 5 gL-1)
3 - Hyposaline (5 - 20 gL-1)
4 - Mesosaline (20 - 40 gL-1)
5 - Hypersaline (40 - 80 gL-1)
6 - Extreme hypersaline (>80 gL-1)
d
e
WL (% fresh mass)
WC (% water to fresh mass)
log Max. HC
(mOsmol kg-1)


E. (L.) ochropterus
E. (L.) salomonis
E. (L.) testaceus
E. (L.) bicolor
E. (L.) jesusarribasi
E. (L.) politus
E. (L.) halophilus
E. (L.) quadripunctatus
Water loss
Max. hyposmotic capacity
Water loss rate (% fresh mass)
Max. hyposmotic capacity (mOsmol kg-1)
Time (Ma)
Salinity category
Freshwater (≤0.5 gL-1)
Hyposaline (5 - 20 gL-1)
Mesosaline (20 - 40 gL-1)
Hypersaline (40 - 80 gL-1)


E. (L.) ochropterus
E. (L.) salomonis
E. (L.) testaceus
E. (L.) bicolor
E. (L.) jesusarribasi
E. (L.) politus
E. (L.) halophilus
E. (L.) quadripunctatus
Water content
Max. hyposmotic capacity
Water content (% mass)
Max. hyposmotic capacity (mOsmol kg-1)
Time (Ma)
Salinity category
Freshwater (≤0.5 gL-1)
Hyposaline (5 - 20 gL-1)
Mesosaline (20 - 40 gL-1)
Hypersaline (40 - 80 gL-1)