



PEARL

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

Pallarés, Susana; Arribas, Paula; Bilton, David T.; Millán, Andrés; Velasco, Josefa; Ribera, Ignacio

Published in:
Molecular Ecology

DOI:
[10.1111/mec.14334](https://doi.org/10.1111/mec.14334)

Publication date:
2017

Link:
[Link to publication in PEARL](#)

Citation for published version (APA):

Pallarés, S., Arribas, P., Bilton, D. T., Millán, A., Velasco, J., & Ribera, I. (2017). The chicken or the egg? Adaptation to desiccation and salinity tolerance in a lineage of water beetles. *Molecular Ecology*, 0(0). <https://doi.org/10.1111/mec.14334>

All content in PEARL is protected by copyright law. Author manuscripts are made available in accordance with publisher policies. Wherever possible please cite the published version using the details provided on the item record or document. In the absence of an open licence (e.g. Creative Commons), permissions for further reuse of content should be sought from the publisher or author.

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

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

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

Running title: Evolution of hyporegulation in water beetles

1 ABSTRACT

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

24

25 INTRODUCTION

26 How organisms acquire novel traits or undergo adaptive trait divergence are central
27 questions in evolutionary ecology, as these processes facilitate niche shifts and the
28 colonisation of novel environments (Heard & Hauser 1995; Hunter 1998; Moczek
29 2008). In the aquatic realm, the evolution of hydric and osmotic regulation mechanisms
30 was a key innovation allowing transitions from marine to freshwater habitats in some
31 animal groups like fishes or crustaceans (e.g. Faria *et al.* 2011; McNamara & Faria
32 2012; Schultz & McCormick 2012). Similarly, but in the opposing direction, the
33 evolution of these mechanisms in inland aquatic lineages has allowed for transitions
34 from fresh to saline inland waters, a recurrent phenomenon in a number of aquatic
35 insect orders (e.g. Bradley *et al.* 2011). Most interestingly, such transitions to saline
36 waters seem to be much more frequent in some taxa than others, with closely related
37 genera either being entirely restricted to freshwaters, or spanning the fresh-hypersaline
38 gradient (see e.g. Arribas *et al.* 2014 for beetles; Carbonell *et al.* 2012 for water bugs; or
39 Herbst 1999 for flies). The physiological and evolutionary processes that may facilitate
40 the colonisation of extreme habitats such as saline waters remain poorly understood,
41 however, and require the study of relevant organismal traits within a phylogenetic
42 context (Cheng & Chen 1999; Tobler *et al.* 2011).

43 In insects, the main osmoregulatory adaptations are a highly impermeable cuticle and
44 a rectum capable of producing hyperosmotic excreta. These are ancestral characters,
45 found in virtually all insect lineages and are clearly essential to their success on land,
46 where desiccation is a major physiological stress factor. In contrast, tolerance to the
47 osmotic stress produced by a saline aquatic medium seems to be a very specialized
48 secondary adaptation, only present in a few insect orders (Bradley *et al.* 2009). In general,

49 insect species that show tolerance to salinities above that of seawater are efficient
50 hyporegulators, i.e. they are able to maintain the concentration of haemolymph below
51 that of the external medium and within a narrow range regardless of the external
52 osmotic concentration (e.g. Tones & Hammer 1975; Herbst *et al.* 1988; Pallarés *et al.*
53 2015). Ultimately, hyporegulation has the same physiological basis as mechanisms
54 dealing with dehydration in air, as both desiccation and hyperosmotic stress alter ionic
55 and water balance, with similar effects at the cellular level (Evans 2008; Bradley 2009;
56 Cohen 2012). Their common physiological basis likely lies in ion transport and cell
57 volume regulation processes (Beyenbach 2016; Griffith 2017), which in most insects
58 involve the activity of excretory organs, such as Malpighian tubules and the rectum, and
59 the control of cuticular permeability (Dow & Davies 2006; Gibbs & Rajpurohit 2010;
60 Larsen *et al.* 2014). Given the physiological similarities between mechanisms to cope
61 with salinity and desiccation stress and the frequent spatial and temporal co-occurrence
62 of both stressors, tolerance to them may be evolutionarily linked in some insect
63 lineages. In such cases, selection on the osmoregulatory system to deal with desiccation
64 stress could have secondarily facilitated hyporegulation at high salinities, or the other
65 way around.

66 The relationship between tolerance to salinity and desiccation has been mostly
67 studied in plants (e.g. Barrieu *et al.* 1999; Cayuela *et al.* 2007; Hossain *et al.* 2013) and
68 to a lesser extent in animal taxa (Gómez-Mestre & Tejedo 2005; Faria *et al.* 2017).
69 Despite the relevance of such relationship, to our knowledge, no previous studies have
70 addressed the potential evolutionary links between mechanisms to deal with salinity and
71 desiccation. However, recent studies on salinity tolerance in aquatic insects point to
72 their close association. Firstly, beetle adults (Pallarés *et al.* 2017) and dipteran larvae
73 (Elnitsky *et al.* 2009) sequentially exposed to salinity and desiccation showed cross-

74 tolerance responses (Sinclair *et al.* 2013; Todgham & Stillman 2013), suggesting a
75 mechanistic link between the response to both stressors. Secondly, a recent study
76 reconstructing the colonisation of saline waters by *Enochrus* water beetles
77 (Hydrophilidae) suggested that salinity tolerance arose during periods of global
78 aridification, when multiple independent transitions from fresh to saline waters
79 apparently occurred (Arribas *et al.* 2014). These authors also found a positive
80 correlation between the salinity of the preferred habitat of a species and the aridity of
81 the region over which it is distributed. Finally, in agreement with this ecological
82 correlation, Pallarés *et al.* (2016) revealed a positive relationship between desiccation
83 resistance and salinity tolerance in species of *Enochrus* in the laboratory.

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

97 The relationship between aridity and salinity demonstrated by Arribas *et al.* (2014)
98 was based only on ecological data (species habitat occupancies and regional climates),

99 which do not always fully reflect the potential physiological tolerance of species
100 (Carbonell *et al.* 2012; Céspedes *et al.* 2013). Mismatches between realised and
101 fundamental niches may result when physiological tolerance evolved as a result of prior
102 exposure to different stressors, since in such cases species may retain the ability to deal
103 with conditions different from those in their current habitats. Disentangling the
104 evolution of hyporegulation and desiccation resistance in organisms spanning the fresh-
105 saline spectrum is thus not straightforward, and requires an integrative approach, based
106 on the measurement of ecological and organismal traits within a sound phylogenetic
107 context – something which has not been attempted to date in any lineage.

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

117 1) The hyporegulation ability allowing the colonisation of saline waters was co-opted
118 from physiological mechanisms evolved originally for desiccation resistance.

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

121 3) Desiccation resistance and hyporegulation ability evolved in correlation.

122 In the first case, all species living in meso- or hypersaline waters should be efficient
123 hyporegulators and tolerant to desiccation, but the reverse needs not to be true (i.e. there
124 may be desiccation resistant species with low or no hyporegulation ability). In addition,
125 there could be species with high desiccation resistance and hyporegulation ability
126 primarily living in fresh - hyposaline waters (i.e. able to tolerate higher salinities even if
127 they -or their ancestors- have never occupied this type of habitat). In the phylogeny,
128 increases in hyporegulation ability may be expected to be preceded by increases in
129 desiccation resistance.

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

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

140 There could be a fourth possibility, namely that there was an independent evolution of
141 desiccation resistance and hyporegulation ability. There is, however, ample evidence for
142 the association between tolerance to desiccation and salinity in *Lumetus* (Arribas *et al.*
143 2014; Pallarés *et al.* 2016, 2017), allowing this possibility to be discarded *a priori*.

144 MATERIAL AND METHODS

145 **Taxon sampling**

146 A total of 220 specimens representing 18 of the 23 known species of the subgenus were
147 used to obtain the phylogeny of *Lumetus* (Table S1). Molecular data were obtained from
148 *de novo* sequencing of 64 specimens plus sequences from previous work (Arribas *et al.*
149 2012, 2013, 2014). Several *Enochrus* species of the subgenera *Methydrus*, *Enochrus*
150 and *Hugoscottia* and a related genus (*Helochares*) were used as outgroups, with two
151 more distantly related genera of Hydrophilidae, *Hydrobius* and *Arabhydrus* (Short &
152 Fikáček 2013) used to root the tree, resulting in a phylogeny of 43 species.

153 Data on hyporegulation ability and desiccation resistance were obtained
154 experimentally from adults of a representative subset of nine species (Table S2).
155 Studied species included at least one from each of the main *Lumetus* clades obtained in
156 preliminary phylogenetic analyses and one outgroup species from the subgenus
157 *Methydrus* (*Enochrus coarctatus*).

158 **Phylogeny of *Lumetus***

159 DNA from the new collected specimens was extracted and sequenced following the
160 methodology of Arribas *et al.* (2013, 2014). We sequenced five mitochondrial genes:
161 two non-overlapping fragments of the cytochrome c oxidase I gene corresponding to the
162 5' (cox1-A) and the 3' end (cox1-B); an internal fragment of the cytochrome b gene
163 (cyt b); and a fragment spanning three genes (5' end of the large ribosomal subunit plus
164 Leucine transferase and the 5' end of NADH dehydrogenase subunit 1; rrnL+trnL+
165 nad1). From nuclear DNA we sequenced an internal fragment of the large ribosomal
166 unit, 28S rRNA (LSU) and an internal fragment of the internal transcribed spacer 2
167 (ITS2) (Table S3).

168 Sequences were assembled and edited with Geneious 5.5.9 (Biomatters Ltd.
169 Auckland, New Zealand), using Ns (missing data) for ambiguous positions. Alignments
170 were obtained with the online version of MAFFT v.7 (Kato & 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

193 trees from both runs and to obtain 1,000 randomly resampled postburnin trees. The
194 consensus tree was estimated with Treeannotator (Drummond *et al.* 2012). The 25 %
195 initial trees were discarded as a burnin fraction, after checking for convergence in
196 Tracer v1.6 (Drummond *et al.* 2012).

197 **Ecological data, hyporegulation ability and desiccation resistance**

198 To track habitat transitions across the salinity gradient, each *Lumetus* species was
199 assigned a qualitative salinity category according to our field data or bibliographic data
200 on the salinity of their most frequently occupied habitats. We followed the same criteria
201 and categorization done by Arribas *et al.* (2014), with special attention to the records of
202 populations in habitats with the highest salinities, as these may better reflect species'
203 tolerance limits (Carbonell *et al.* 2012; Céspedes *et al.* 2013). Six categories were used,
204 freshwater (≤ 0.5 g/L), mineralized (0.5–5 g/L), hyposaline (5–20 g/L), mesosaline (20–
205 40 g/L), hypersaline (40–80 g/L) and extreme hypersaline (>80 g/L).

206 To determine the hyporegulation ability of the nine selected species (Table S2),
207 haemolymph osmolalities were measured in individuals exposed for 48 h to different
208 salinities within their specific tolerance ranges (as determined by pilot trials or previous
209 work, Pallarés *et al.* 2015). All species were exposed to at least two common
210 hyposmotic treatments (0.3 and 12 g L⁻¹) and a hyperosmotic one (35 g L⁻¹) to obtain
211 comparable osmolality measurements. For each species, the treatment in which
212 mortality exceeded 50% of the tested individuals was considered as the upper lethal
213 limit (e.g. Faria *et al.* 2017) (Table S4). From each treatment, we obtained haemolymph
214 samples from a minimum of three of the exposed individuals (Table S4), as pilot trails
215 showed low intraspecific variation within salinity treatments. Osmolality of the
216 haemolymph and the saline media were measured using a calibrated nanolitre

217 osmometer (Otago Osmometers, Dunedin, New Zealand). For each treatment, we
218 estimated the hyper- or hyposmotic capacity, i.e. the difference between the osmotic
219 concentration of the haemolymph and the external medium, which represents an
220 integrated measure of the physiological ability to compensate for the osmotic gradient
221 between internal and external media (Charmantier *et al.* 1984; Calosi *et al.* 2005). The
222 hyposmotic capacity at 35 g L⁻¹ (hyposmotic capacity hereafter) and the maximum
223 hyposmotic capacity (i.e. that measured at the highest salinity tolerated by each species)
224 showed the highest variation between species and were therefore used for subsequent
225 analyses.

226 Controlled desiccation experiments were conducted as described by Pallarés *et al.*
227 (2016). Specimens were exposed to desiccation at 20±5 % RH (relative humidity),
228 20±1°C for 6 h. For each specimen, we measured the initial and final fresh mass (i.e.
229 specimen mass before and after desiccation treatments) as well as dry mass. From these
230 measurements, we obtained the initial water content as the % wet mass (difference
231 between fresh and dry mass) relative to initial fresh mass and water loss as the % of
232 water lost relative to initial fresh mass. These variables, and in particular water loss,
233 have previously been shown to be relevant for desiccation resistance in *Lumetus* species
234 (Pallarés *et al.* 2016, 2017). Specimens were allowed to recover at freshwater conditions
235 for 24 h after desiccation. Mortality was assessed after both desiccation and the
236 recovery period. These estimates were obtained for 20-30 specimens per species (Table
237 S4).

238 After each experiment, specimens were sexed by examining genitalia under a Leica
239 M165C stereomicroscope. Further details of the experimental procedures are indicated
240 in the supplementary material (Data S1).

241 **Habitat transitions, evolution of desiccation resistance and osmoregulatory**
242 **capacity**

243 *Ancestral trait reconstruction.* We tested different models of trait evolution (Brownian
244 motion – BM and Ornstein-Uhlenbeck – OU) (Kaliontzopoulou *et al.* 2016) to
245 reconstruct ancestral values of habitat salinity (considered as a semi-continuous
246 variable), hyposmotic capacity and desiccation resistance traits. Intraspecific variation,
247 missing observations and small tree size can profoundly affect the performance of such
248 models (Boettiger *et al.* 2012; Cooper *et al.* 2016). To account for this, we used a
249 Monte-Carlo based approach to assess the power of our data to distinguish between the
250 models tested. We compared the distribution of δ (i.e. the difference in log likelihood of
251 observing the data under the two maximum likelihood estimate models) from Monte
252 Carlo simulations (n= 1,000 replicates) using *pmc* (Phylogenetic Monte Carlo) in R
253 (Boettiger *et al.* 2012). When there was insufficient power to distinguish between
254 models, the simplest (i.e. BM) was used. Ancestral trait reconstructions were made
255 using the R function *phylopars* (package Rphylopars, Bruggeman *et al.* 2009; Goolsby
256 *et al.* 2016), which uses a maximum likelihood-based method to estimate trait
257 covariance across (phylogenetic covariance) and within species (phenotypic covariance)
258 for datasets with missing data and multiple within-species observations (e.g. Pollux *et*
259 *al.* 2014). This method provides predicted trait values and variances for ancestral nodes
260 and unmeasured extant species (Penone *et al.* 2014). Trees were pruned to keep one
261 representative specimen per putative species in order to fix the species level resolution
262 of the physiological traits. Outgroup species with missing physiological and ecological
263 data were excluded. Multiple trait observations per species were included to account for
264 inter-individual variation and measurement error (Bruggeman *et al.* 2009).

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

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

289 *Relationships between traits.* Phylogenetic generalized least squares (PGLS) were
290 applied, using the R function *ppls* (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

312 clade of saline species (the *E. bicolor* group, clade C3) from one including three
313 subclades of Nearctic and Palaeartic species (clades C4-C6). Within these groups, both
314 short branches and node age estimations suggest rapid diversification in the Oligocene-
315 Miocene, around 27–5 Ma. The *E. quadripunctatus* group (clade C6) was formed of 6
316 recently diverged lineages (the *E. quadripunctatus* complex) with well characterised
317 geographical distributions. These included (A) a coastal Mediterranean clade; (B)
318 another containing a single specimen from Canada; two Eurasian clades, one (C) widely
319 distributed and another (D) restricted to Bulgaria and Turkey; (E) a clade apparently
320 restricted to Italy; and (F) an Ibero-Moroccan clade. Sequence length, number of
321 variable sites and the estimated substitution rates for each partition are provided in
322 Table S5.

323 **Hyporegulation ability and desiccation resistance**

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

334 **Habitat transitions, evolution of desiccation resistance and hyporegulation** 335 **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 ($423 \text{ mOsmol kg}^{-1}$ at
346 35 g L^{-1} , Figs 2b and S5), but within a limited salinity range (maximum estimated
347 hyposmotic capacity of $1,000 \text{ mOsmol kg}^{-1}$, 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-Palaeartic 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 ($\lambda = 0$), indicating a significant phylogenetic signal (Table 2). For hyposmotic
373 capacity and water content, estimated λ s 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. $\lambda = 1$) 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

385 relationships were strongly influenced by the outlier values that one species, *E.*
386 *salomonis*, showed for these variables. After removing this species from PGLS, the
387 relationship with water loss was not significant and the relationship with water content
388 became stronger and significantly positive for all the analyzed trees (Table S7, Fig. 3d-
389 e).

390 When the relative order of appearance of changes in desiccation resistance and
391 maximum hyposmotic capacity was traced across individual branches of the phylogeny
392 (Figs 4 and 5), increases in hypoosmotic capacity were not clearly preceded by
393 increases in desiccation resistance nor *vice versa*. Among the species with the highest
394 hyporegulation ability (*E. testaceus*, *E. bicolor* and *E. jesusarribasi*), the increase in
395 hyposmotic capacity along their evolutionary path was coupled with parallel decreases
396 in water loss and increases in water content, suggesting an associated increase in
397 desiccation resistance. On the contrary, increases in desiccation resistance were not
398 always associated with an increase in hyposmotic capacity, as in e.g. *E. ochopterus* and
399 *E. quadripunctatus* in Fig. 4, or *E. salomonis* in Fig. 5.

400 **DISCUSSION**

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

406 The ancestral reconstruction of water loss suggests that the most common recent
407 ancestor of *Lumetus* had similar desiccation resistance to extant species of the subgenus.
408 Water loss did not change abruptly through the evolutionary history of the lineage, but

409 had instead apparently remained relatively stable, as suggested by the lack of
410 phylogenetic signal in this trait. The control of water loss has been previously reported
411 as essential for survival in some *Lumetus* species (Pallarés *et al.* 2016), which show
412 comparable water loss rates to those reported for the highly desiccation resistant aquatic
413 beetle *Peltodytes muticus* (Arlan & Staiger 1979). The hypersaline *Enochrus*
414 *jesusarribasi* has much lower water loss rates and higher resistance to desiccation than
415 hypersaline diving beetles studied to date (Pallarés *et al.* 2017), which seem to have
416 more permeable cuticles than *Enochrus* species (Botella-Cruz *et al.* 2017). Our data
417 suggest a high resistance to desiccation in the whole *Lumetus* subgenus, something
418 which could be a plesiomorphic character present in the wider genus *Enochrus*, or even
419 the Hydrophilidae itself. Despite the lack of data on desiccation resistance of other
420 hydrophilids, the unusually frequent transitions between terrestrial and aquatic
421 environments within this family (Bernhard *et al.* 2006; Short & Fikacek 2013) would be
422 in agreement with this hypothesis.

423 The ancestor of *Lumetus* was inferred to have lived in mineralized waters, and to
424 have had moderate hyporegulation ability. In contrast to the low variation in water loss,
425 hyporegulation ability underwent large and, in some cases, accelerated changes through
426 the evolutionary history of *Lumetus*, most of these being associated with habitat
427 transitions across the salinity gradient. Arribas *et al.* (2014) found that transitions to
428 saline habitats in the *E. bicolor* group occurred at a higher rate than habitat transitions in
429 the rest of the lineage. In agreement with this result, we found that transitions from
430 fresh-mineralized to mesosaline waters and the subsequent diversification of these
431 beetles in saline habitats were associated with rapid increases in their hyporegulation
432 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 (Finarely & 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

483 with reduced hyporegulation ability, such as *E. ochropterus*. Nevertheless, further
484 research identifying potential gene expression pathways related with either desiccation
485 (e.g. López-Martínez *et al.* 2009) or salinity stress (e.g. Uyhelhi *et al.* 2016), as well as
486 those common to both stressors, would be needed to shed light on the degree of
487 mechanistic overlap between desiccation and salinity tolerances.

488 Parallel increases in desiccation resistance and salinity tolerance could have been
489 strengthened instead as a response to aridification during the radiation of *Lumetus*.
490 According to Arribas *et al.* (2014), and in agreement with our results, desiccation
491 resistance and hyporegulation ability in the *E. bicolor* group started to increase in
492 parallel in the Late Eocene, a period of global aridification (Mosbrugger *et al.* 2005;
493 Bosboom *et al.* 2014). Temporary habitats were presumably more abundant during
494 such arid periods, which, together with an increase in the mineralization of the surface
495 waters in some populations of these *Lumetus* species, could have posed a strong
496 selective pressure on a further development of existing mechanisms to deal with saline
497 stress and periodic exposure to desiccation. Other studies have proposed that global
498 aridification events promoted diversification of several aquatic taxa (e.g. Pinceel *et al.*
499 2013; Dorn *et al.* 2014). Aridification, by enhancing the linked tolerance of desiccation
500 and salinity, could have also been a key driver in the diversification of *Lumetus*.

501 Euryhalinity is also an important source of evolutionary diversity (Schultz &
502 McCormick 2012; Brauner *et al.* 2013). However, the process of adaption to saline
503 inland waters seems to be a unidirectional path, likely reflecting trade-offs between
504 competitive ability and tolerance to osmotic stress (Dunson & Travis 1991; Herbst
505 2001; Latta *et al.* 2012). In general, species of *Lumetus* (and other beetle genera) typical
506 of hypersaline waters are almost absent from freshwater habitats, despite been able to
507 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

533 show here that the linked evolution of stress resistance traits could have been key for
534 developing tolerance to extreme salinities.

535 **Acknowledgements**

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

545 **REFERENCES**

- 546 Adams DC (2013) Comparing evolutionary rates for different phenotypic traits on a
547 phylogeny using likelihood. *Systematic Biology*, **62**, 181-192.
- 548 Albers MA, Bradley TJ (2011) On the evolution of saline tolerance in the larvae of
549 mosquitoes in the genus *Ochlerotatus*. *Physiological and Biochemical Zoology*,
550 **84**, 258-267.
- 551 Andújar C, Serrano J, Gómez-Zurita J (2012) Winding up the molecular clock in the
552 genus *Carabus* (Coleoptera: Carabidae): assessment of methodological decisions
553 on rate and node age estimation. *BMC Evolutionary Biology*, **12**, 40.
- 554 Arlian L, Staiger T (1979) Water balance in the semiaquatic beetle, *Peltodytes muticus*.
555 *Comparative Biochemistry and Physiology Part A: Molecular & Integrative*
556 *Physiology*, **62A**, 1041-1047.
- 557 Arribas P, Andújar C, Abellán P *et al.* (2014) Tempo and mode of the multiple origins
558 of salinity tolerance in a water beetle lineage. *Molecular Ecology*, **23**, 360-373.
- 559 Arribas P, Andújar C, Sánchez-Fernández D *et al.* (2013) Integrative taxonomy and
560 conservation of cryptic beetles in the Mediterranean region (Hydrophilidae).
561 *Zoologica Scripta*, **42**, 182-200.

- 562 Arribas P, Velasco J, Abellán P *et al.* (2012) Dispersal ability rather than ecological
563 tolerance drives differences in range size between lentic and lotic water beetles
564 (Coleoptera: Hydrophilidae) *Journal of Biogeography*, **39**, 984–994.
- 565 Barrett RD, Schluter D (2008) Adaptation from standing genetic variation. *Trends in*
566 *Ecology and Evolution*, **23**, 38–44.
- 567 Barrieu F, Marty-Mazars D, Thomas D *et al.* (1999) Desiccation and osmotic stress
568 increase the abundance of mRNA of the tonoplast aquaporin BobTIP26-1 in
569 cauliflower cells. *Planta*, **209**, 77–86.
- 570 Bernhard D, Schmidt C, Korte A *et al.* (2006) From terrestrial to aquatic habitats and
571 back again – molecular insights into the evolution and phylogeny of
572 Hydrophiloidea (Coleoptera) using multigene analyses. *Zoologica Scripta*, **35**,
573 597–606
- 574 Beyenbach KW (2016) The plasticity of extracellular fluid homeostasis in insects.
575 *Journal of Experimental Biology*, **219**, 2596–2607.
- 576 Boettiger C, Coop G, Ralph P (2012) Is your phylogeny informative? Measuring the
577 power of comparative methods. *Evolution*, **66**, 2240–2251.
- 578 Bosboom RE, Abels HA, Hoorn C *et al.* (2014) Aridification in continental Asia after
579 the Middle Eocene Climatic Optimum (MECO). *Earth and Planetary Science*
580 *Letters*, **389**, 34–42.
- 581 Botella-Cruz M, Villastrigo A, Pallarés S *et al.* (2017) Cuticle hydrocarbons in saline
582 aquatic beetles. *PeerJ*, **5**, e3562.
- 583 Bradley TJ (2009) *Animal osmoregulation*. Oxford University Press, New York, NY,
584 USA.
- 585 Bradley TJ, Briscoe AD, Brady SG *et al.* (2009) Episodes in insect evolution.
586 *Integrative and Comparative Biology*, **49**, 590–606.
- 587 Brauner CJ, Gonzales RJ, Wilson JM (2013) Extreme environments; hypersaline,
588 alkaline and ion poor waters. In: *Fish Physiology, Vol. 32, Euryhaline Fishes* (ed.
589 by S.D. McCormick, A.P. Farrell & C.J. Brauner), pp. 433–474. Elsevier, New
590 York, NY, USA.
- 591 Bruggeman J, Heringa J, Brandt BW (2009) PhyloPars: estimation of missing parameter
592 values using phylogeny. *Nucleic Acids Research*, **37**, 179–184.
- 593 Calosi P, Ugolini A, Morritt D (2005) Physiological responses to hyposmotic stress in
594 the supralittoral amphipod *Talitrus saltator* (Crustacea: Amphipoda) *Comparative*
595 *Biochemistry and Physiology A*, **142**, 267–275.
- 596 Carbonell JA, Millán A, Velasco J (2012) Concordance between realised and
597 fundamental niches in three Iberian *Sigara* species (Hemiptera: Corixidae) along a
598 gradient of salinity and anionic composition. *Freshwater Biology*, **57**, 2580–2590.

- 599 Cayuela E, Muñoz□Mayor A, Vicente□Agulló F *et al.* (2007) Drought pretreatment
600 increases the salinity resistance of tomato plants. *Journal of Plant Nutrition and*
601 *Soil Science*, **170**, 479-484.
- 602 Céspedes V, Pallarés S, Arribas P *et al.* (2013) Water beetle tolerance to salinity and
603 anionic composition and its relationship to habitat occupancy. *Journal of Insect*
604 *Physiology*, **59**, 1076–1084.
- 605 Charmantier G, Charmantier-Daures M, Aiken DE (1984) Variation des capacités
606 osmoregulatrices des larves et postlarves de *Homarus americanus* Milnes-
607 Edwards, 1837 (Crustacea, Decapoda) *Comptes Rendus de l'Académie des*
608 *Sciences*, **299**, 863–866.
- 609 Cheng CHC, Chen L (1999) Evolution of an antifreeze glycoprotein. *Nature*, **401**, 463-
610 464.
- 611 Cohen E (2012) Roles of aquaporins in osmoregulation, desiccation and cold hardiness
612 in insects. *Entomology, Ornithology and Herpetology*, **S1**, 1–17.
- 613 Cooper N, Thomas GH, Venditti C *et al.* (2016) A cautionary note on the use of
614 Ornstein Uhlenbeck models in macroevolutionary studies. *Biological Journal of*
615 *the Linnean Society*, **118**, 64–77.
- 616 Divino JN, Monette MY, McCormick SD *et al.* (2016) Osmoregulatory physiology and
617 rapid evolution of salinity tolerance in threespine stickleback recently introduced
618 to fresh water. *Evolutionary Ecology Research*, **17**, 179–201.
- 619 Dorn A, Musilová Z, Platzer M *et al.* (2014) The strange case of East African annual
620 fishes: aridification correlates with diversification for a savannah aquatic group?
621 *BMC Evolutionary Biology*, **14**, 210.
- 622 Dow JAT, Davies SA (2006) The Malpighian tubule: rapid insights from post-genomic
623 biology. *Journal of Insect Physiology*, **52**, 365-378.
- 624 Dowse R, Palmer CG, Hills K *et al.* (2017) The mayfly nymph *Austrophlebioides*
625 *pusillus* Harker defies common osmoregulatory assumptions. *Royal Society Open*
626 *Science*, **4**, 160520.
- 627 Drummond AJ, Suchard MA, Xie D *et al.* (2012) Bayesian Phylogenetics with BEAUti
628 and the BEAST 1.7. *Molecular Biology and Evolution*, **29**, 1969–1973.
- 629 Dunson WA, Travis J (1991) The role of abiotic factors in community organization. *The*
630 *American Naturalist*, **138**, 1067-1091.
- 631 Elnitsky MA, Benoit JB, Lopez-Martinez G *et al.* (2009) Osmoregulation and salinity
632 tolerance in the Antarctic midge, *Belgica antarctica*: seawater exposure confers
633 enhanced tolerance to freezing and dehydration. *Journal of Experimental Biology*,
634 **212**, 2864–2871.
- 635 Evans DH (2008) *Osmotic and ionic regulation: cells and animals*. CRC Press, Boca
636 Raton, FL, USA.

- 637 Faria SC, Augusto AS, McNamara JC (2011) Intra- and extracellular osmotic regulation
638 in the hololimnetic *Caridea* and *Anomura*: a phylogenetic perspective on the
639 conquest of fresh water by the decapod Crustacea. *Journal of Comparative*
640 *Physiology B*, **181**, 175–186.
- 641 Faria SC, Provete DB, Thurman CL *et al.* (2017) Phylogenetic patterns and the adaptive
642 evolution of osmoregulation in fiddler crabs (*Brachyura*, *Uca*) *PLoS ONE*, **12**,
643 e0171870.
- 644 Finarelli JA, Goswami A (2013) Potential pitfalls of reconstructing deep time
645 evolutionary history with only extant data, a case study using the Canidae
646 (Mammalia, Carnivora) *Evolution*, **67**, 3678–3685.
- 647 Freckleton RP, Harvey PH, Pagel M (2002) Phylogenetic analysis and comparative
648 data: a test and review of evidence. *American Naturalist*, **160**, 712–726.
- 649 Gibbs AG, Rajpurohit S (2010). Cuticular lipids and water balance. In: *Insect*
650 *Hydrocarbons: Biology, Biochemistry, and Chemical Ecology* (ed. by G. J.
651 Blomquist & A. G. Bagnères), pp. 100-120. Cambridge University Press,
652 Cambridge, UK.
- 653 Glenner H, Thomsen PF, Hebsgaard MB *et al.* (2006) The origin of insects. *Science*,
654 **314**, 1883–1884.
- 655 Gómez-Mestre I, Tejedo M (2005) Adaptation or exaptation? An experimental test of
656 hypotheses on the origin of salinity tolerance in *Bufo calamita*. *Journal of*
657 *Evolutionary Biology*, **18**, 847–855.
- 658 Goolsby EW, Bruggeman J, Ane C (2017) Rphylopar: Fast multivariate phylogenetic
659 comparative methods for missing data and within-species variation. *Methods in*
660 *Ecology and Evolution*, **8**, 22–27.
- 661 Griffith MB (2017) Toxicological perspective on the osmoregulation and ionoregulation
662 physiology of major ions by freshwater animals: teleost fish, crustacea, aquatic
663 insects, and mollusca. *Environmental Toxicology and Chemistry*, **36**, 576–600.
- 664 Hadley NF (1994) *Water relations of terrestrial arthropods*. Academic Press, San
665 Diego, CA, USA.
- 666 Heard SB, Hauser DL (1995) Key evolutionary innovations and their ecological
667 mechanisms. *Historical Biology*, **10**, 151–173.
- 668 Hebert PDN, Remigio EA, Colbourne JK *et al.* (2002) Accelerated molecular evolution
669 in halophilic crustaceans. *Evolution*, **56**, 909-926.
- 670 Herbst DB, Conte FP, Brookes VJ (1988) Osmoregulation in an alkaline salt lake insect,
671 *Ephydra* (*Hydropyrus*) *hians* Say (Diptera: Ephydridae) in relation to water
672 chemistry. *Journal of Insect Physiology*, **34**, 903– 909.
- 673 Herbst DB (1999) Biogeography and physiological adaptations of the brine fly genus
674 *Ephydra* (Diptera: Ephydridae) in saline waters of the Great Basin. *Great Basin*
675 *Naturalist*, **59**, 127–135.

- 676 Herbst DB (2001) Gradients of salinity stress, environmental stability and water
677 chemistry as a template for defining habitat types and physiological strategies in
678 inland salt waters. *Hydrobiologia*, **466**, 209–219.
- 679 Hossain MA, Mostofa MG, Fujita M. (2013) Cross Protection by Cold-shock to Salinity
680 and Drought Stress-induced Oxidative Stress in Mustard (*Brassica campestris* L.)
681 Seedlings. *Molecular Plant Breeding*, **4**, 50-70.
- 682 Hunter JP (1998) Key Innovations and the Ecology of Macroevolution. *Trends in*
683 *Ecology and Evolution* **13**, 31–36.
- 684 Kaliontzopoulou A, Adams DC (2016) Phylogenies, the comparative method, and the
685 conflation of tempo and mode. *Systematic Biology*, **65**, 1–15.
- 686 Katoh K, Toh H (2008) Recent developments in the MAFFT multiple sequence
687 alignment program. *Briefings in Bioinformatics*, **9**, 286–298.
- 688 Kellermann V, Overgaard J, Loeschcke V *et al.* (2013) Trait associations across
689 evolutionary time within a *Drosophila* phylogeny: correlated selection or genetic
690 constraint? *PLoS ONE*, **8**, e72072.
- 691 Lanfear R, Calcott B, Ho SY *et al.* (2012) Partition Finder: combined selection of
692 partitioning schemes and substitution models for phylogenetic analyses.
693 *Molecular Biology and Evolution*, **29**, 1695–1701.
- 694 Larsen EH, Deaton LE, Onken H *et al.* (2014) Osmoregulation and Excretion.
695 *Comprehensive Physiology*, **4**, 405-573.
- 696 Latta LC, Weider LJ, Colbourne JK *et al.* (2012) The evolution of salinity tolerance in
697 *Daphnia*: a functional genomics approach. *Ecology Letters*, **15**, 794–802.
- 698 Lee CE, Kiergaard MG, Gelembiuk W *et al.* (2011) Pumping ions: rapid parallel
699 evolution of ionic regulation following habitat invasions. *Evolution*, **65**, 2229–
700 2244.
- 701 Lopez-Martinez G, Benoit JB, Rinehart JP *et al.* (2009) Dehydration, rehydration, and
702 overhydration alter patterns of gene expression in the Antarctic midge, *Belgica*
703 *antarctica*. *Journal of Comparative Physiology B*, **179**, 481–491.
- 704 McNamara J, Faria S (2012) Evolution of osmoregulatory patterns and gill ion transport
705 mechanisms in the decapod Crustacea: A review. *Journal of Comparative*
706 *Physiology B*, **182**, 997-1014.
- 707 Miller MA, Pfeiffer W, Schwartz T (2010) Creating the CIPRES Science Gateway for
708 inference of large phylogenetic trees. *Proceedings of the Gateway Computing*
709 *Environments Workshop (GCE), 14 November 2010*, pp. 1–8. New Orleans, LA.
- 710 Moczek AP (2008) On the origins of novelty in development and evolution. *Bioessays*,
711 **30**, 432–447.

- 712 Mosbrugger V, Utescher T, Dilcher DL (2005) Cenozoic continental climatic evolution
713 of Central Europe. *Proceedings of the National Academy of Sciences of the USA*,
714 **102**, 14964–14969.
- 715 Pagel M (1999) Inferring the historical patterns of biological evolution. *Nature*, **401**,
716 877–884
- 717 Pallarés S, Arribas P, Bilton DT *et al.* (2015) The comparative osmoregulatory ability
718 of two water beetle genera whose species span the fresh-hypersaline gradient in
719 inland waters (Coleoptera: Dytiscidae, Hydrophilidae) *PLoS ONE*, **10**, e0124299.
- 720 Pallarés S, Botella-Cruz M, Arribas P *et al.* (2017) Aquatic insects in a multistress
721 environment: cross-tolerance to salinity and desiccation. *Journal of Experimental*
722 *Biology*, doi: 10.1242/jeb.152108
- 723 Pallarés S, Velasco J, Millán A *et al.* (2016) Aquatic insects dealing with dehydration:
724 do desiccation resistance traits differ in species with contrasting habitat
725 preferences? *PeerJ*, **4**, e2382.
- 726 Papadopoulou A, Anastasiou I, Vogler AP (2010) Revisiting the insect mitochondrial
727 molecular clock: the mid-Aegean trench calibration. *Molecular Biology and*
728 *Evolution*, **27**, 1659–1672.
- 729 Penone C, Davidson AD, Shoemaker KT *et al.* (2014) Imputation of missing data in
730 life-history datasets: which approach performs the best? *Methods in Ecology and*
731 *Evolution*, **5**, 961–970.
- 732 Pinceel T, Brendonck L, Larmuseau MHD *et al.* (2013) Environmental change as a
733 driver of diversification in temporary aquatic habitats: Does the genetic structure
734 of extant fairy shrimp populations reflect historic aridification? *Freshwater*
735 *Biology*, **58**, 1556–1572.
- 736 Pollux BJA, Meredith RW, Springer MS *et al.* (2014) The evolution of the placenta
737 drives a shift in sexual selection in live bearing fish. *Nature*, **513**, 233 – 236.
- 738 Revell LJ (2012) phytools: An R package for phylogenetic comparative biology (and
739 other things). *Methods in Ecology and Evolution*, **3**, 217–223.
- 740 Rezende E, Diniz-Filho J (2012) Phylogenetic analyses: Comparing species to infer
741 adaptations and physiological mechanisms. *Comprehensive Physiology*, **2**, 639-
742 674.
- 743 Ribera I, Fresneda J, Bucur R *et al.* (2010) Ancient origin of a Western Mediterranean
744 radiation of subterranean beetles. *BMC Evolutionary Biology*, **10**, 29.
- 745 Roesti M, Gavrillets S, Hendry AP *et al.* (2014) The genomic signature of parallel
746 adaptation from shared genetic variation. *Molecular Ecology*, **23**, 3944–3956.
- 747 Schultz ET, McCormick SD (2012) Euryhalinity in An Evolutionary Context. *Fish*
748 *Physiology*, **32**, 477-533.

- 749 Short AEZ, Fikáček M (2013) Molecular phylogeny, evolution and classification of the
750 Hydrophilidae (Coleoptera). *Systematic Entomology*, **38**, 723–752.
- 751 Sinclair BJ, Ferguson LV, Salehipour-shirazi G *et al.* (2013) Cross-tolerance and cross-
752 talk in the cold: relating low temperatures to desiccation and immune stress in
753 insects. *Integrative and Comparative Biology*, **53**, 545–556.
- 754 Smith KE, Raymond SL, Valenti ML *et al.* (2010) Physiological and pharmacological
755 characterizations of the larval *Anopheles albimanus* rectum support a change in
756 protein distribution and/or function in varying salinities. *Comparative*
757 *Biochemistry and Physiology A*, **157**, 55–62.
- 758 Smith KE, VanEkeris LA, Okech BA *et al.* (2008) Larval anopheline mosquito recta
759 exhibit a dramatic change in localization patterns of ion transport proteins in
760 response to shifting salinity: a comparison between anopheline and culine larvae.
761 *Journal of Experimental Biology*, **211**, 3067–3076.
- 762 Takeda S, Matsumasa M, Kikuchi S *et al.* (1996) Variation in the branchial formula of
763 semiterrestrial crabs (Decapoda: Brachyura: Grapsidae and Ocypodidae) in
764 relation to physiological adaptations to the environment. *Journal of Crustacean*
765 *Biology*, **16**, 472–486.
- 766 Tobler M, Plath M (2011) Living in extreme habitats. In: *Ecology and evolution of*
767 *Poeciliid fishes*. (ed. by J. Evans, A. Pilastro & I. Schlupp), pp. 120–127.
768 University of Chicago Press, Chicago, IL, USA.
- 769 Todgham AE, Stillman JH (2013) Physiological responses to shifts in multiple
770 environmental stressors: Relevance in a changing world. *Integrative and*
771 *Comparative Biology*, **53**, 539–544.
- 772 Tones PI (1977) Osmoregulation in adults and larvae of *Hygrotus salinarius* Wallis
773 (Coleoptera, Dytiscidae) *Comparative Biochemistry and Physiology A*, **60**, 247–
774 250.
- 775 Tones PI, Hammer UT (1975) Osmoregulation in *Trichocorixa verticalis interiores*
776 Sailer (Hemiptera, Corixidae) -an inhabitant of Saskatchewan saline lakes,
777 Canada. *Canadian Journal of Zoology*, **53**, 1207–1212.
- 778 Uyhelji HA, Cheng C, Besansky NJ (2016) Transcriptomic differences between
779 euryhaline and stenohaline malaria vector sibling species in response to salinity
780 stress. *Molecular Ecology*, **25**, 2210–2225.

781 DATA ACCESSIBILITY

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

787 AUTHOR CONTRIBUTIONS

788 Conceived the study: all authors
789 Field collection 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 $\lambda = 1$ (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

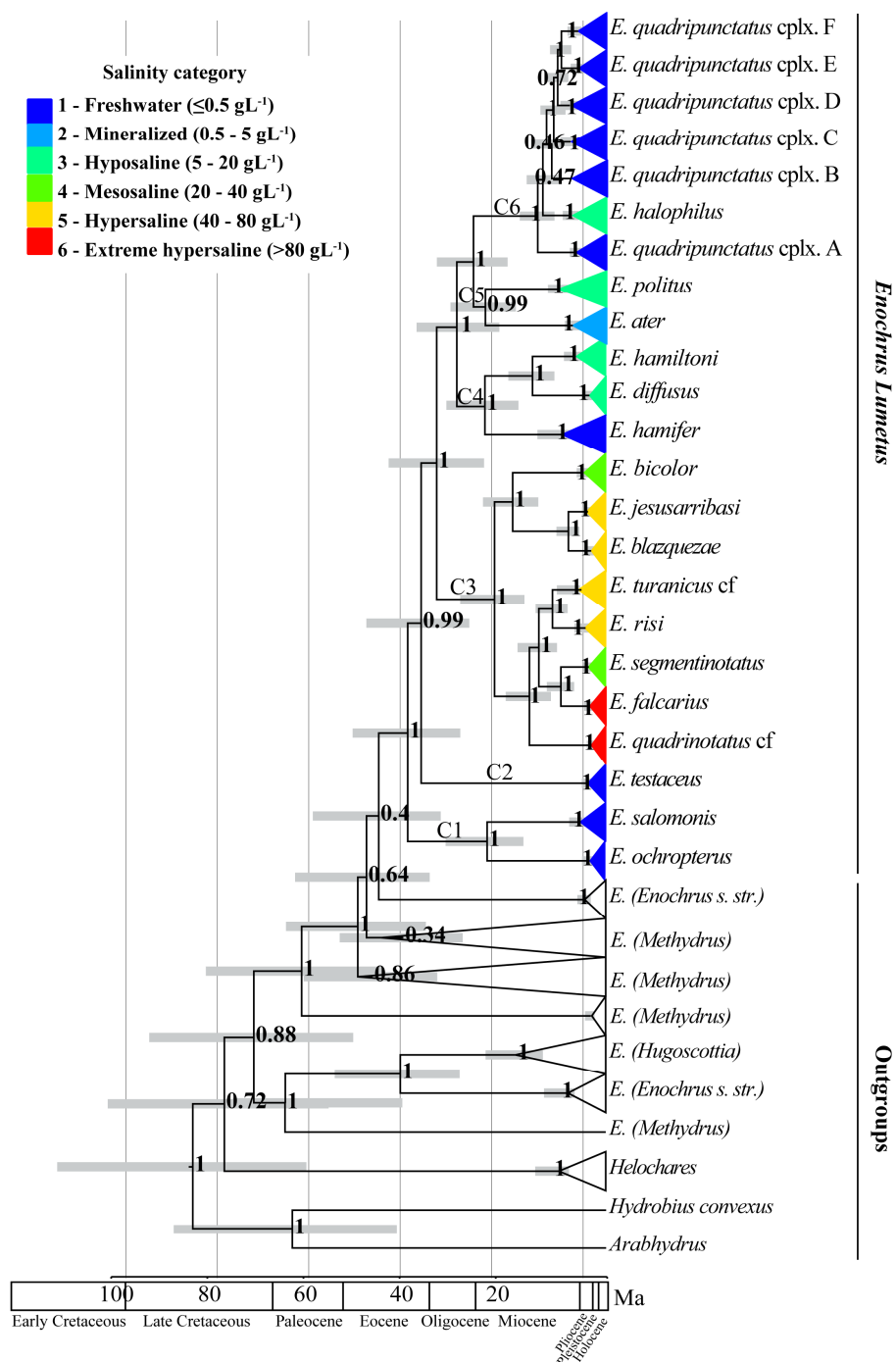


Figure 1. Dated phylogeny of *Lumetus*. Node numbers: posterior probabilities; bars on nodes: 95% confidence intervals for node ages; letters: main clades as referred to in the text. Terminals are collapsed to reflect species-level relationships (see Fig. S1 and Table S1 for details on terminals).

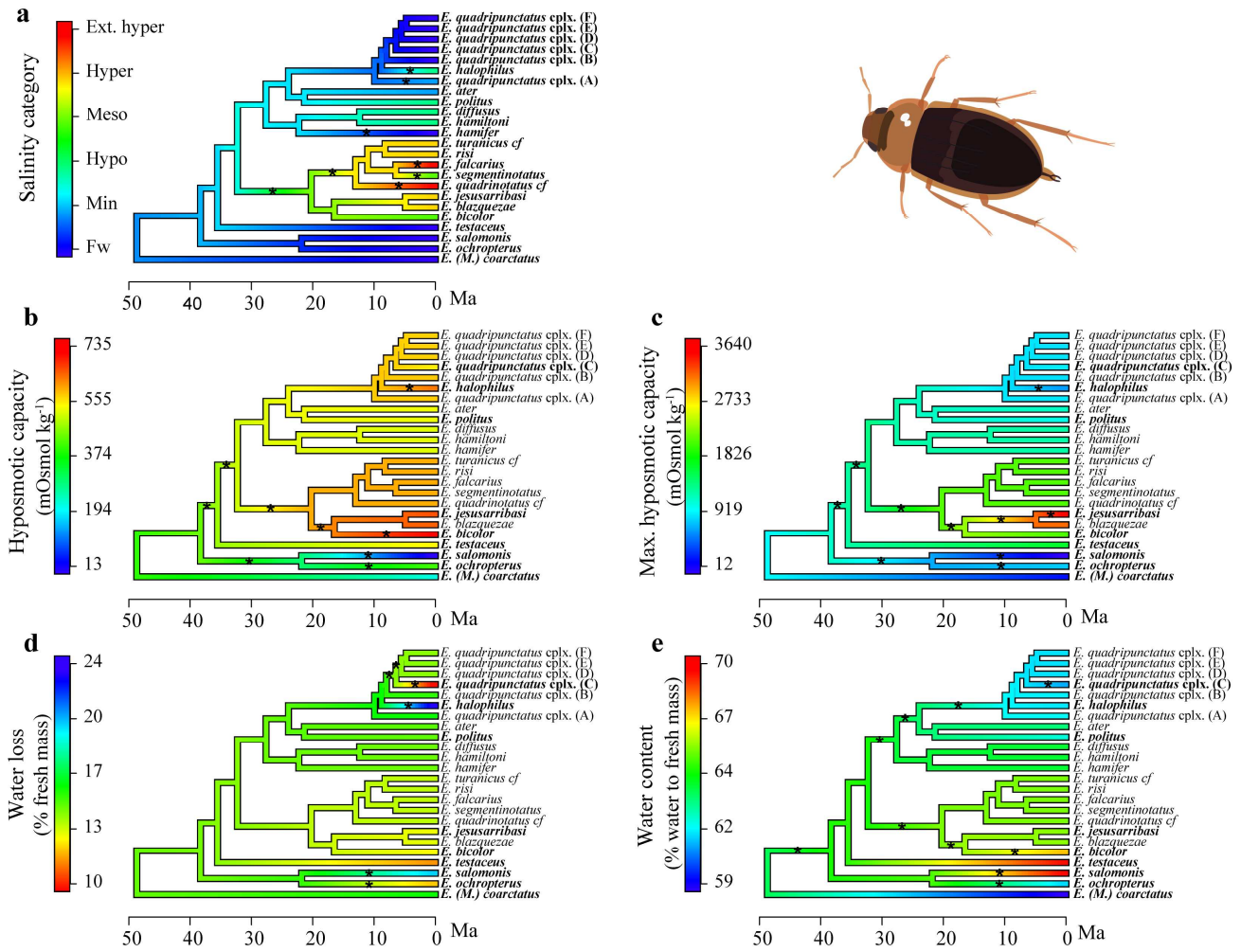


Figure 2. Ancestral reconstruction of desiccation and osmoregulation traits. The warmer (red) colours indicate higher resistance to desiccation or salinity than cooler (blue) colours. Branches where significantly accelerated increases or decreases in the rate of phenotypic change were identified (see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

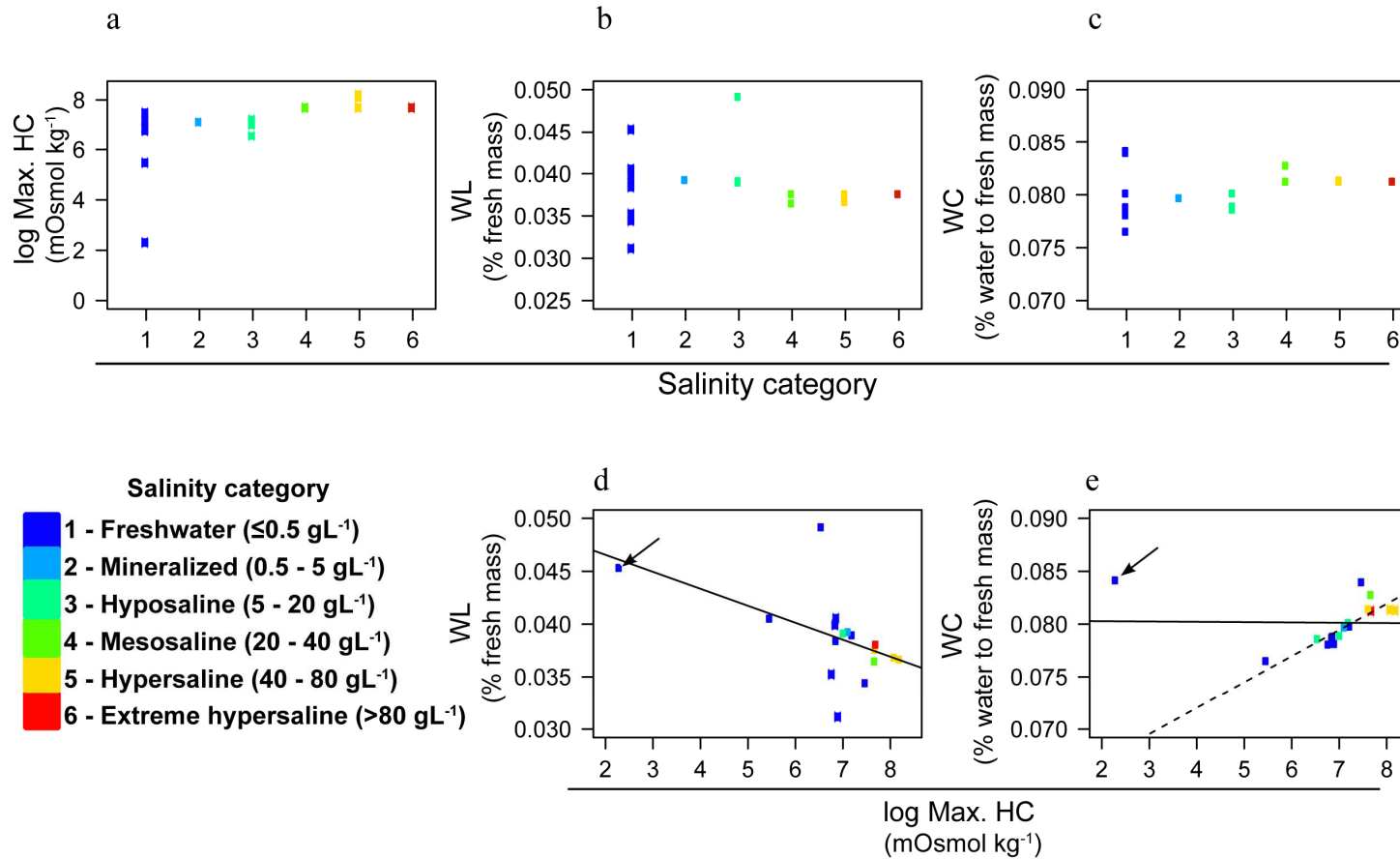


Figure 3. Relationships between habitat salinity, hyposmotic capacity and desiccation traits. Regression lines are shown for significant relationships in PGLS (see Table S6). Dashed line for regressions excluding *E. salomonis* (indicated by arrow). Max. HC: maximum hyposmotic capacity, WL: water loss,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

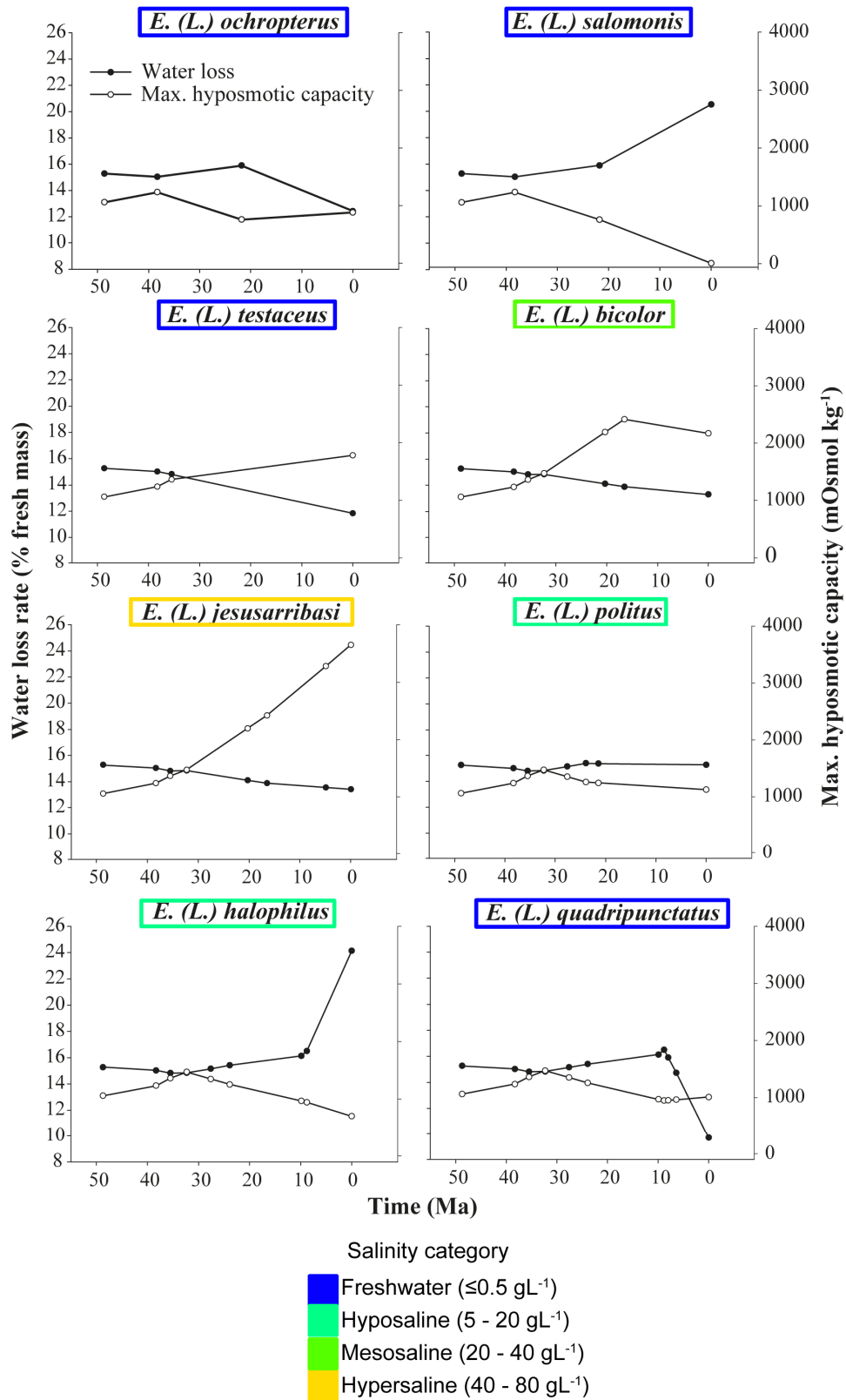


Figure 4. Values of water loss and maximum hyposmotic capacity through the full evolutionary path of the *Lumetus* species used in desiccation and osmoregulation experiments.

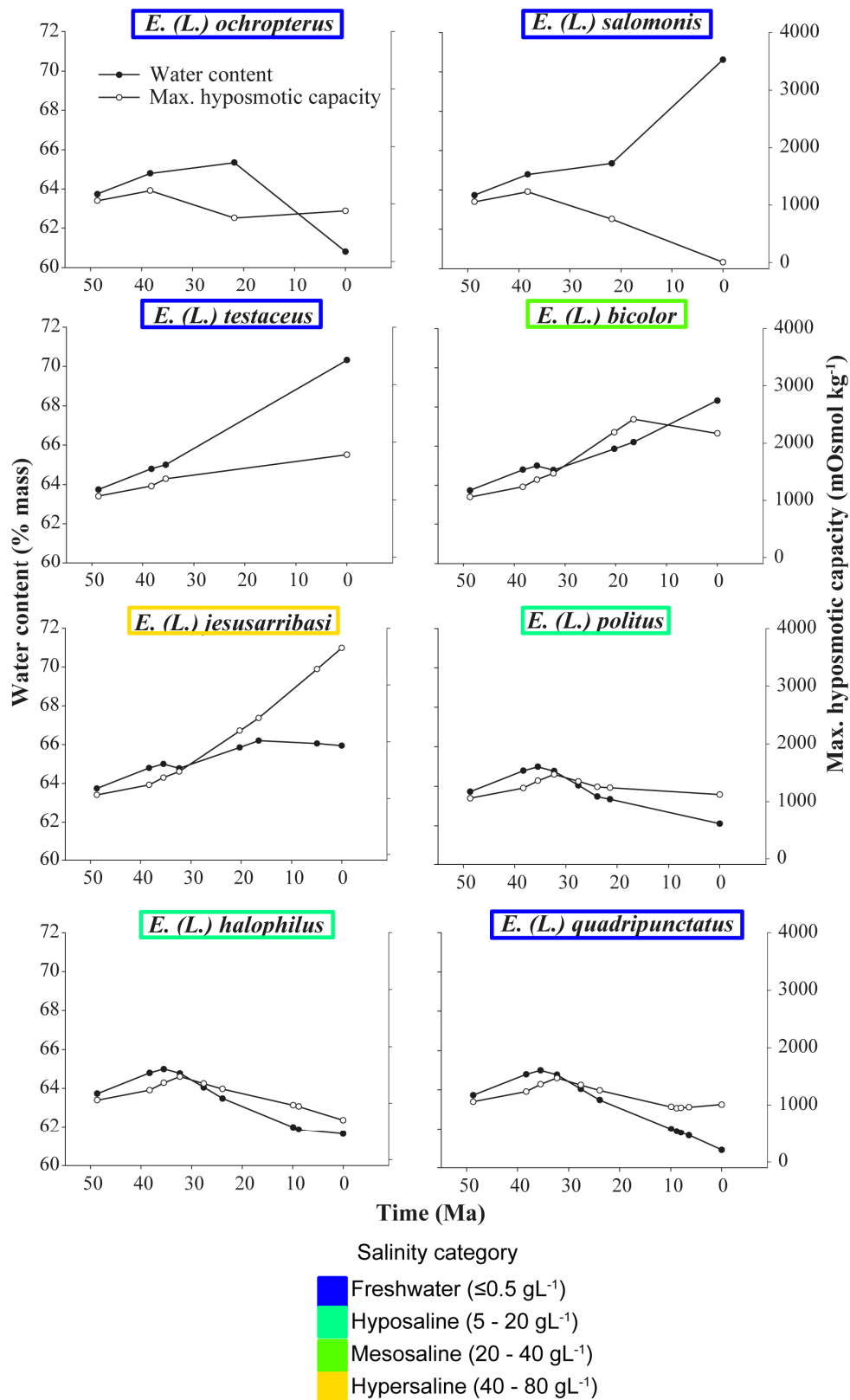


Figure 5. Values of water content and maximum osmotic capacity trough the full evolutionary path of the *Lumetus* species used in desiccation and osmoregulation experiments.

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 trees.

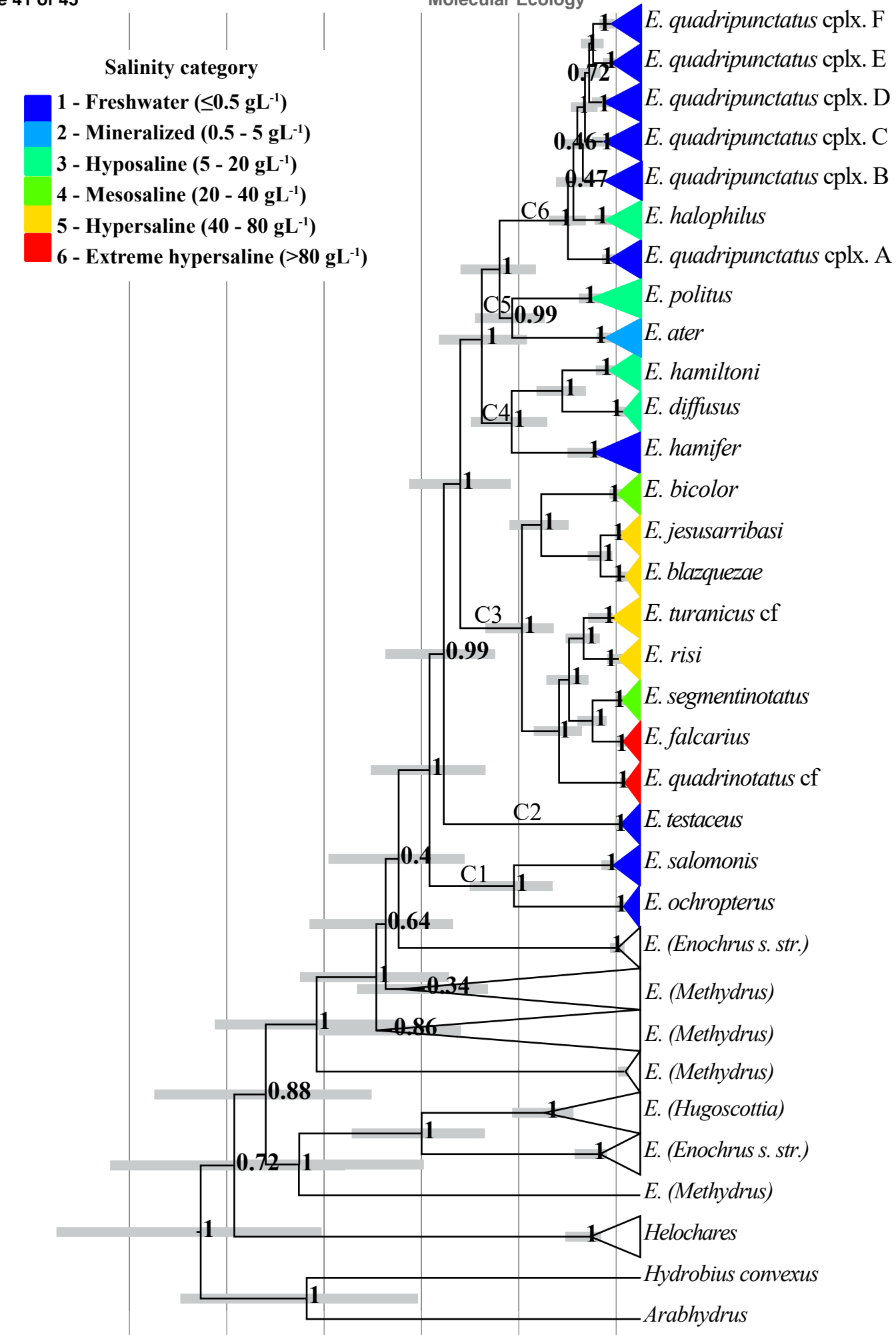
trait	σ^2	pairwise comparison	LRT _{df=1}	P	AIC _c
Max. HC	0.021 – 0.049				
WL	0.001 – 0.004	Max. HC vs. WLR	27.4 – 36.4	< 0.001	obs = 54.2 – 67.4 cons = 82.5 – 100.9
WC	0.00003 – 0.00007	Max. HC vs. WC	121.1 – 125.5	< 0.001	obs = -40.3 – -25.2 cons = 78.8 – 97.9

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	< 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

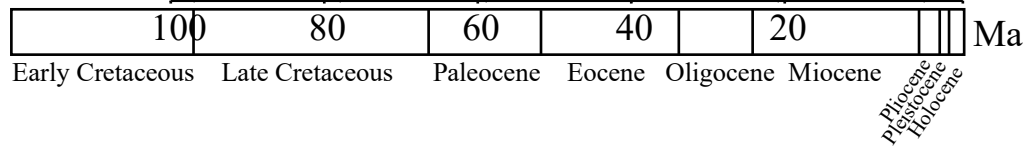
Salinity category

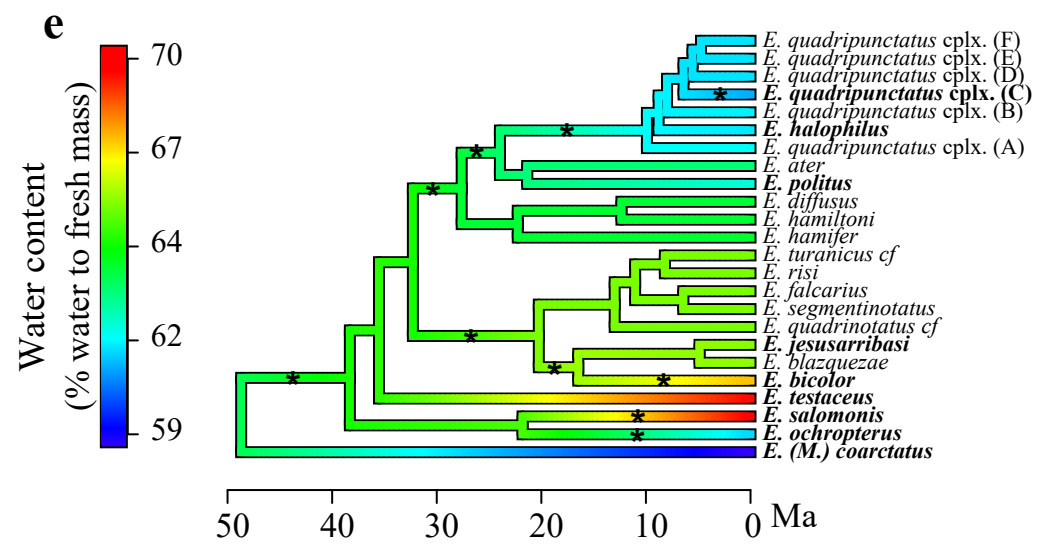
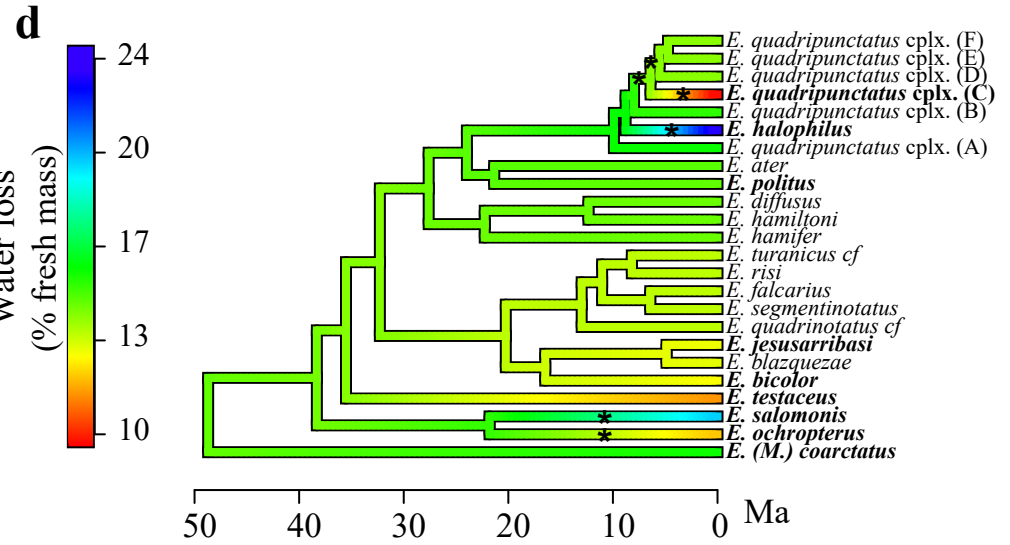
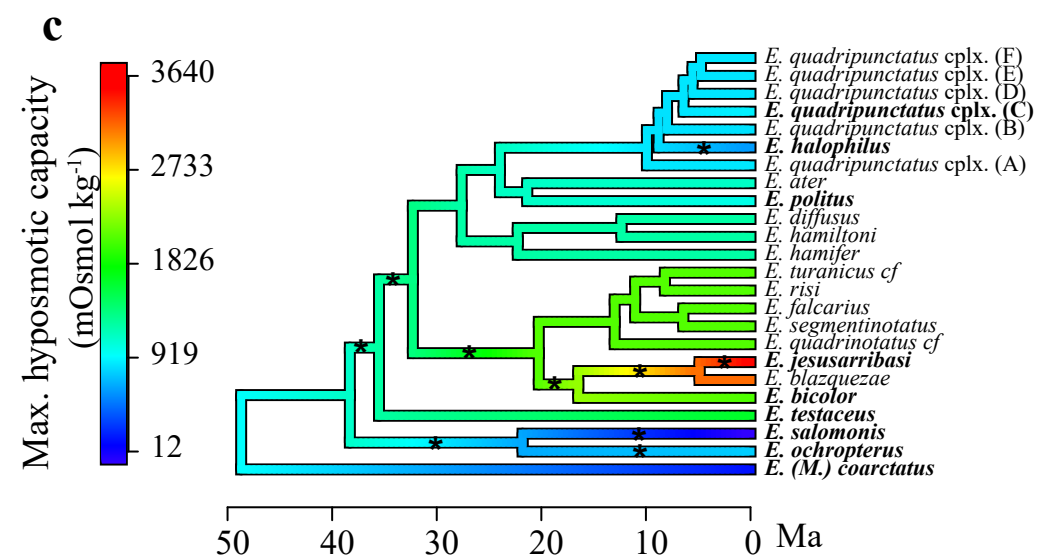
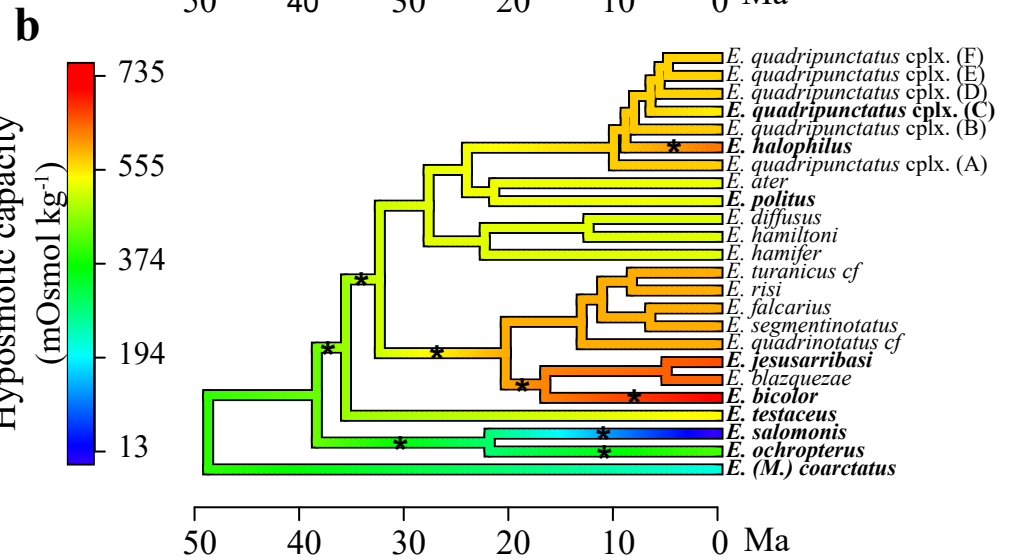
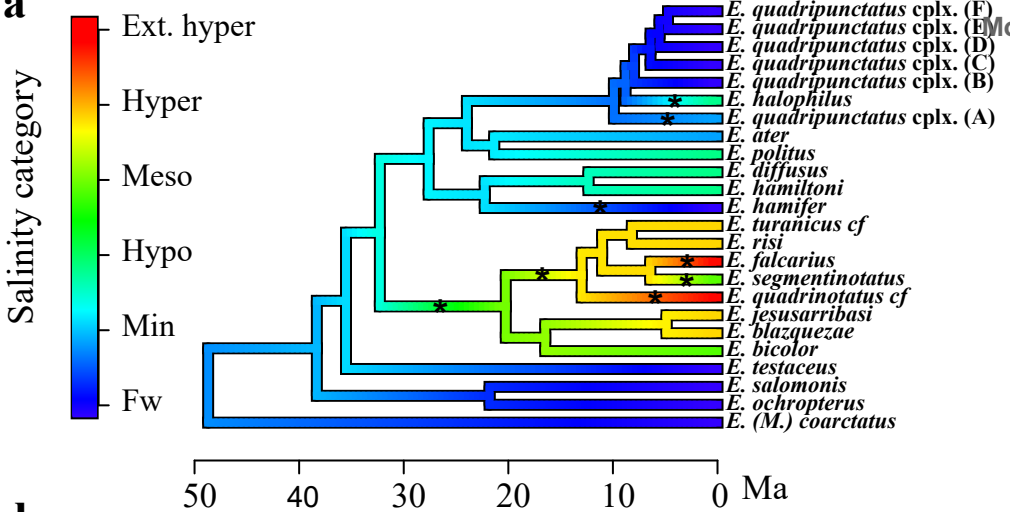
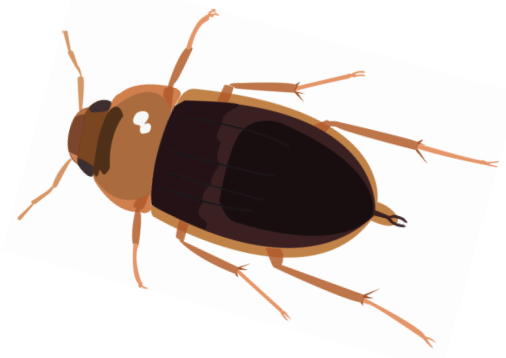
- 1 - Freshwater ($\leq 0.5 \text{ gL}^{-1}$)
- 2 - Mineralized ($0.5 - 5 \text{ gL}^{-1}$)
- 3 - Hyposaline ($5 - 20 \text{ gL}^{-1}$)
- 4 - Mesosaline ($20 - 40 \text{ gL}^{-1}$)
- 5 - Hypersaline ($40 - 80 \text{ gL}^{-1}$)
- 6 - Extreme hypersaline ($>80 \text{ gL}^{-1}$)



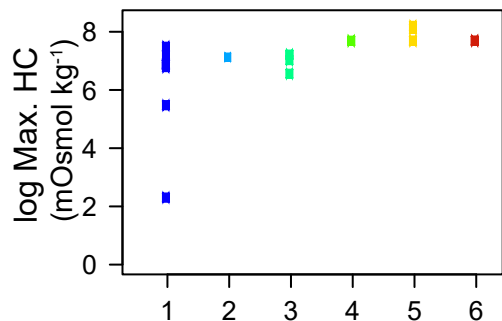
Enochrus Lumetus

Outgroups

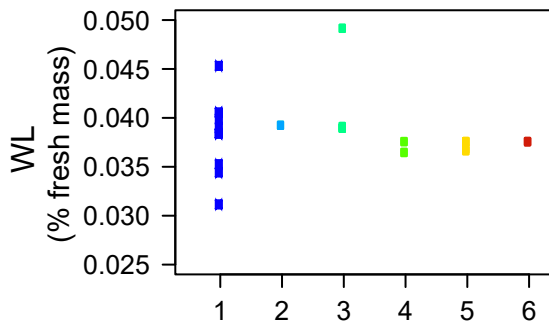




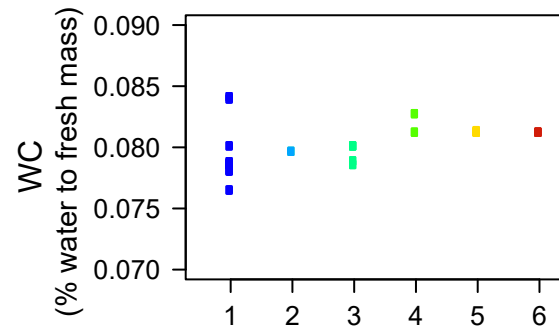
a



b

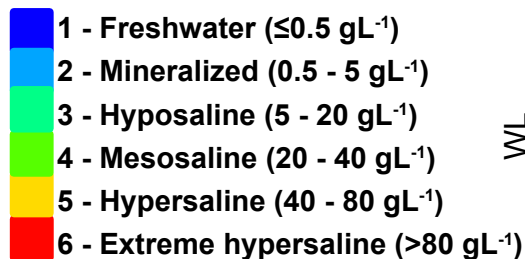


c

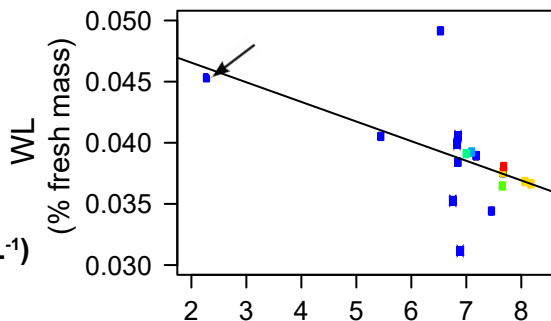


Salinity category

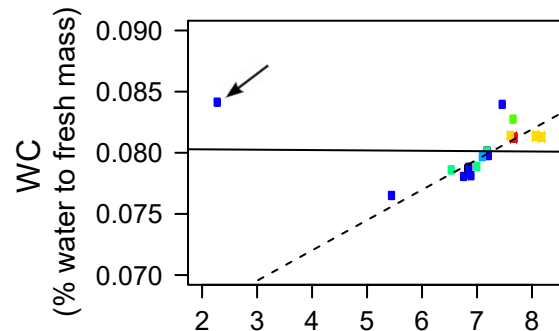
Salinity category

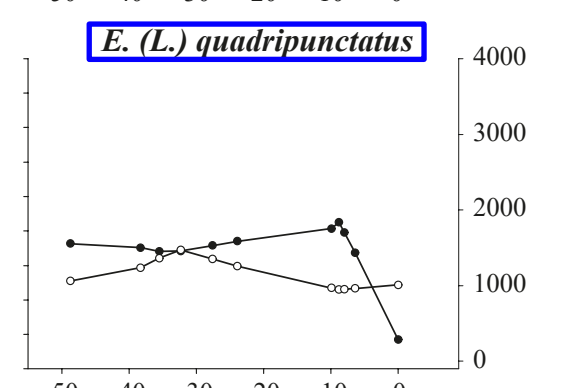
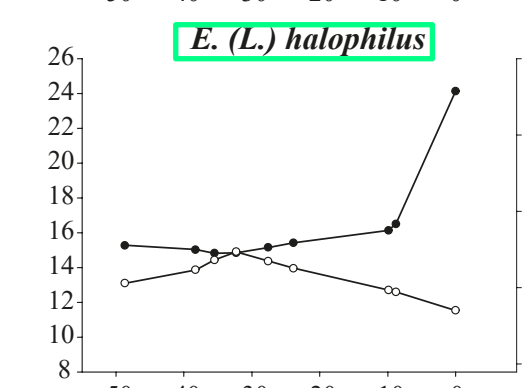
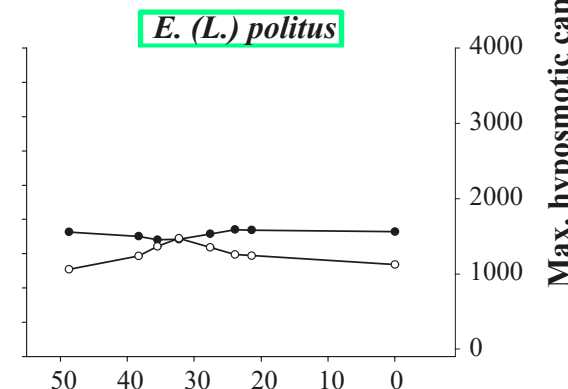
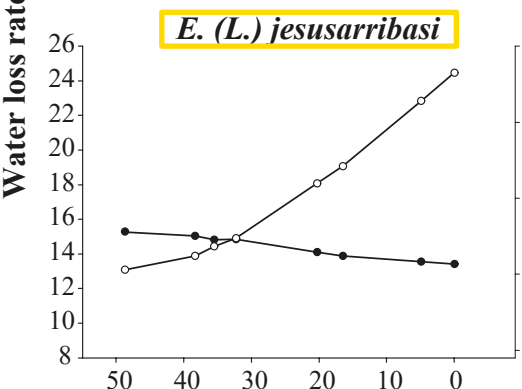
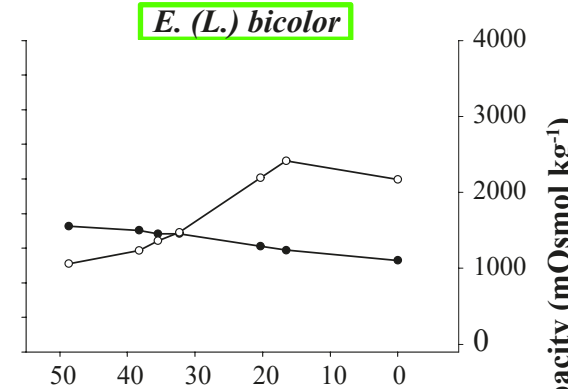
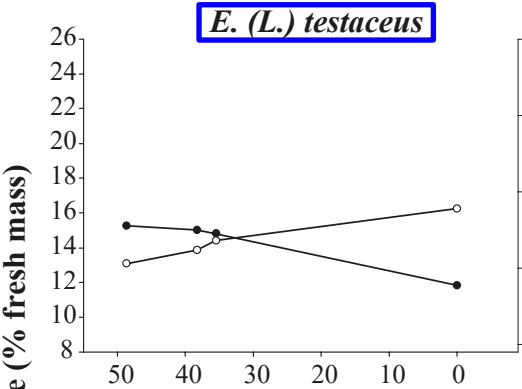
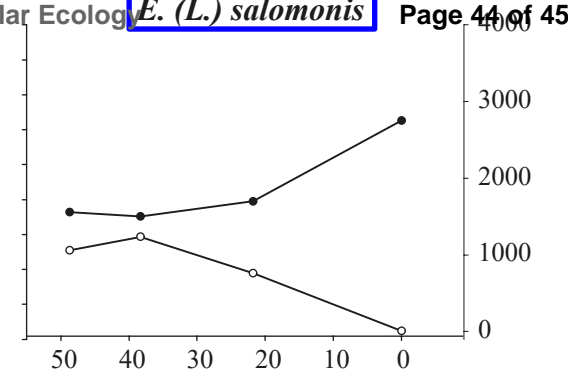
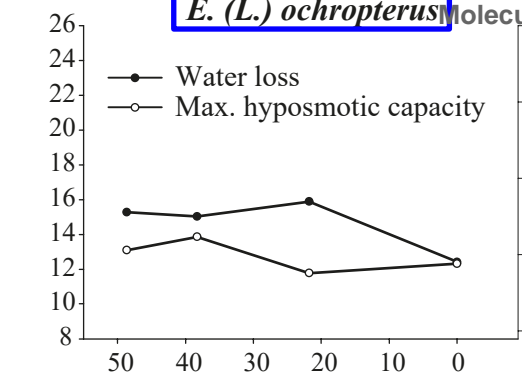


d



e

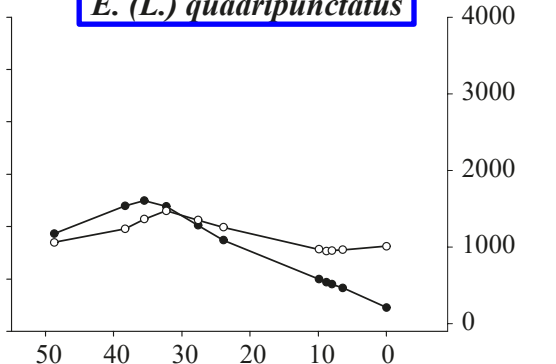
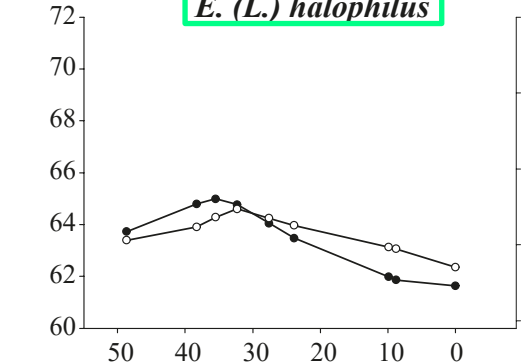
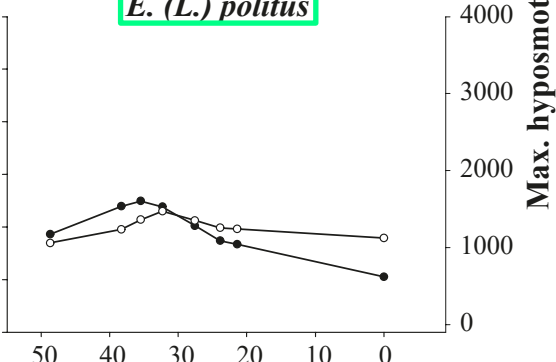
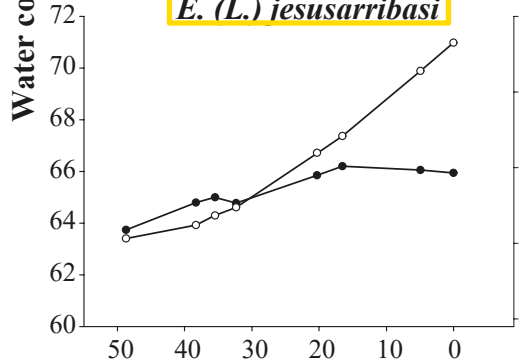
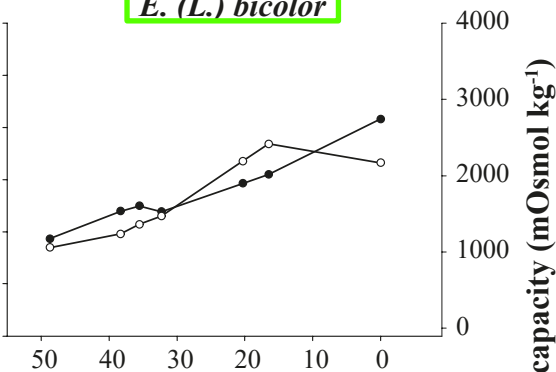
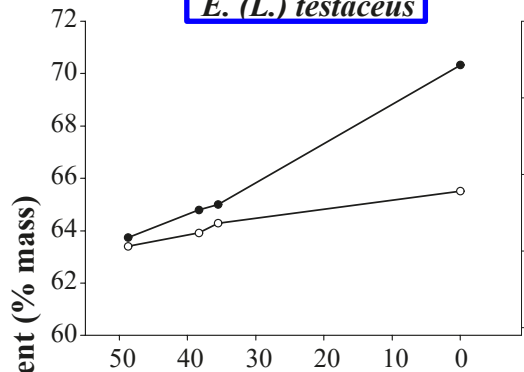
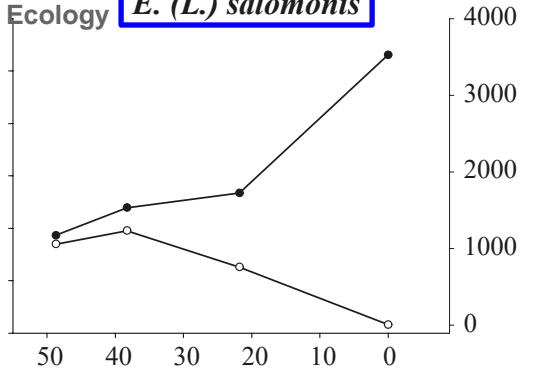
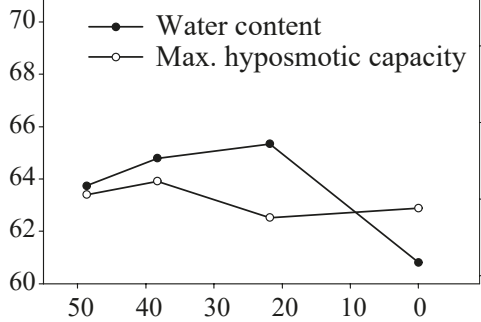
log Max. HC
(mOsmol kg⁻¹)



Time (Ma)

Salinity category

- Freshwater ($\leq 0.5 \text{ gL}^{-1}$)
- Hyposaline ($5 - 20 \text{ gL}^{-1}$)
- Mesosaline ($20 - 40 \text{ gL}^{-1}$)
- Hypersaline ($40 - 80 \text{ gL}^{-1}$)



Time (Ma)

Salinity category

- Freshwater (≤ 0.5 gL⁻¹)
- Hyposaline (5 - 20 gL⁻¹)
- Mesosaline (20 - 40 gL⁻¹)
- Hypersaline (40 - 80 gL⁻¹)