Tempo and mode of the multiple origins of salinity tolerance in a water beetle lineage

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Abstract

Salinity is one of the most important drivers of the distribution, abundance and diversity of organisms. Previous studies on the evolution of saline tolerance have been mainly centred on marine and terrestrial organisms, while lineages inhabiting inland waters remain largely unexplored. This is despite the fact that these systems include a much broader range of salinities, going from freshwater to more than six times the salinity of the sea (i.e. >200 g/L). Here, we study the pattern and timing of the evolution of the tolerance to salinity in an inland aquatic lineage of water beetles (*Enochrus* species of the subgenus *Lumetus*, family Hydrophilidae), with the general aim of understanding the mechanisms by which it was achieved. Using a time-calibrated phylogeny built from five mitochondrial and two nuclear genes and information about the salinity tolerance and geographical distribution of the species, we found that salinity tolerance appeared multiple times associated with periods of global aridification. We found evidence of some accelerated transitions from freshwater directly to high salinities, as reconstructed with extant lineages. This, together with the strong positive correlation found between salinity tolerance and aridity of the habitats in which species are found, suggests that tolerance to salinity may be based on a co-opted mechanism developed originally for drought resistance.

Keywords: aquatic insects, aridity, drought resistance, exaptation, inland saline waters, salinity tolerance evolution

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Introduction

Salinity is one of the main environmental factors constraining the fundamental niche of the species. Salts present in the environment where species live and evolve are fundamental to supply necessary compounds for the metabolism, but they also act as cytotoxic ions that affect the water balance (Evans 2009). Accordingly, salinity represents one of the main natural sources of stress shaping biodiversity of ecosystems (Odum 1985; Millán *et al.* 2011). The physiological tolerance to the effect of salt (i.e. salinity tolerance) limits the habitats that different organisms can occupy in a salinity stress

gradient, determining community turnover and beta-diversity along this gradient (Gutiérrez-Cánovas *et al.* 2013). The physiological tolerance to extreme physico-chemical conditions can become a double-edge sword, as this allows to escape from the adverse effect of predation and competition in the more diverse communities in habitats with less stressful conditions, but at the same time requires an extra metabolic cost (Herbst 2001).

Studies on the evolution of habitat preference across salinity gradients have been mainly centred on terrestrial organisms, especially on plants (e.g. Flowers *et al.* 2010). In the aquatic medium, most of the studies focus on the colonization from marine to freshwater habitats (e.g. Whitehead *et al.* 2013), while in inland waters remains largely unexplored. In these systems the range of salinity is much broader than in coastal ecosystems,

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going from freshwater to salinities that sextuplicate that of the sea (i.e. >200 g/L; Oren 2002; Millán *et al.* 2011). The high diversity of inland saline habitats, together with their isolation, seem thus to offer a unique system to study the evolution of salinity tolerance.

In the aquatic medium, the effect of dissolved salts is more pronounced than in terrestrial ecosystems, and consequently, salinity has been recognized as one of the most important drivers influencing the distribution, abundance and diversity of inland aquatic organisms (Velasco *et al.* 2006; Gutiérrez-Cánovas *et al.* 2013). Insects, and in particular Coleoptera and Diptera, are among the few aquatic lineages with species across the full salinity gradient of inland waters, being able to live in hypersaline environments too toxic for any aquatic vertebrate (Millán *et al.* 2011). Although their species richness is usually lower than that of freshwater systems, saline communities have high levels of endemicity and cryptic diversity, including a high number of exclusive species that represents a disproportionately large contribution to global biodiversity (Abellán *et al.* 2007; Muñoz *et al.* 2008; Arribas *et al.* 2013). Within aquatic lineages, plesiomorphic traits are usually associated with freshwater conditions and apomorphic traits with high tolerance to solute concentration, often found in the terminal branches of a phylogeny (Bradley *et al.* 2009). However, few studies have focused on the evolution of salinity tolerance in lineages of inland aquatic organisms (as e.g. mosquitoes, Grueber & Bradley 1994; Albers & Bradley 2011), and there has been no research on the evolutionary trajectory of salinity tolerance. In particular, little is known about how progressive was the transition or its temporal framework. One possibility is that freshwater species adapted sequentially first to low mineralized waters, then to increasing salinities and finally to hypersaline waters. This would suggest that the regulatory mechanisms were progressively improved to be able to cope with increasing concentrations of salt and would likely require a long evolutionary time and a continuous, directional selective pressure. An alternative scenario would be a direct switch from freshwater to high salt concentration. If this were found, it would suggest either that the regulatory mechanism did not appear progressively and was thus likely to be simple enough to have evolved in a short time without intermediary steps, or that this regulatory mechanism did not evolve as an adaptation to salinity but for some other purpose, in which case it would be an exaptation (Gould & Vrba 1982). Thus, it has been suggested that because both tolerance to salinity and to desiccation rely on a similar physiological basis to avoid internal water loss, it could be hypothesized that the mechanism for salinity tolerance could arise as an exaptation in the process of adaptation to drought in arid and hyper-arid climatic conditions (Gómez-Mestre & Tejedo 2005). Salinity tolerance might have then evolved in correlation with the climatic niche.

Here, we study the pattern and timing of the evolution of the tolerance to salinity in an inland aquatic lineage of water beetles, with the general aim of understanding the mechanisms by which it was achieved. We focus on the *Enochrus* species of subgenus *Lumetus* (family Hydrophilidae), which includes representatives across the full gradient of salinity in the Holarctic region (Hansen 1999). We used molecular data from five mitochondrial and two nuclear genes to build a calibrated phylogeny of the lineage, and information about salinity tolerance and geographical distribution of its species to address the following specific objectives: i) establish the directionality and progressivity of change, that is, if transitions were direct from freshwater to hypersaline (or vice versa) or through intermediate stages; ii) determine the relationship between the salinity tolerance of the species and their climatic niche in a phylogenetic framework; and iii) obtain an estimation of the time framework in which these processes occurred and their possible relation to past climatic changes.

Materials and methods

Study group

Enochrus (family Hydrophilidae) has ca. 200 species occurring across all the zoogeographic regions (Hansen 1999). Despite its broad distribution and the fact that it includes representatives in many aquatic communities, a general taxonomic revision is not currently available, probably due to the high plasticity of some morphological characters and the large amount of cryptic diversity found in some species (Arribas *et al.* 2013). Within *Enochrus,* there are six recognized subgenera (Hansen 1990, 1999), with one of them, *Lumetus*, distributed across the Holarctic region and inhabiting a variety of aquatic habitats along the salinity gradient, from freshwater (<0.5 g/L) to extreme hypersaline (>80 g/L). In a recent, comprehensive phylogeny of Hydrophilidae including five of the six subgenera, both *Enochrus* and *Lumetus* were recovered as monophyletic (Short & Fikácek 2013). The last world catalogue of Hydrophilidae (Hansen 1999) recognized 23 species of *Lumetus* (see Table S1 for a checklist), after transferring some previously counted in *Lumetus* to other subgenera (mainly *Methydrus*). Twenty of these species occur in the Palaearctic region, with only one well-defined species group recognized among them, the *Enochrus bicolor* group. It includes 8 species, all of them inhabiting saline waters of different concentrations (Schödl 1998; Arribas *et al.* 2013). The remaining Palaearctic species

occupy a variety of aquatic habitats across vast geographical areas, including some of the most frequent species of standing waters in central and northern Europe (e.g. *Enochrus testaceus* or *Enochrus halophilus*). Within the Nearctic region, only three species are found, *Enochrus hamiltoni*, *E. difussus* and *E. reflexipennis*, which form a species complex inhabiting brackish waters across this area (Short 2004).

Taxon sampling and DNA sequencing

A total of 108 specimens of *Lumetus* representing 19 of the 23 known species were studied (mean of 11 specimens per species; Table S2). Missing species were *E. sinuatus* (Middle East, included in the *E. bicolor* group, Schödl 1998), *E. subsignatus* and *E. asiaticus* (east Asia, related to *Enochrus fuscipennis*, Kuwert 1893; Jia & Wang 2010) and *E. reflexipennis*, closely related to the other two Nearctic species (Short 2004). Several species of *Enochrus* belonging to the subgenera *Methydrus*, *Enochrus* and *Hugoscottia* were included in the analyses as outgroups, together with a related genus (*Helochares*). Trees were rooted in other more distantly related genera of Hydrophilidae (*Hydrobius*, *Arabhydrus*) (Short & Fikácek 2013; Table S2).

DNA was extracted using Invisorb Spin Tissue Mini Kit (Invitek, Berlin, Germany), following the manufacturer's recommendations. From the mitochondrial genome, we sequenced five genes: two nonoverlapping fragments of the cytochrome c oxidase 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 (3′ end of the large ribosomal unit plus the Leucine transferase and the 5′ end of NADH dehydrogenase subunit 1, *rrnL+trnL+nad1*). From the nuclear DNA, we sequenced two genes: an internal fragment of the large ribosomal unit, 28S rRNA, (*LSU*); and an internal fragment of the internal transcribed spacer 2 gene (*ITS2*) (see Table S3 for the primers used). Sequencing was conducted using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA), and sequenced products were electrophoresed on ABI 310 and 3700 automated sequencers (Applied Biosystems). Sequences were assembled and edited with Sequencher 4.7 (GeneCodes Corporation, Ann Arbor, MI, USA), using Ns (missing data) for the few heterozygous positions found in nuclear loci (see Table S2 for GenBank Accession nos.).

Phylogentic analyses

Alignments were obtained with the online version of MAFFT v.6 (Katoh *et al.* 2009) using the EINS-i option for protein coding and QINS-i for ribosomal genes, with other parameters as defaults. For the protein coding genes, the correct translation to amino acids was checked, and they were subsequently divided into two codon partitions (*12p*, first and second codon positions; *3p*, third codon positions).

Bayesian phylogenetic analyses on the concatenated DNA matrix were implemented in BEAST 1.7 (Drummond *et al.* 2012) for the joint estimation of the topology of the tree and the ages of diversification. Analyses were conducted applying the best fitting model of substitution to each gene or codon partition as estimated prior to the analyses with jModelTest (Posada 2008). A Yule speciation prior was applied and analyses were run for 25 million generations, sampling one tree every 2000 generations. To calibrate the tree, we used the rates of molecular evolution obtained for the same DNA fragments in another Coleoptera genus (*Carabus*, family Carabidae), which were estimated using a comprehensive analytical procedure employing multiple fossils and geological events (Andújar *et al.* 2012, 2013). Rates were applied conservatively, using as confidence intervals a uniform probability function adjusted to the 95% HPD obtained for *Carabus* (see Andújar *et al.* 2012). These confidence intervals are in good agreement with most of the rates estimated for the same gene fragments in other families of Coleoptera (e.g. Papadopoulou *et al.* 2010; Ribera *et al.* 2010), and with the standard arthropod mitochondrial clock of Brower (1994). For the partition by genes, we applied an uncorrelated lognormal (ULN) clock to the nuclear genes and a strict clock (SC) to each mitochondrial gene. Analyses were conducted for the data set including all outgroups ('complete' data set) and for a data set including only *Lumetus* species ('*Lumetus*-only' data set).

Consensus trees were estimated with Treeanotator (Drummond & Rambaut 2007) discarding the 20% initial trees as a burning fraction, after checking effective sample size of likelihood, evolutionary rates and root age values and ensuring that the plateau in tree likelihood values had been reached. Posterior probabilities were considered as a measure of node support.

Evolution of the tolerance to salinity

Salinity tolerance of the species of Lumetus. The salinity tolerance of each *Lumetus* species was assigned using data of the salt concentration of the habitats in which they were found whenever available (Table S4); otherwise, we used a qualitative assessment of the habitat from our own field data set or bibliographic data on their habitat occupation (e.g. Pirisinu 1981; Hebauer 1997, 2004; Ribera *et al.* 1997; Schödl 1997, 1998; Short 2004; Arribas *et al.* 2013). Special attention was given to

the records of large and stable populations in habitats with the highest salinities, as these may better reflect their tolerance limits. In aquatic insects, the adaptation to saline stress is generally associated to an increase in saline tolerance, as all studies exploring the physiological performance of saline organisms have shown a high survival on freshwater conditions (e.g. Albers & Bradley 2011; Carbonell *et al.* 2012; Céspedes *et al.* 2013). However, species tend to be restricted to the habitats with the highest salinities that they can tolerate, as this may allow them to escape from the stronger predation and competition pressures found in the more diverse communities of low mineralized habitats (O'Brien & Blinn 1999; Herbst 2001).

We first classified the species of *Lumetus* into six categories ('6-states' categorization), corresponding to previously defined main types of aquatic systems across the water salinity gradient (Herbst 2001; Arribas *et al.* 2009): (1) strictly freshwater (≤ 0.5 g/L), (2) mineralized waters (>0.5–5 g/L), (3) hyposaline waters (>5–20 g/L), (4) mesosaline waters (>20–40 g/L), (5) hypersaline waters (>40–80 g/L) and (6) extreme hypersaline waters (>80 g/L) (Fig. 1). We used these six categories as a semi-continuous variable representing species salinity tolerance. We then pooled these categories to create first a '3-states' categorization in freshwater (including categories 1 and 2 above), hyposaline (category 3) and saline (categories 4, 5 and 6); and finally a '2-states' categorization considering only freshwater (categories 1 and 2) and saline (categories 3, 4, 5 and 6) (Fig. 1 and Table 1). In both the '3-states' and the '2-states' categorizations, variables were considered as discrete. The resultant species categorizations are congruent with previous qualitative assessments (e.g. Ribera *et al.* 1997; Schödl 1997, 1998; Velasco *et al.* 2006), as well as with other field observations (A. Millán & I. Ribera unpublished data).

Models of evolution and phylogenetic signal of salinity tolerance. We used continuous-time Markovian models of trait evolution to test different hypotheses for the evolution of salinity tolerance in our lineage of water beetles with likelihood methods. To account for topological uncertainties, we resampled 1000 trees from the postburning fraction of the BEAST analysis (*Lumetus*-only data set) using Logcombiner (Drummond & Rambaut 2007), pruned to keep one example per species in some of the analyses.

For the 2 and 3-states categorizations, we tested three competing nested models of character transitions (Pagel 1999): (i) an equal-rates model, which estimates a single rate of transition among all salinity-tolerance states (i.e. all state changes occur at the same underlying rate); (ii) a symmetrical-rates model, where forward and reverse transitions between each pair of salinity-tolerance states share the same parameter value while transition rates among states may differ (i.e. with three different transition rates in the 3-states categorization; for the 2-states categorization, this is the same as the equal-rate model); and (iii) an all-rates-different model, where transition rates between each combination of salinity-tolerance states were assumed to be different (i.e. with four parameters in the 2-states and six in the 3-states categorizations). The three models were compared using the fitDiscrete function in the Geiger package (Harmon *et al.* 2008) in R version 2.11.1 (R Development Core Team 2011) using a likelihood ratio test. When differences were nonsignificant the model with the lower number of estimated parameters was preferred.

In the case of the semi-continuous categorization (6-states), we tested two alternative models of continuous character evolution, the Brownian motion (evolution of a continuous trait as a random walk process) and the Ornstein–Uhlenbeck model (a random walk with a central tendency as expected under adaptive evolution, Butler & King 2004).

We also determined whether salinity tolerance showed significant phylogenetic signal (i.e. whether it exhibits a significant tendency for related species to resemble each other). We calculated the maximum likelihood value of Pagel's lambda (λ; Pagel 1999) using the functions 'fitDiscrete' and 'fitConstinuous' in the Geiger package with salinity tolerance as a discrete character (2- and 3-states categorization) and as a semi-continuous character (6-states categorization), respectively. A λ value of 1 is consistent with a model of character evolution entirely in agreement with the phylogeny (i.e. the probability of shared inheritance is strictly proportional to relatedness), whereas a λ value of 0 suggests an evolution of the character with independence of the phylogenetic relationships (Freckleton *et al.* 2002). For the 2 and 3-states categorizations, we used the best evolutionary model as estimated above. In all cases, the sample of 1000 postburnin trees from BEAST was used. We used a likelihood ratio test to compare the fitted maximum likelihood value of λ with a model assuming either no phylogenetic signal ($\lambda = 0$) or a perfectly congruent evolution along the tree ($\lambda = 1$).

Reconstruction of ancestral salinity tolerance. We reconstructed the ancestral states for salinity tolerance in BEAST 1.7, which simultaneously estimates the phylogeny and assigns trait states to all its nodes considering topological uncertainty (Drummond *et al.* 2012). We used the *Lumetus*-only data set and salinity tolerance as a discrete (2-states and 3-states categorizations) and as a semi-continuous character (6-states categorization). Both for the discrete and continuous trait analyses, we
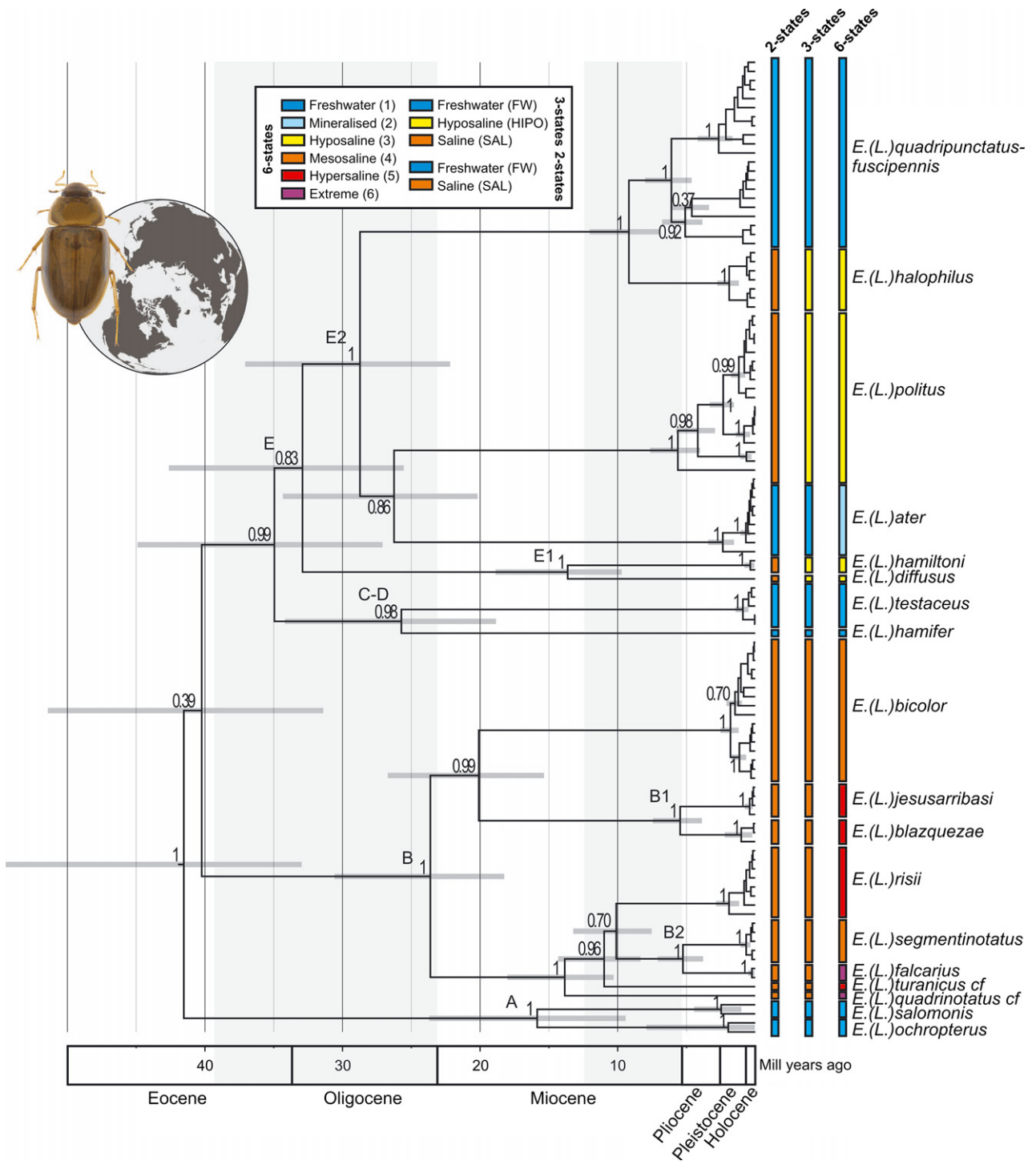
Fig. 1 Ultrametric time-calibrated tree for the subgenus *Lumetus* (*Lumetus*-only data set). Numbers above nodes: posterior probabilities. Grey bars on nodes: 95% confidence intervals for node ages (Ma). Letters: main clades as referred to in the text. Coloured vertical bars: salinity tolerance of each species according to the three proposed categorizations. Shaded areas: events of global aridification in the northern hemisphere. Habitus figure: *Enochrus falcarius* (photo: P.Arribas). See Fig. S2 (Supporting information) for details on the terminals.

applied the estimated optimal evolutionary model. Other parameters of the BEAST runs were as in the phylogenetic and calibration analyses above.

Relationships between salinity tolerance and climatic niche. We tested for the association between salinity tolerance and climatic niche of the species in the subgenus

Table 1 Mean and maximum salinities for large stable populations of the different species within the subgenus *Enochrus* (*Lumetus*) and the salinity tolerance assignation for each species according to the three proposed categorizations.

Species	Mean Salinity (g/L)	Max Salinity (g/L)	2-states category	3-states category	6-states category
Enochrus ater	2.1	3.9	Freshwater	Freshwater	Mineralized (2)
Enochrus bicolor	21.0	43.0	Saline	Saline	Mesosaline (4)
Enochrus blazquezae	45.6	68.2	Saline	Saline	Hypersaline (5)
Enochrus diffusus	18.0	–	Saline	Hyposaline	Hyposaline (3)
Enochrus falcarius	55.0	97.0	Saline	Saline	Hypersaline ext (6)
Enochrus fuscipennis	0.0	0.5	Freshwater	Freshwater	Freshwater (1)
Enochrus halophilus	5.8	20.0	Saline	Hyposaline	Hyposaline (3)
Enochrus hamiltoni	13.0	14.0	Saline	Hyposaline	Hyposaline (3)
Enochrus jesusarribasi	43.2	80.0	Saline	Saline	Hypersaline (5)
Enochrus ochropterus	0.0	–	Freshwater	Freshwater	Freshwater (1)
Enochrus politus	11.4	43.0	Saline	Hyposaline*	Hyposaline (3*)
Enochrus quadripunctatus	0.1	0.3	Freshwater	Freshwater	Freshwater (1)
Enochrus risii	48.5	51.2	Saline	Saline	Hypersaline (5)
Enochrus salomonis	0.4	1.0	Freshwater	Freshwater	Freshwater (1)
Enochrus segmentinotatus	34.4	36.4	Saline	Saline	Mesosaline (4)
Enochrus testaceus	0.2	–	Freshwater	Freshwater	Freshwater (1)

E. politus was additionally considered as mesosaline in complementary 3-states and 6-states analyses, see Figs S4 and S5 (Supporting information).

Lumetus using distributional information in a phylogenetically controlled framework. First, occurrence data from European and Moroccan species were compiled from different regional databases (ESACIB, Sánchez-Fernández *et al.* 2008; CKmap, Ruffo & Stoch 2005; Balfour-Browne Club NBN Gateway: http://data.nbn.org. uk, accessed December 2011), global databases (GBIF: www.gbif.net, accessed December 2011), as well as from extensive sampling and literature surveys. A total of 777 occurrence data of 14 *Lumetus* species were obtained, mainly representing the European and North African distribution of each species (Table S5 and Fig. S1, Supporting information). We could not obtain detailed data of *E. quadrinotatus*, *E. turanicus* cf, *E. hamiltoni* and *Enochrus diffusus*, which were excluded from the analyses. For the species with a wide distribution across the Holarctic or Palaearctic regions, the occurrence data (restricted to Europe and North Africa) intended to be representative of their geographic and ecological ranges. We extracted climate data (19 bioclimatic variables at 5′ resolution) for each georeferenced locality from the Worldclim database (http://www. worldclim.org).

We then used the bioclimatic information associated with the species occurrences to characterize the climatic niche of each species with an Outlying Mean Index analysis (OMI, Doledec *et al.* 2000). OMI is a multivariate analysis that allows the ordination of species niches in community analyses and has been previously used for the estimation of species niche separation across lineages (e.g. Thuiller *et al.* 2004). OMI estimates the mean position of the species in the environmental space, which represents a measure of the distance between the mean conditions of the habitat used by the species and the mean conditions of the study area (Doledec *et al.* 2000). OMI analyses were here used to identify the main climatic gradients separating niches among the species of *Lumetus* and to estimate the location of each species along them.

We tested the significance of the relationship between the salinity tolerance of each species (6-states categorization) and their location in each of the main axes of the OMI analyses using phylogenetic generalized least squares (PGLS) regressions. PGLS were run using the Caper package in the statistical program R using the best evolutionary model based on the outcome of our model fitting tests. To account for phylogenetic uncertainty, PGLS were calculated with the set of 1000 postburnin trees of the BEAST analysis (*Lumetus*-only data set), which were pruned to contain one tip per each species with climate niche data.

Results

Phylogenetic analyses

Sequences for mitochondrial protein coding genes (*cox1–A*, *cox1–B*, *cytb* and *nad1*) showed no length variation and correctly translated into amino acids for the complete and *Lumetus*-only data sets. The ribosomal *rrnL+trnL* gene showed some length variation, resulting in 38 and 15 gapped positions for the complete and

Lumetus-only data sets, respectively. The alignment of the conserved nuclear ribosomal gene *LSU* required 9 and no gapped positions respectively, whereas the alignment of the more variable *ITS2* nuclear gene resulted in a relatively high proportion of gapped positions (235 of 567) for the complete data set, lower (38 of 439) for the *Lumetus*-only data set. The best-fitting substitution models and additional information on the studied loci and alignments are provided in Table S6. The median estimated rates of evolution (substitutions per site per million years per lineage) for the different fragments were: 0.0147 for *cox1–A*, 0.0198 for *cox1–B*, 0.0369 for *cytb*, 0.0022 for *rrnL+trnL+nad1*, 0.0020 for *LSU* and 0.0081 for *ITS2*.

We obtained a well-resolved phylogeny with strong support for most nodes, including basal splits in the evolution of *Enochrus* and the diversification of *Lumetus* (Figs 1 and S2, Supporting information). The complete data set recovered the subgenus *Lumetus* as monophyletic (Clade 1; posterior probability (pp) = 1), whereas the subgenus *Methydrus* was paraphyletic and divided into four clades, one of them (a species from North America) sister to *Lumetus* (Clade 2; pp = 0.97). The relationships among the outgroups did not conform with current taxonomy (Fig. S2, Supporting information), but the incomplete sampling prevents any further interpretation.

Within subgenus *Lumetus* both data sets (complete and *Lumetus*-only) resulted in highly similar topologies, posterior probabilities and estimated node ages (Figs 1 and S2, Supporting information). The origin of *Lumetus* was dated at 50 Ma (95% HPD 41.7–60.0) in the complete data set, followed by an initial split at 41 Ma (95% HPD 34.1–49.4) of the lineage formed by *Enochrus salomonis* and *Enochrus ochropterus* (Clade A; pp = 1). The remaining *Lumetus* species were divided in four clades (Clades B-E), with uncertain relationships and short branches among them, suggesting a fast diversification around 31–37 Ma (95% HPD 25.7–44.5). Clade B (pp = 1) included all the species of the *E. bicolor* group; clades C and D were, respectively, formed by *Enochrus testaceus* and *E. hamifer* (which were sisters in the *Lumetus*-only data set with pp = 0.98); and clade E (pp = 0.83) grouped the Nearctic species (*Enochrus hamiltoni* and *Enochrus diffusus*; clade E1; pp = 1) with the Palaearctic *Enochrus politus*, *Enochrus quadripunctatus* (including *Enochrus fuscipennis*), *Enochrus halophilus* and *Enochrus ater* (clade E2; pp = 1). These two subclades (E1 and E2) split at 31.2 Ma (95% HPD 25.7–37.5). Within the *E. bicolor* group (clade B), two of the nodes included a south European-north African split (B1 and B2), estimated to have occurred 5.1 Ma (95% HPD 3.7–6.6) and 5.0 Ma (95% HPD 3.8–6.4), respectively, coherent with a post-Messinian vicariant origin.

Evolution of the tolerance to salinity

Models of evolution and phylogenetic signal of salinity tolerance. For the 2-states categorization of salinity tolerance, there were no differences between the two tested models in 100% of the trees (range of *P* = 0.16–0.41), and thus, a symmetrical model was selected. For the 3-states categorization both the symmetrical and all-rates-different models were significantly better than the equal-rates model for 100% of the trees (*P* = 0.015–0.047 and *P* = 0.006–0.020, respectively). Although the all-rates-different model showed a better fit than the symmetrical-rates model for all the trees, differences were not significant (*P* = 0.19–0.29), and hence, the latter was preferred.

The maximum-likelihood estimates of Pagel's lambda for the 2-states categorization were better than those obtained when the phylogenetic structure was erased ($\lambda = 0$) for 93.8% of the trees (*P* = 0.02–0.06). On the contrary, there were no significant differences with respect to a model where the phylogenetic structure was left intact ($\lambda = 1$) for 100% of the trees (*P* = 0.999–1), indicating a significant phylogenetic signal in the trait. Similar results were found for the 3-states and 6-states categorizations, with a significant difference with the model without phylogenetic signal for 100% of trees (*P* = <0.001–0.001)) and no differences with the model with $\lambda = 1$ for 100% of trees (*P* = 0.999–1). The best fitting model for trait evolution for the 6-states categorization was the Ornstein–Uhlenbeck, but without significant differences with a Brownian model for 100% of the trees (*P* = 0.06–0.62).

Reconstruction of the ancestral salinity tolerance. When salinity tolerance was reconstructed as a discrete variable using the 2-states or the 3-states categorizations, the most recent common ancestor of the subgenus *Lumetus* was estimated to be a freshwater species (pp = 0.57/0.76 for the 2- and 3-states, respectively) (Fig. 2a, b). Salinity tolerance independently developed in four lineages during the diversification of the subgenus (Fig. 2a,b). The first transition to saline habitats took place at the origin of the *E. bicolor* species group (clade B), when a direct switch from freshwater to saline was inferred (pp = 0.95/0.98). The other three most recent transitions involved the Nearctic *E. hamiltoni* and *E. diffusus* (clade E1) and the origins of *E. politus* and *E. halophilus*, with a transition from freshwater to hyposaline habitats in all cases (or to saline in the 2-states categorization, all pp >0.92) (Fig. 2a,b). The estimated rate of character evolution in the favoured symmetric model for the transition between freshwater and hyposaline for the 3-states categorization was 1.8 changes/Ma, higher than between freshwater and saline (0.6 changes/Ma) or hyposaline and saline (0.5

changes/Ma). The single transition rate estimated for the 2-states model was 1.0 changes/Ma.

The ancestral state reconstructions using the 6-states categorization showed a similar pattern, but the most recent common ancestor of the subgenus *Lumetus* was inferred as occupying mineralized waters (ancestral habitat type 2.5, i.e. mineralized, 95% HPD 1.91–2.98) with a subsequent colonization of saline waters in the same four clades than in the discrete reconstruction, and the maintenance of the mineralized-freshwater habitat for the rest of the lineages (Fig. 2c). The rates of evolution of the salinity tolerance were estimated to be 0.016 and 0.015 changes/lineage/Ma but were not homogeneous across the tree (Fig. 2d). They were accel-

erated especially on the origin of the *E. bicolor* group, but also in some terminal branches, such as those leading to *E. halophilus* and *Enochrus falcarius*. The plot of the branch lengths with the absolute range of the salinity transitions for the 6-states ancestral reconstruction showed those accelerated sections as outliers, beyond the upper 99% confidence interval of the significant regression line ($P < 0.005$) (Fig. 2d).

Relationship between tolerance to salinity and climatic niche

The two axes of the OMI analysis explained the 92.1% of the total variability (86.5% axis 1, 5.6% axis
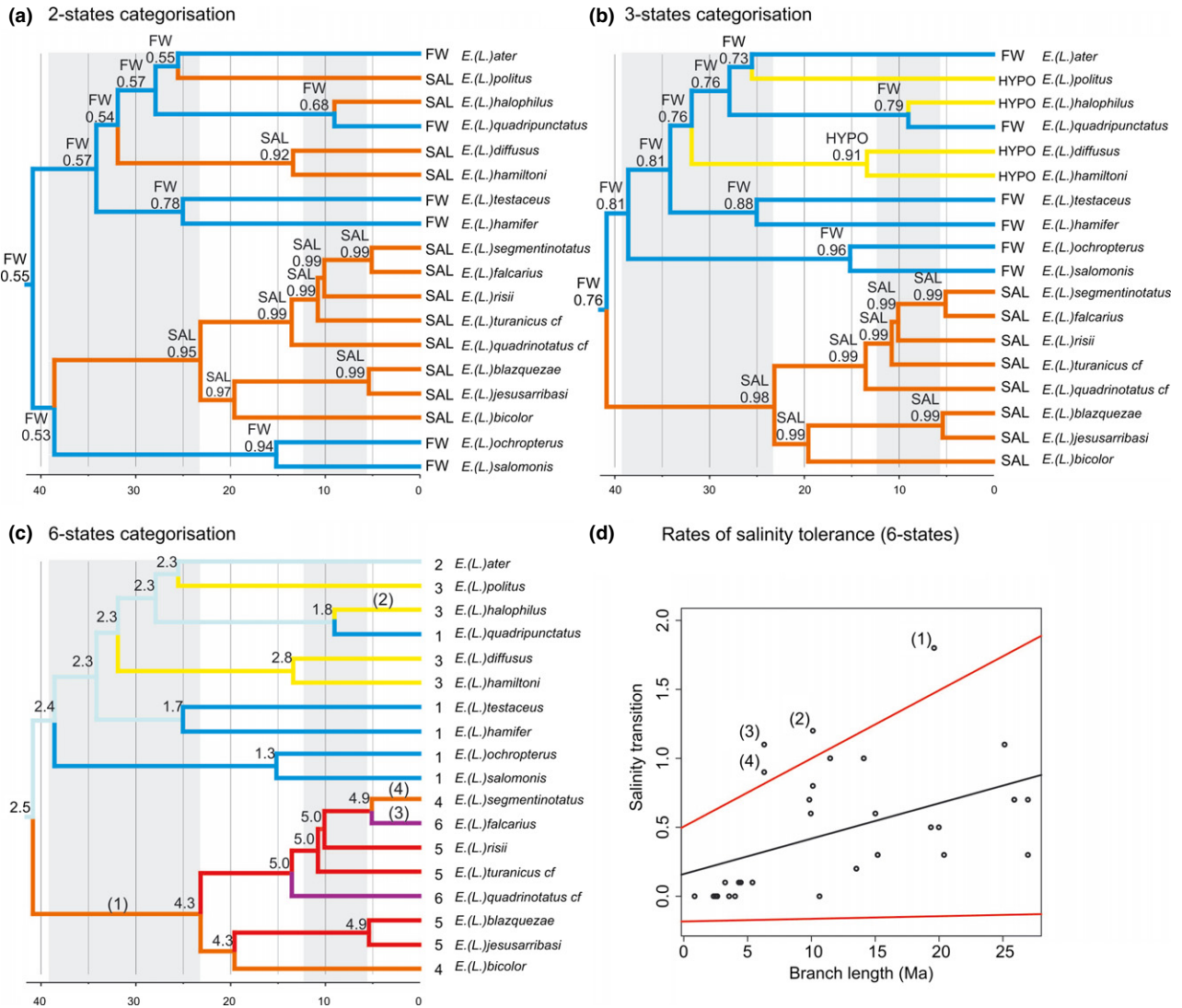

Fig. 2 Reconstruction of ancestral salinity tolerance using an ultrametric time-calibrated tree for the subgenus *Lumetus* for each of the three categorizations (a–c) (the represented topologies are those of the best tree in each of the respective analyses). Codes above nodes (numbers for 6-states) and colour branches as in Fig. 1. Numbers above nodes: posterior probability of the node discrete state. (d) Plot of the branch lengths with the absolute range of the salinity transitions for the 6-states ancestral reconstruction. Red lines: 99% confidence intervals of the significant regression line (black line), numbers: branches indicated in (c).

2) and discriminated the species climatic niche in two main gradients (Figs 3 and S3, Supporting information). The first axis was strongly negatively correlated with the mean temperature of the warmest annual quarter ($r = -0.94$) and positively correlated with the precipitation of the warmest quarter ($r = 0.93$), thus reflecting a thermal-aridity gradient from hot and dry (negative values) to cold and wet conditions (positive values) (Figs 3 and S3, Supporting information). The second climatic gradient was positively correlated with temperature seasonality ($r = 0.86$) and annual range ($r = 0.77$), that is, continentality (low seasonality and narrow temperature range in the negative values, and high seasonality and wide temperature range in the positive ones) (Figs 3 and S3, Supporting information).

The PGLS analyses showed a significant correlation between salinity tolerance, as measured with the 6-states categorization, and species position along the first OMI axis (thermal gradient) for 100% of the sampled trees (range of $P = 0.002$–0.003, $R^2 = 0.52$–0.53). No significant relationships were found between salinity tolerance and the second OMI axis ($P = 0.053$–0.055, $R^2 = 0.20$–0.23) (Fig. 3).
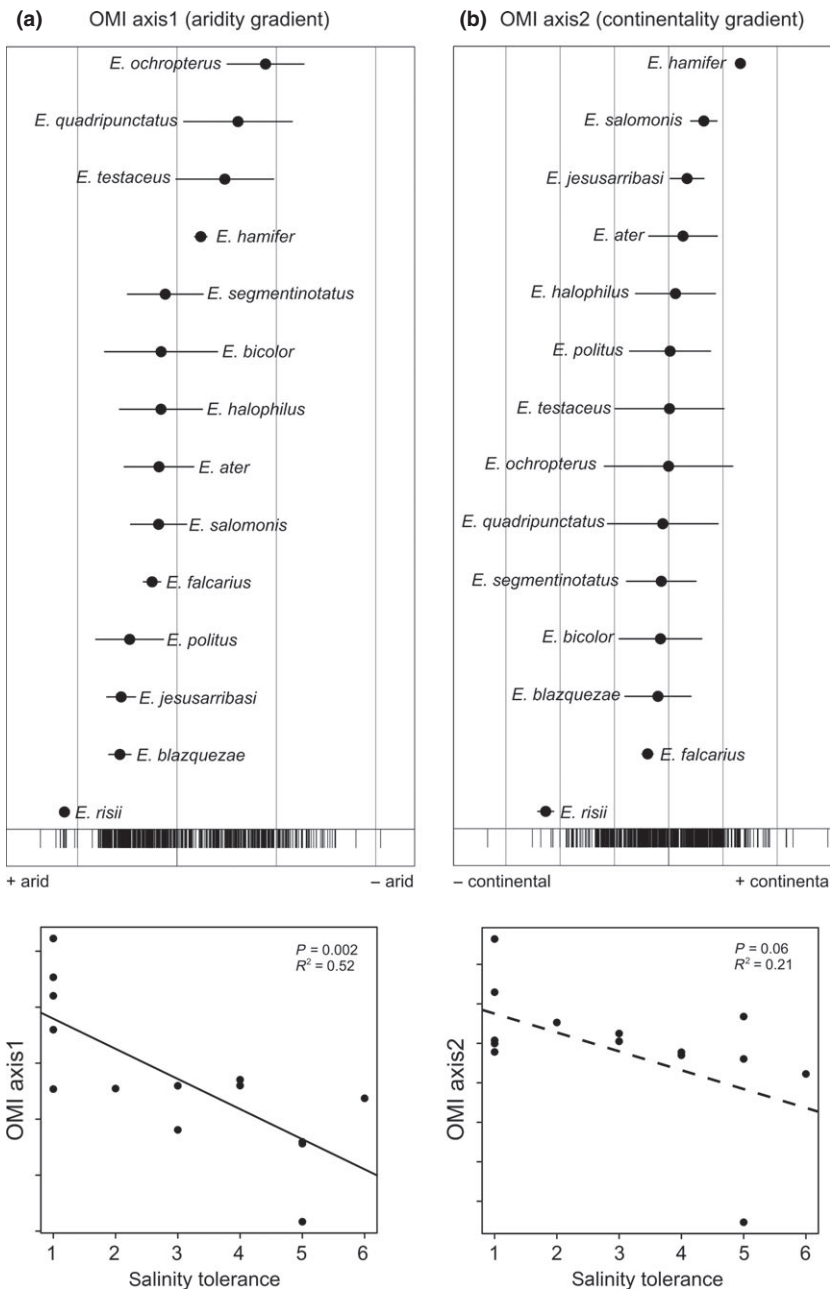

Fig. 3 Position of the niche of the species of *Lumetus* along the OMI axes (a: aridity, b: continentality) and their relationship with salinity tolerance (6-states categorization). Horizontal bars: niche breadth (standard deviation from the mean). Vertical bars below the OMI panels: position of all sites along the axes. Scatterplot graphs: PGLS correlation between salinity tolerance and OMI axes scores and their significance for each species according to the consensus phylogenetic tree (*Lumetus*-only data set).

Discussion

We have identified four independent transitions from the ancestral freshwater to saline habitats in the species of *Lumetus*, three of them at the end of the Eocene-early Oligocene and the fourth during the late Miocene. Most of them were one-step transitions, from freshwater or mineralized (depending on the resolution of the categorization used) to hyposaline, but at least one, at the origin of the *Enochrus bicolor* group, was reconstructed to be a direct switch to saline. The transition rates estimated for the stem branch of the *E. bicolor* group (and for many of the branches within) and for *Enochrus halophilus* were significantly higher than that of other parts of the tree, pointing to accelerated increases in salinity tolerance. In fact, the transition leading to *Enochrus politus* could also be considered a more direct change in salinity tolerance than estimated, as even if most of their stable populations were found in hyposaline habitats, some of them were in water with a higher salinity (Tables 1 and S4, Supporting information). We opted for considering *E. politus* as hyposaline since this criterion is conservative with respect to our conclusions (as seen below), specially considering the presence of likely cryptic lineages within this species and the lack of comprehensive data on the salinity of their habitats.

The possibility of direct transitions between fresh and saline waters suggests that the regulatory mechanisms leading to salinity tolerance not only can develop progressively, as expected under continuous, directional selection, but could also change directly in a single step from freshwater (or lowly mineralized) to able to tolerate saline water. These direct transitions could be said to be an artefact simply due to the lack of intermediate forms in our phylogeny, either because of extinction or undersampling. However, some of these events had accelerated transition rates, and extinct or missing intermediate forms (for which there is no evidence) could only increase them, at least in the transition at the base of the *E. bicolor* group. The significance of these increased transition rates did not change when *E. politus* was considered as mesosaline, due to the long stem branch of this species (Fig. S4, Supporting information).

The direct, accelerated transitions to saline habitats could be explained if the regulation mechanism was simple and labile enough to evolve quickly. It has been proposed that in some organisms the physiological response to salinity may rely on common stress-tolerance pathways (Munns & Tester 2008), mainly controlled by constitutive gene expression patterns (Latta *et al.* 2012). Such mechanisms are by definition labile, because few mutations in the expression regulatory system could promote transitions on the tolerance to salt. However, the saline aquatic insects that have been studied so far have specialized and complex mechanisms to deal with salinity rather than common stress pathways, mainly osmoconformism and osmoregulation (Bradley 2008). The strong phylogenetic signal and the generally slower transition rate of saline tolerance found in the complete lineage are more in agreement with the presence of a complex trait rather than a labile, generalist mechanism.

An alternative explanation for the direct evolution of salinity tolerance in *Lumetus* is that it was co-opted from a regulatory mechanism developed for different purposes, as happens frequently in the process of character evolution (Gould & Vrba 1982). A potential candidate is the physiological mechanism to avoid internal water loss, which is similar to that for the tolerance to salinity (Cloudsley-Thompson 1975; Gómez-Mestre & Tejedo 2005). Although osmoconformism has been observed in some insects able to inhabit coastal waters, osmoregulation has been proposed as the most recurrent adaptation to salinity in aquatic insects, especially on those lineages occupying the full salinity gradient (Bradley 2008), such as the subgenus *Lumetus*. The evolutionary steps allowing a freshwater species to evolve enhanced osmoregulator capacity include the up-regulation of the activity of the second rectal segment transporting ions into the rectal lumen and recovering water to the haemolymph (Bradley 2008). Despite being not functional on freshwater insects, the physiological basis of the osmorregulatory mechanism producing hyperosmotic urine in the rectum is a plesiomorphic character in insects (Bradley *et al.* 2009), as it is one of the main evolutionary steps that allowed their survival in terrestrial conditions. Among terrestrial insects living in the most arid conditions, an up-regulation of the capacity to produce hyperosmotic excreta and the enlargement and fusion of cuticular segments have been reported as some of the main adaptations for the regulation of the water loss (Cloudsley-Thompson 1975, 2001). Because of the similar physiological basis of the desiccation and osmotic tolerances, it seems reasonable to hypothesize that the development of drought tolerance in freshwater lineages (mainly up-regulation of rectum activity, i.e. water absortion and ion expulsion) could facilitate the colonization of saline waters. If this were the case, the origin of salinity tolerance would be an exaptation.

The strong relationship that we found between salinity tolerance and the aridity gradient is consistent with the hypothesis of a linked evolution of the osmotic and drought tolerances. Again, the consideration of *E. politus* as mesosaline would only strengthen this relationship, as it has score values for the first axis more similar to those of saline species (Figs 3 and S5, Supporting information). The results of the calibration of our phylogeny showed that the four transitions to

salinity tolerance occurred in periods of global aridification, three of them during the late Eocene or early Oligocene. During this period, there was a progressive fall in temperature and a global aridification that lasted until the end of the Oi-1 glaciation (Mosbrugger *et al.* 2005). There were important changes in the vegetation of Europe and the Mediterranean region, when tropical and subtropical plants disappeared and plants associated with dry climates and open country increased and established until the beginning of the Miocene (about 23 Ma, Postigo-Mijarra *et al.* 2009). Similarly, the fourth transition to salinity tolerance was estimated to have occurred during the last global aridification event of the Middle to late Miocene (Bruch *et al.* 2007).

Our results on the calibrated phylogeny and trait evolution in the subgenus *Lumetus* are concordant with a process of exaptation under a non progressive pattern. During periods of global aridification, in the freshwater populations of the subgenus *Lumetus,* most exposed to the increase in aridification (likely southern populations), selection for controlling water loss could secondarily lead to the possibility to tolerate saline waters and subsequently diversify in these habitats. Other studies have proposed that these global aridification events promoted the diversification of other lineages (e.g. Pepper *et al.* 2011; Pittermann *et al.* 2012), also relating them with the colonization of inland saline habitats (Pinceel *et al.* 2013).

We did not find any evidence of a reversal from saline to freshwater, other than the changes between freshwater mineralized in the reconstruction using the 6-states categorization. Despite this, in both the 2- and 3-states categorization the preferred models were symmetrical, that is, with the same estimated probability for both transitions. The lack of inferred reversals may be the consequence of the initial constraint of the freshwater origin of the group and the low global rate of change, resulting in an insufficient number of transitions in the phylogeny. But there are also physiological arguments favouring a reduced probability of reversal from saline to freshwater conditions. The cost of homoeostatic adjustments to stressful conditions will likely be detrimental to other metabolic functions (Southwood 1988). A trade-off between competitive ability and tolerance to osmotic stress has been suggested for multiple groups (e.g. Herbst 2001; Latta *et al.* 2012), with tolerant species displaying a selective disadvantage on more competitive habitats, such as those with less stressful conditions.

Our results were robust to topological uncertainty, as shown with the use of the 1000 postburnin trees, but some concerns could be raised as to the number of missing species, the assignation to salinity tolerances and the use of concatenated sequences. Despite the important problems of morphological identification of most *Lumetus* species, the main clades of the phylogeny

are concordant with most of the described species. Of the four known missing species of *Lumeus*, two (*E. subsignatus* and *E. asiaticus*) are morphologically close to *Enochrus quadripunctatus* complex (Kuwert 1893; Jia & Wang 2010), a freshwater species, and although there is few data on their habitat, they also do not seem to inhabite saline waters (Yano *et al.* 1983). The missing *E. reflexipennis* is closely related to the other two Nearctic species and found in similar habitats (Short 2004), so again it inclusion would not likely modify our conclusions. The situation within the *E. bicolor* group is more complex, as although there is only one missing species (*E. sinuatus*) recent work has shown that there is potentially a high level of cryptic diversity, and the number and identity of the species recognized in it is likely to vary (Schödl 1998; Arribas *et al.* 2013). However, this group is morphologically well characterized, and all known members are found in saline to hypersaline waters, so any rearrangement or addition of new taxa would not alter our main conclusions.

Conclusions

We have shown that salinity tolerance evolved multiple times in a group of water beetles, always associated with periods of global aridification. In some of these transitions, we found evidence of direct accelerated increases in tolerance to salinity, as reconstructed with extant lineages. The strong correlation of salinity tolerance with the aridity of the habitats in which species are found, and the possibility of multiple and direct transitions from freshwater to saline, suggest that tolerance to salinity may be based on a co-opted mechanism developed originally for drought resistance.

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P.Ar. and I.R. conceived the work; P.Ar., A.M. and I.R. led the specimen and data collection; P.Ar. and C.A. obtained the molecular data; P.Ar., C.A. and P.Ab analysed the data; P.Ar led the writing and all authors contributed to the discussion of results and the writing.

Data accessibility

DNA sequences: GenBank Accessions nos. are given in Table S2 in Supporting Material; primers used in Table S3; sampling localities information in Table S2 and Table S4; climate data in Worldclim database (http://www.worldclim.org/); species occurrences in Table S5 and Fig. S1; sequence alignment and tree files as Supporting Information.

Supporting information

Additional supporting information may be found in the online version of this article.

Table S1 Checklist of species in the subgenus *Enochrus (Lumetus)*.

Table S2 List of sequenced specimens for the subgenus *Enochrus (Lumetus)*.

Table S3 Primers used in reconstructing the phylogeny of subgenus *Enochrus (Lumetus)*.

Table S4 Localities and associated salinities for populations of the different species of the subgenus *Enochrus (Lumetus)*.

Table S5 Occurrence data of 14 *Lumetus* species used in the Outlying Mean Index analysis.

Table S6 Information on individual gene, codon partition, and combined data sets from aligned sequence data in the subgenus *Enochrus (Lumetus)*.

Fig. S1 Occurrence data of 14 *Lumetus* species used in the Outlying Mean Index analysis.

Fig. S2 Ultrametric time-calibrated tree for the subgenus *Lumetus* including outgroups (complete data set).

Fig. S3 Ordination diagrams on the first two axes of outlying mean index analysis (OMI).

Fig. S4 Reconstructions of ancestral salinity tolerance using *Enochrus politus* as a mesosaline species.

Fig. S5 Relationship between niche position and salinity tolerance (6-states categorization) using *Enochrus politus* as a mesosaline species.

Data S1 Sequence alignment file for the specimens in the subgenus Enochrus (Lumetus).

Data S2 Phylogenetic tree file for the specimens in the subgenus Enochrus (Lumetus).