

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

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Background. Desiccation resistance shapes the distribution of terrestrial insects at multiple spatial scales. However, responses to drying stress have been poorly studied in aquatic groups, despite their potential role in constraining their distribution and diversification, particularly in arid and semi-arid regions. **Methods.** We examined desiccation resistance in adults of four congeneric water beetle species (*Enochrus*, family Hydrophilidae) with contrasting habitat specificity (lentic vs lotic systems and different salinity optima from fresh- to hypersaline waters). We measured survival, recovery capacity and key traits related to desiccation resistance (fresh mass, % water content, % cuticle content and water loss rate) under controlled exposure to desiccation, and explored their variability within and between species. **Results.** Meso- and hypersaline species were more resistant to desiccation than freshwater and hyposaline ones, showing significantly lower water loss rates and higher water content. No clear patterns in desiccation resistance traits were observed between lotic and lentic species. Intraspecifically, water loss rate was positively related to specimens' initial % water content, but not to fresh mass or % cuticle content, suggesting that the dynamic mechanism controlling water loss is mainly regulated by the amount of body water available. **Discussion.** Our results support previous hypotheses suggesting that the evolution of desiccation resistance is associated with the colonization of saline habitats by aquatic beetles. The interspecific patterns observed in *Enochrus* also suggest that freshwater species may be more vulnerable than saline ones to drought intensification expected under climate change in semi-arid regions such as the Mediterranean Basin.

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2 **differ in species with contrasting habitat preferences?**

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25 **Abstract**

26 **Background.** Desiccation resistance shapes the distribution of terrestrial insects at multiple
27 spatial scales. However, responses to drying stress have been poorly studied in aquatic groups,
28 despite their potential role in constraining their distribution and diversification, particularly in
29 arid and semi-arid regions.

30 **Methods.** We examined desiccation resistance in adults of four congeneric water beetle species
31 (*Enochrus*, family Hydrophilidae) with contrasting habitat specificity (lentic vs lotic systems and
32 different salinity optima from fresh- to hypersaline waters). We measured survival, recovery
33 capacity and key traits related to desiccation resistance (fresh mass, % water content, % cuticle
34 content and water loss rate) under controlled exposure to desiccation, and explored their
35 variability within and between species.

36 **Results.** Meso- and hypersaline species were more resistant to desiccation than freshwater and
37 hyposaline ones, showing significantly lower water loss rates and higher water content. No clear
38 patterns in desiccation resistance traits were observed between lotic and lentic species.
39 Intrinspecifically, water loss rate was positively related to specimens' initial % water content, but
40 not to fresh mass or % cuticle content, suggesting that the dynamic mechanism controlling water
41 loss is mainly regulated by the amount of body water available.

42 **Discussion.** Our results support previous hypotheses suggesting that the evolution of desiccation
43 resistance is associated with the colonization of saline habitats by aquatic beetles. The
44 interespecific patterns observed in *Enochrus* also suggest that freshwater species may be more
45 vulnerable than saline ones to drought intensification expected under climate change in semi-arid
46 regions such as the Mediterranean Basin.

47 **Introduction**

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

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

63 However, a large knowledge gap still exists in the way aquatic macroinvertebrates respond to
64 droughts (Robson et al. 2011). In the case of aquatic insects, most studies are focused on
65 desiccation-resistant eggs or dormant larvae stages (e.g. Benoit 2010; Juliano et al. 2002; Woods
66 and Singer 2001), but little is known on the effects of dryness on species which lack these
67 resistant stages (e.g. most aquatic beetles, Strachan et al. 2015). Although some species of water
68 beetles resist the dry phase of temporary waters in microrefuges *in situ*, as adult or larvae stages
69 (e.g. Davy-Bowker 2002; Stubbington et al. 2016), winged adults of many species show a more

70 resilient strategy, dispersing by flying from drying sites to more favourable wet habitats (Bilton
71 2014; Strachan et al. 2015). The duration of exposure to drying stress during dispersal depends
72 on specific biological traits (e.g. flight ability) and landscape configuration, i.e. the availability of
73 suitable habitats that may serve as wet refuges and the distance and connectivity between them
74 (Datry et al. 2016; Larned et al. 2010). But even short exposures to drying stress are challenging
75 for flying aquatic insects, because flight activity is associated to a strong dehydration (Dudley
76 2000).

77 Studies of geographical variation in responses to desiccation stress in terrestrial insects have
78 typically demonstrated that species and populations from xeric environments show a greater
79 ability to deal with dehydration than those from mesic areas (e.g. Chown 1993; Gibbs and
80 Matzkin 2001; Le Lagadec et al. 1998; Schultz et al. 1992). Different responses to desiccation
81 have also been related to patterns of habitat and microhabitat choice in arthropods (e.g. Chown
82 1993; De Vito et al. 2004; Gereben 1995; Lapinski and Tschapka 2014), including aquatic
83 species (e.g. Wissinger et al. 2003; Yoder et al. 2015). In general, these studies show that some
84 physiological mechanisms linked to drying stress such as the control of water loss rate have an
85 important plastic and adaptive component, whilst others, such as the tolerance of water loss, are
86 less variable across species (Chown et al. 1999; Hoffmann and Harshman 1999). In inland water
87 ecosystems, even congeneric macroinvertebrate species show remarkable differences in the
88 strategies and particular traits to deal with desiccation, and such variability is strongly associated
89 with the frequency and duration of droughts in their habitats (Strachan et al. 2015).

90 In Mediterranean inland waters, a number of genera of water beetles belonging to different
91 families contain species which are adapted to different parts of the fresh – hypersaline gradient
92 (Millán et al. 2011). Organisms dealing with osmotic stress in saline waters face similar

93 physiological challenges to those imposed by desiccation; i.e. maintaining water balance and
94 compensating for the increase in the osmotic concentration of internal fluids (Bradley 2009). In
95 light of this, interspecific differences in desiccation resistance may correlate with salinity
96 tolerance, so that species inhabiting saline waters are expected to be potentially more resistant to
97 desiccation than those in lower salinity ranges (Arribas et al. 2014). In addition, species living in
98 relatively short-lived small lentic water bodies, which are more unstable systems over
99 evolutionary and ecological time-scales than lotic systems (see Ribera 2008 for details), may also
100 be expected to have higher desiccation resistance compared to related lotic taxa. These
101 predictable differences have already been found between lotic and lentic congeneric beetle
102 species in other traits such as dispersal capacity (Arribas et al. 2012), behavioural responses to
103 acute thermal stress (Pallarés et al. 2012) and salinity tolerance (Céspedes et al. 2013).

104 Together with behavioural responses (e.g. use of microrefuges, burrowing) or aestivation,
105 invertebrates have evolved a variety of physiological adaptations to cope with desiccation stress
106 (Hershkovitz and Gasith 2013; Strachan et al. 2015). These fall under two main strategies
107 (Chown and Nicolson 2004; Edney 1997): 1) avoiding desiccation through the reduction of water
108 loss and increases in body water content (*desiccation resistance*, e.g. Gray and Bradley 2005)
109 and 2) withstanding the loss of a significant proportion of body water (*desiccation tolerance*, e.g.
110 Benoit et al. 2007; Suemoto et al. 2004). In insects, mechanisms regulating cuticle permeability
111 are the major component of desiccation resistance because the cuticle represents their main
112 avenue for water loss (Benoit et al. 2010; Chown and Nicolson 2004; Hadley 1994). Cuticle
113 permeability is related with the cuticle thickness (Crowson 1981; Harrison et al. 2012;
114 Reidenbach et al. 2014), but can be actively regulated through changes in the amount and
115 composition of surface lipids (Gibbs and Rajpurohit 2010; Stinziano et al. 2015). Water loss has

116 shown to be non-linear following exposure to desiccation in a range of taxa (e.g. Benoit et al.
117 2007; Arlian and Staiger 1979). Greater water loss rates occur during initial hours of exposure
118 and decrease as body water content approaches lethal levels, suggesting that water loss is
119 actively regulated by dynamic mechanisms. As a consequence, the water content of an individual
120 at a particular moment could influence its water loss dynamics and ultimately its survival under
121 drying stress. On the other hand, body size can affect desiccation resistance in arthropods in a
122 number of ways. In general, larger body mass allows a higher proportion of water and lipid
123 content (Lighton et al.1994; Prange and Pinshow 1994), and additionally smaller insects may
124 show higher mass-specific water loss rates due to higher surface area - volume ratios (Chown et
125 al. 1998; Schmidt-Nielsen 1984; Williams and Bradley 1998). Such effects of size on water loss
126 rates have been seen both inter- (e.g. Chown and Klok 2003; Le Lagadec et al. 1998) and
127 intraspecifically (e.g. Renault and Coray 2004).

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

139 **Material and methods**

140 *Study species*

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

155 *Experimental procedures*

156 Adult specimens (approx. 50 per species) were collected from different localities all in
157 southeastern Spain and representing the optima salinity conditions of each species (see Table 1).
158 For logistic reasons, we used specimens from one single location per species. Such locations
159 were selected minimizing distances between each other and so have a comparable climatic
160 regime. All species were collected during the spring 2014, therefore the specimens used for the

161 experiments were all mature adults from the winter generation and presumably had not been
162 previously exposed to desiccation stress in natural conditions.

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

170 The experimental protocol and variables recorded in controlled desiccation experiments are
171 showed in Fig. S1. For each specimen studied we obtained the initial fresh mass (M_0) as a
172 surrogate of size, initial water content (WC_0 ; % wet mass to initial fresh mass), cuticle content as
173 a surrogate of cuticle thickness (CC; % of cuticle mass to initial fresh mass), water loss rates
174 (WLR; % of water lost to initial fresh mass per unit time) and total water loss after the
175 corresponding treatment (WL, % of water loss to total water content). For this, groups of 20-25
176 individuals of each species were dried on blotting paper, weighed on a balance accurate to 0.01
177 mg and placed individually into clean 15 mL open glass vials. These were kept for 6 h in a glass
178 desiccator containing silica gel (Sigma-Aldrich, Madrid, Spain) at $20\pm 1^\circ\text{C}$. Relative humidity,
179 monitored with a hygrometer (OM-EL-USB-2-LCD, Omega Engineering, Seville, Spain),
180 dropped from approx. 40% (laboratory humidity) to $20\pm 5\%$ within the first 2 h and remained
181 stable within this range until the end of the trial. The experimental conditions were optimized
182 through pilot trials in order to detect differences among species, within their tolerance limits and
183 in a reasonable experimental time (to avoid additional stress such as starvation). The remaining

184 specimens (N=10-20 individuals per species) were used as a control under no desiccation stress.
185 They were kept in glass vials placed in a closed tank with deionized water in the base, producing
186 a relative humidity $\geq 90\%$. After 6 h, surviving specimens from control and test groups were re-
187 weighed for estimation of water loss rates and allowed to recover for 24 h in 100 mL containers
188 with 40 mL of the dilute solution. Some studies have shown that rehydration may result in an
189 excessive increase in specimens' water content (overhydration stress, e.g. Lopez-Martinez et al.
190 2009; Yoder et al. 2015). However, we checked in pilot trials (data not shown) that the species
191 here studied recovered their initial water content after rehydration, with no significant water
192 gains. Mortality was monitored after desiccation exposure and after the recovery period.
193 Specimens were then dried at 50°C for 48 h and re-weighed for estimation of the initial water
194 content. A subgroup of 20 individuals per species from the test group were also immersed in 4
195 mL of 2M NaOH(aq.) for 48 h at room temperature to allow tissue digestion, rinsed in distilled
196 water, dried and weighed again for estimation of cuticle content (Harrison et al. 2012).
197 Specimens were sexed after the experiment by examining genitalia under a Leica M165C
198 stereomicroscope.

199 *Data analyses*

200 *Interspecific comparison of desiccation traits*

201 Fresh mass, water loss rate, water content and cuticle content were compared among species
202 using generalized linear models (GLMs) with species as factor, followed by Bonferroni post-hoc
203 tests. Gaussian error distribution and identity link function were used for fresh mass, water
204 content and cuticle content models; and gamma distribution for water loss rate which showed a
205 positively skewed distribution. To account for the potential effects of sex and body size in
206 desiccation resistance, sex and the interaction of sex and species were included as predictors, as

207 well as fresh mass in comparisons of water loss rate, water content and cuticle content (e.g.
208 Addo-Bediako et al. 2001; Terblanche et al. 2005). Model residuals were checked for normality
209 and homoscedasticity assumptions.

210 *Relationships between desiccation resistance traits within species*

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

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

218 **Results**

219 *Interspecific comparison of desiccation traits*

220 The water and cuticle contents of the four studied species ranged from 60 to 68 % and 12 – 23 %
221 M_0 , respectively. Mean water loss rates of specimens exposed to desiccation ranged from 2.22 to
222 3.57% $M_0 h^{-1}$, with a total water loss after 6 h of desiccation exposure of 19.3 – 39.1 % WC_0 .
223 Specimens in the control group showed very little water loss (approx. 0.5% $M_0 h^{-1}$ and a
224 maximum water loss of 6 % WC_0) (see Table S2 for species comparative data).

225 All desiccation resistance traits differed significantly between species (Table 2). Females showed
226 higher fresh mass and water content than males in all species (see sex and sex x species effects in

227 Table 2). Despite significant interspecific differences in mean fresh mass (Fig. 1A), the effect of
228 initial body mass on the other trait comparisons was not significant (Table 2).

229 The species living in fresh–subsaline waters (*E. halophilus*) showed a significantly higher water
230 loss rate, but this did not differ significantly amongst the other three species (Fig. 1B). Initial
231 water content was higher in the meso and hypersaline species (*E. bicolor* and *E. jesuarribasi*)
232 than in the subsaline and hyposaline ones (*E. halophilus* and *E. politus*) (Fig. 1C). The species
233 showed similar cuticle contents, except for *E. halophilus* which had the highest value (Fig.1D).
234 No consistent patterns between lotic and lentic species were observed for any of the measured
235 traits.

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

241 *Relationships between desiccation resistance traits within species*

242 In general, the desiccation resistance traits showed high inter-individual variability in all species
243 studied (see Figs. 1 and 2). A significant positive relationship was found between individual
244 water loss rates and water content in all species except for *E. halophilus* (Fig. 2A). In contrast,
245 cuticle content was not related to water loss rate in any species (Fig.2B), and these were also
246 independent of initial body mass (Fig.2C).

247 **Discussion**

248 On the basis of our investigations, desiccation resistance in *Enochrus* water beetles appears to be
249 associated with habitat salinity, but does not differ between species occupying lotic and lentic
250 water bodies. The more saline-tolerant species studied (*E. bicolor*, *E. jesuarribasi* and *E.*
251 *politus*) showed lower water loss rates than the freshwater-subsaline species (*E. halophilus*).
252 Furthermore, within these three saline species, the meso and hypersaline ones (*E. bicolor* and *E.*
253 *jesuarribasi*) had significantly higher initial water content than the hyposaline *E. politus*.
254 Indeed, these interspecific differences in water control efficiency seem to be relevant in terms
255 of survival under drying stress, as *E. halophilus* was also the most sensitive species to the
256 conditions tested here. In consequence, assuming that the species may tolerate similar levels of
257 water loss (Chown et al. 1999; Hoffmann and Harshman 1999), the studied saline tolerant
258 species showed a clear physiological advantage over freshwater ones under desiccation
259 conditions.

260 Arribas et al.(2014) suggested that salinity tolerance in water beetles could be based on a co-
261 opted mechanism originally developed for desiccation resistance, relying on the temporal
262 correlation of global aridification events and the phylogenetic ages of saline lineages. The pattern
263 found here of stronger desiccation resistance in aquatic species living in saline waters is clearly
264 consistent with this hypothesis and emphasizes the important role that traits associated with
265 coping with osmotic and desiccation stress could have in shaping the ecological diversification
266 of *Enochrus*. Also in line with the relationship between desiccation and salinity tolerance seen
267 across the beetles studied here, intraspecific studies of corixid populations found similar
268 responses to the two stressors (e.g. Cannings 1981), and salinity acclimation was showed to
269 confer desiccation resistance in an Antarctic midge (Elnitsky et al. 2009). Salinity imposes
270 similar stress on aquatic organisms as that resulting from desiccation during air exposure at the

271 cellular level (i.e. water loss and increase of the osmotic pressure) (Evans 2008; Bradley 2009).

272 In consequence, shared genetic and physiological mechanisms might underlie resistance to these
273 two factors, as found with other related stressors such as desiccation and cold (e.g. Everatt et al.
274 2014; Holmstrup et al. 2002; Levis et al. 2012).

275 Our study found no direct association between desiccation resistance and the lotic/lentic habitat
276 divide. Previous studies on water beetles have shown that lentic taxa have a higher colonization
277 ability (i.e. the ability of a species to disperse and establish new populations) than lotic related
278 species, resulting in larger geographical ranges and lower population genetic structure (Abellán
279 et al.2009; Hof et al. 2012; Ribera 2008). Dispersal capacity and thermal tolerance seem to be
280 the main traits driving this lotic/lentic pattern in water beetles (e.g. Hjalmarsson et al. 2015) and
281 particularly in two of the species here studied, *E. jesusarribasi* and *E. bicolor* (Arribas et al.
282 2012; Pallarés et al. 2012). The two lotic species studied here are restricted to the Iberian
283 Peninsula and Morocco whilst the lentic ones are distributed across larger areas, including
284 northern Europe (Millán et al. 2014), but no clear patterns in desiccation resistance traits were
285 found accordingly. Therefore, desiccation resistance could play a secondary role to differences in
286 dispersal capacity in shaping the colonization ability of water beetles. In this point it should be
287 noted that *Enochrus* species' occurrence across different habitat types will be also constrained by
288 the limited desiccation resistance of eggs and larvae, being the latter likely the most desiccation-
289 sensitive stage because of their thinner cuticles. In addition, desiccation resistance might show
290 inter-population variability (e.g. Hoffmann & Harshman 1999) as a result of physiological
291 plasticity or local adaptations. Despite our study on adults from populations on similar climatic
292 regimes but different habitats allows for a robust comparison across species, further studies on

293 multiple stages and populations are needed to deeply understand the relationship between habitat
294 occupation and resistance to desiccation in this group.

295 Beetles are one of the groups of arthropods best adapted to desiccation, with species from desert
296 or semi-desert areas typically representing the extremes in tolerance to dehydration. For
297 example, the terrestrial spider beetle *Mezium affine* shows daily water losses as little as 0.3% per
298 day and the ability to survive up to 3 months with no food or water (Benoit et al. 2005).

299 Surprisingly, the highest tolerance to water loss (89% of the body water content) has been
300 reported for a fully aquatic beetle, the haliplid *Peltodytes muticus* (Arlan and Staiger 1979).

301 Since they occupy the shallow margins of waterbodies, *Enochrus* species may be expected to be
302 intermediate in desiccation resistance between strictly terrestrial beetles and those occupying
303 deeper water such as many diving beetles (Dytiscidae) (Beament 1961; Holdgate 1956;
304 Wigglesworth 1945). However, it is difficult to establish a comparative framework because of
305 the few existent data on desiccation resistance traits in adult aquatic insects and the multiple and
306 contrasting approaches and/or experimental conditions used to measure them. The water contents
307 of the four *Enochrus* species (60-68% of fresh mass) were consistent with the typical 62% of
308 most beetles (Hadley 1994). Water loss rates, ranging from 2.2 to 3.6% of initial mass at 20%
309 RH, appear to be comparable to those reported for the extraordinary desiccation resistant *P.*
310 *muticus*, which lost ca. 5.4% of initial mass per hour under more severe conditions (0% RH)
311 (Arlan and Staiger 1979). Nevertheless, the total water losses that the studied species reached
312 after the desiccation treatment (Table S2) were close to the limit of dehydration tolerance of
313 most insects (20 - 30% of water content) (Hadley 1994). Although such water loss was measured
314 under an unrealistic humidity in natural conditions (20% RH), a combination of high
315 temperatures (>30°C) and low humidity (40-50% RH) is frequent in the natural habitats of these

316 species. Prolonged exposures to such conditions in nature may result in extremely stressful
317 conditions and high mortalities of local populations of the studied species, but further research is
318 needed to identify desiccation level and duration thresholds under natural conditions for each
319 particular species.

320 The analysis of traits at the individual level is essential for further exploration of the mechanisms
321 regulating water loss rate. In *Enochrus* species, water loss rates were positively related to the
322 specimens' initial water content. These relationships were relatively weak ($r^2 < 0.5$) due to high
323 inter-individual variation in both traits, which might be associated to age, sex or the
324 physiological state of the individuals (e.g. Chown et al. 1999; Lyons et al. 2014; Matzkin et al.
325 2007). Despite this variability, resistance to water loss seems to be partly a function of individual
326 water content, as beetles with a higher initial proportion of water lost it faster than those with
327 lower values. This suggests that a critical level of water loss may induce active mechanisms for
328 water conservation (e.g. changes in cuticular permeability), which might be "relaxed" when
329 organismal water content rises above this threshold. Such regulation is concordant with the
330 nonlinearity of water loss following exposure to desiccation found in many fully terrestrial
331 insects (e.g. Arlian and Staiger 1979; Benoit et al. 2007).

332 Although we used cuticle content as a potential surrogate of cuticle permeability, since increased
333 cuticle thickness is associated with desiccation resistance in insects adapted to arid conditions
334 (Crowson 1981; Elias 2010), this trait showed no relationship with water loss rates in any
335 *Enochrus* species. In addition, in interspecific comparisons, the species with the highest mean
336 water loss rate had the highest cuticle content. A recent study also showed that cuticle thickness
337 in adult mosquitoes appeared not to affect desiccation resistance (Reidenbach et al. 2014).
338 Therefore, the validity of cuticle thickness as proxy for cuticular permeability could be very

339 different across taxa and may perhaps have low resolution for intra-generic comparisons. In
340 some terrestrial insects, changes in the composition and quantity of cuticular hydrocarbons
341 appear to be the main mechanism through which they can modulate cuticular permeability (e.g.
342 Hadley 1978; Nelson and Lee 2004; Stinziano et al.2015; Toolson 1982). In aquatic insects,
343 similar mechanisms may shape responses to dehydration occurring both in exposure to air or
344 hyperosmotic aquatic medium but to date even basic cuticular properties in such taxa have
345 received little study (but see Alarie et al. 1998 for an example).

346 Despite the fact that many previous studies suggest that body size affects water loss rate in
347 arthropods (e.g. Chown et al.1998; Lighton et al. 1994; Prange and Pinshow1994) our results
348 suggest that both interspecific and inter-individual size differences do not significantly affect
349 desiccation resistance in these water beetles. Although large size (lower area-to-volume ratio)
350 might be expected to be beneficial for survival under desiccating conditions (Chown et al. 1998;
351 Schmidt-Nielsen 1984), important trade-offs could arise as a result of increases in body size
352 (Chown and Gaston 2010; Chown and Klok 2003). This could be particularly true in the case of
353 aquatic insects living in fluctuating or temporary waters, such as the beetles studied here, where
354 rapid larvae development and small body size are common, alongside other r-selected traits
355 (Millán et al. 2011; Williams 1985).

356 **Conclusions**

357 This study is the first to explore both interspecific and inter-individual variation in desiccation
358 resistance traits within a group of closely related aquatic insects. Our results suggest that control
359 of both water loss rate and water content may be key mechanisms for dealing with desiccation
360 stress in adult water beetles and suggest an association between salinity tolerance and desiccation

361 resistance. Further studies are required to evaluate the ecological and evolutionary consequences
362 of interspecific variation in key desiccation resistance traits, but our results point to habitat-
363 mediated differences (saline vs. freshwater) in the vulnerability of water beetle species to a
364 higher frequency and intensity of droughts expected in semi-arid regions.

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368 helping with experimental procedures. The manuscript was greatly improved by the suggestions
369 of Tomas Ditrich and two other anonymous reviewers.

370 **Figure captions**

371 **Fig 1.** Interspecific comparison of desiccation resistance traits in *Enochrus* species. Letters below the
372 boxes indicate significant differences between species (Bonferroni post-hoc tests, $P < 0.05$). Boxplots
373 represent Q25, median and Q75, whiskers are Q10 and Q90 and dots are outliers.

374 **Fig 2.** Relationships between individual water loss rates (WLR) and initial water content (WC_0), cuticle
375 content (CC) and fresh mass (M_0) for *Enochrus* species. P-values and deviance (D^2) are showed for the
376 statistically significant relationships ($P < 0.05$).

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Table 1 (on next page)

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

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

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

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

3 ^b Ranges of conductivity of each category (mS cm⁻¹): Freshwater: < 1, Subsaline: 1–10, Hyposaline: 10–30, Mesosaline: 30–60, Hypersaline: > 60 (Montes and
4 Martino 1987).

Table 2 (on next page)

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

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

3

4 Trait	Predictors	df	F-value/ χ^2 ^a (Explained deviance) ^b	P
5 M_0 (mg)	Sp	3	37.627	< 0.001
	Sex	1	14.206	< 0.001
6	Sp x Sex	3	0.607 (0.651)	0.613
7				
8 WLR (% M_0 h ⁻¹)	Sp	3	2.718	< 0.001
	M_0	1	0.126	0.161
	Sex	1	0.004	0.799
9	Sp x Sex	3	0.007 (0.397)	0.990
10				
11 WC_0 (% M_0)	Sp	3	22.086	< 0.001
	M_0	1	1.387	0.243
12	Sex	1	4.736	0.033
	Sp x Sex		0.335 (0.519)	0.800
13				
14				
15 CC (% M_0)	Sp	3	27.019	< 0.001
16	M_0	1	3.067	0.085
17	Sex	1	0.027	0.870
18	Sp x Sex	3	1.629 (0.593)	0.192
19				
20				
21				
22				

23

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

24

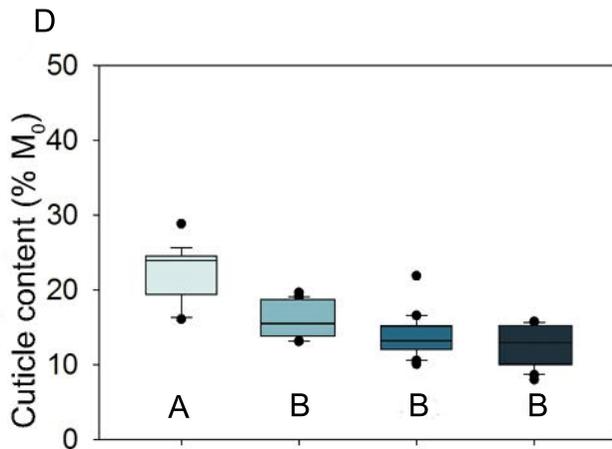
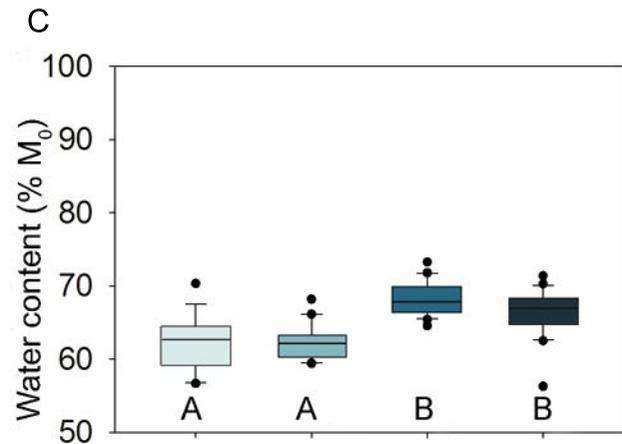
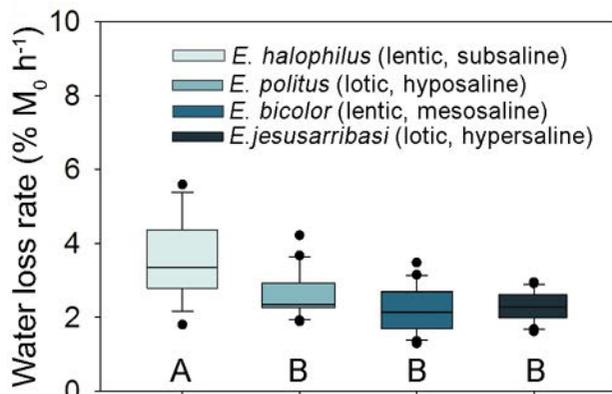
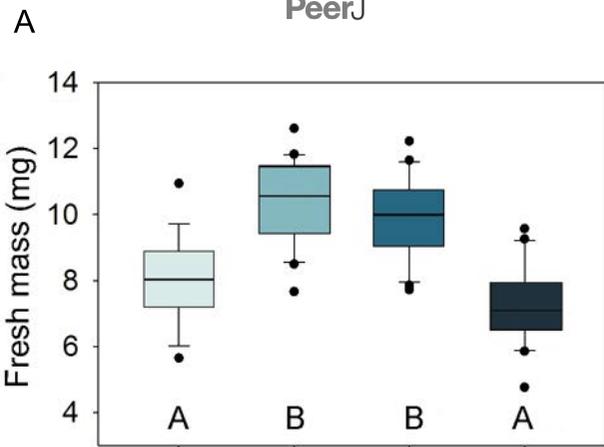
^b (null deviance – residual deviance/null deviance)

25

Figure 1 (on next page)

Interspecific comparison of desiccation resistance traits in *Enochrus* species.

Letters below the boxes indicate significant differences between species (Bonferroni post-hoc tests, $P < 0.05$). Boxplots represent Q25, median and Q75, whiskers are Q10 and Q90 and dots are outliers.



species

Figure 2 (on next page)

Relationships between individual water loss rates (WLR) and initial water content (WC_0), cuticle content (CC) and fresh mass (M_0) for *Enochrus* species.

P-values and deviance (D^2) are showed for the statistically significant relationships ($P < 0.05$).

