



# The effects of salinity on the distribution and survival of two exotic ostracods in the Iberian Peninsula

Alexandre Mestre<sup>1</sup> · Raül Sorlí ·  
Francesc Mesquita-Joanes<sup>1</sup>

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**Abstract** Recent research highlighted the need to include experimental estimates of tolerance limits to varying environmental conditions when investigating what factors limit species distributions. However, most niche approaches are only based on the statistical dependence between environmental and occurrence data. Here, we combined field data with survival experiments to assess the role of salinity as a limiting factor in the distribution of two species of exotic ostracods from the Iberian Peninsula. *Vizcainocypria viator* is a free-living species associated with rice fields and *Ankylocythere sinuosa* is a commensal of the red swamp crayfish (*Procambarus clarkii*). Experiments and field data indicate that the distribution of *V. viator* is limited by adult survival at low and high salinities (below electrical conductivity of 0.6 mS/cm and above 10 mS/cm). In the case of *A. sinuosa*, the analysis of field data shows that its prevalence is negatively affected by high salinity, whereas experiments indicate an optimal survival at high salinities (conductivity above 10.2 mS/cm), thus

suggesting that high salinity may impact *A. sinuosa* distribution indirectly through affecting host traits (e.g. reduced activity). The habitat of close ancestors (marine versus non-marine respectively for *A. sinuosa* and *V. viator*) most likely explains the contrasting differences in salinity tolerance between both ostracod species.

**Keywords** Abiotic niche · Experimental survival · Halotolerance · Invasive Crustacea

## Introduction

The geographic range of a species is particularly difficult to estimate (Grinnell, 1917). It is defined as “the fraction of geographical space where a species is present and interacts non-ephemerally with the ecosystem” (Zunino & Palestini, 1991). The concept involves both the location or type of habitat used by individuals, and the form in which they occur, depending on their life cycle (e.g. butterfly larvae on a host plant or ostracod diapausing eggs in the sediment of a dry pond). Geographic distributions are dynamic over time, undergoing contractions and expansions (Tomiolo & Ward, 2018; Fitt et al., 2019). Many species are experiencing significant range reductions, with habitat alteration being one of the main causes of these declines (Sala et al., 2000; Barnosky et al., 2011). On the other hand, the current globalisation trend has allowed many other

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A. Mestre (✉) · R. Sorlí · F. Mesquita-Joanes  
Cavanilles Institute of Biodiversity and Evolutionary  
Biology, University of Valencia, 46980 Paterna, Spain  
e-mail: alexandre.mestre@uv.es

species to occupy large geographic extensions outside their native range, aided by anthropogenic introductions followed by rapid geographic expansion (Davis, 2009; Mestre et al., 2020). In addition, climate change is driving range shifts in different taxa (e.g. Bridle et al., 2014), also frequently favouring invasive species expansions or amplifying their impacts in a synergistic way (Dukes, 2010).

The geographical distribution of a species and its dynamics can be affected by multiple factors including abiotic, biotic, demographic, spatial and temporal (Pulliam, 2000; Wiens & Graham, 2005; Colwell & Rangel, 2009; Holt, 2009). Firstly, species distributions are constrained by their intrinsic ranges of tolerance to environmental conditions (e.g. temperature, humidity, salinity, etc.; De Candolle, 1855; Good, 1931; MacArthur, 1972; Holt, 2009). Second, interactions with other species may facilitate or hinder the presence of a species in a given location, or influence its dispersal capacity (McGill et al., 2006; Soberón, 2007; HilleRisLambers et al., 2013; Mestre et al., 2020). For example, the geographic distribution of a parasite may be limited by the distribution of its hosts (Colwell et al., 2012), and the spatial distributions of orchids are strongly influenced by their interactions with pollinating insects (Štípková et al., 2020). Third, species experience demographic fluctuations of a stochastic nature (not linked to environmental variation), which can influence local extinction-colonisation dynamics (Pulliam, 2000; Pearson & Dawson, 2003; Huntley et al., 2010). Fourth, dispersal dynamics in space can generate incongruities between the actual and potential distribution of a species (Colwell & Rangel, 2009). On the one hand, a species may be absent from sites with optimal habitats due to dispersal limitations (Kubisch et al., 2014). On the other hand, a species may inhabit unsuitable places due to a permanent flow of immigration from nearby favourable habitats, a phenomenon known as “demographic rescue” (Kanarek et al., 2015). Fifth, a species may occupy a habitat seasonally during favourable periods, and disappear during periods of environmental harshness, through migration or diapause (i.e. “temporary dispersal”; Plue & Cousins, 2013; Wisnoski et al., 2019). Finally, evolution may play a very important role in the geographic distribution of species. Across the range occupied by a species, local populations may differ in their tolerances to environmental conditions due to phenotypic plasticity or

genetic differences driven by local adaptation (Pereira et al., 2017; Bennett et al., 2019). Local adaptation is considered one of the possible mechanisms of geographical expansion of species (Lee-Yaw et al., 2018; Mestre et al., 2020).

The development of new tools, such as ecological niche models and geographic information systems have allowed to better estimate the geographic distribution of species in relation to environmental gradients (Elith et al., 2006). However, obtaining the data needed to apply such models is not always a straightforward task. Due to the complexity of processes associated with geographic distributions, the tolerance limits of species to environmental variables (i.e. their ecological niche; Hutchinson, 1978) cannot be solely inferred from correlations based on geographic distribution data. It requires the design of laboratory survival experiments under controlled conditions (Holt, 2009). Survival experiments are basic approaches for testing ecological and evolutionary theories, largely related to the concept of ecological niche (Soberón & Peterson, 2005). At a more practical level, they allow predictions of species distributions when occurrence data are unavailable or limited, and increase the predictive capacity of models with existing data (Peterson & Soberón, 2012; Kotta et al., 2019). In this study, we combine the analysis of occurrence data with tolerance experiments under laboratory conditions to assess the role of salinity in shaping the geographic distribution of two exotic ostracod species from the Iberian Peninsula.

Freshwater ostracods are among the most frequent invertebrate groups within inland water bodies. Sexual dimorphism is common in podocopid ostracods, in most cases large differences are found in the external features of the shells, and males are usually smaller than females (Cohen & Morin, 1990; Meisch, 2000). Ostracods have proved to be a very useful group in ecological studies, due to their wide distribution, small size, high developmental speed and ease of keeping them alive in aquaria under controlled conditions. For these reasons, they are particularly suitable organisms for addressing questions requiring laboratory experiments (Ganning, 1971; Martens, 1985; Mesquita-Joanes et al., 2012). Ostracods play a very important role in the structure of small aquatic systems (Diner et al., 1986) and are sensitive organisms for ecotoxicological testing (Havel & Talbott, 1995). The presence of ostracods in freshwater

ecosystems is conditioned by the physico-chemical characteristics of the water. Hydroperiod, temperature and salinity are amongst the most influential factors in the distribution and abundance of ostracods (De Deckker, 1981; Neale, 1988; Aladin, 1993; Horne, 1993). In general, the species richness of microcrustaceans is altered by changes in salinity (Jensen et al., 2010). In ostracods, salinity affects the osmotic regulation of individuals and the balance between calcification and ionic regulation (Aladin, 1993; Mezquita et al., 1999). For this reason, a relationship exists between the distribution of ostracod species and the ionic composition of the water, due to their need to calcify their shells. Ostracods have evolved different osmotic regulation mechanisms to tolerate changes in salt content (Mesquita-Joanes et al., 2012). Some freshwater ostracod species tolerate very high salinities (Santamaria et al., 1992), which may be related to their marine origin (Park & Ricketts, 2003). Other factors affecting the survival of ostracods include temperature, depth of the water column, pH, substrate type, feeding, predation, parasitism, dissolved oxygen content, submerged vegetation, photoperiod, amount of dissolved organic matter or water flow velocity (Delorme, 1969; Carbonel et al., 1988; Delorme, 1989; Griffiths & Holmes, 2000).

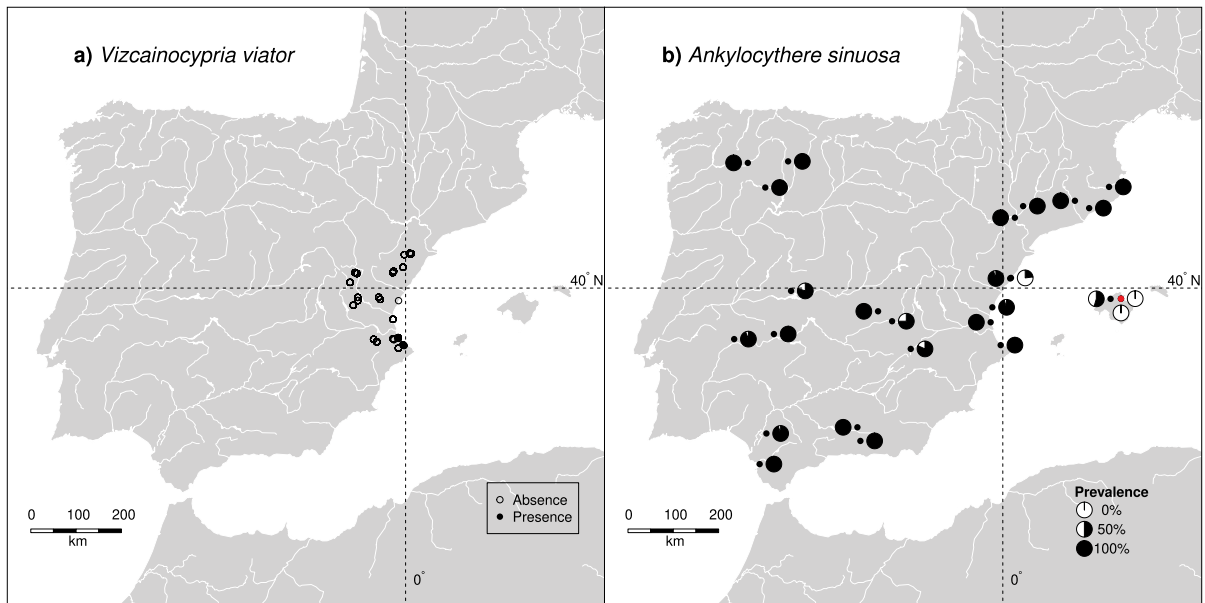
This work assesses the effects of salinity on the distribution of exotic populations of two ostracod species in the Iberian Peninsula. *Vizcainocypria viator* Bisquert-Ribes et al. (2023) is a recently described free-living species, found in rice fields in southern Valencia, and belonging to the Cyclocyprididae (Bisquert-Ribes et al., 2023). Despite having been described from the Iberian Peninsula, molecular data and morphological similarities with other species suggest that *V. viator* is actually an exotic invader in the Iberian Peninsula, originally from North America (Bisquert-Ribes et al., 2023). The other study species, *Ankylocythere sinuosa* (Rioja, 1942), belongs to the family Entocytheridae (Hart & Hart, 1974). Entocytherids are ostracods that are symbionts of other crustaceans. They are small in size (< 600 µm) and show sexual dimorphism in which the female is larger than the male (Aguilar-Alberola et al., 2012). Most species are native to North and Central America where they live in association with crayfish belonging to Astacoidea, as commensals, without any apparent effect on the host (Hart & Hart, 1974). Introduced entocytherids have been discovered associated with

exotic crayfish in areas of Europe and East Asia (Smith & Kamiya, 2001; Aguilar-Alberola et al., 2012; Mestre et al., 2013; Huys et al., 2014; Ohtaka et al., 2017). In particular, the species *A. sinuosa* has established exotic populations in the Iberian Peninsula and Balearic Islands (Aguilar-Alberola et al., 2012), in association with *Procambarus clarkii* (Girard, 1852), an invasive crayfish that has been very successful (Geiger et al., 2005). Populations of *A. sinuosa* on the Iberian Peninsula have been the subject of recent research (Castillo-Escrivá et al., 2013; Mestre et al., 2013, 2014, 2016, 2019). The aims of the present work are: (1) analysing the effect of salinity on the distribution of Mediterranean populations of both exotic ostracods based on published field data (Gálvez et al., 2023; Mestre et al., 2014); (2) testing the effects of salinity on the survival of adults under laboratory conditions; and (3) testing whether the effects of salinity on adult survival differ between males and females.

## Materials and methods

### Occurrence data analyses

First, the role of salinity in the distribution of the two ostracod species (*V. viator* and *A. sinuosa*) was analysed based on field data available from published scientific papers and projects. In our analyses, we considered salinity as the total concentration of dissolved ions in the water, and estimated from electrical conductivity. Occurrence data for the species *V. viator* were obtained from an ostracod database developed as part of the METACOMSET project (Gálvez et al., 2023). The database gathers presence/absence information for *V. viator* at 32 different localities in the eastern Iberian Peninsula (Fig. 1a), and site conductivity data. The species was identified as *Dentocypria* sp. in the dataset prior to its description as a new species by Bisquert-Ribes et al. (2023). For each locality, multiple seasonal surveys (about 4 surveys per year) were conducted during 2018 and 2021. Occurrence (presence-absence) data of *V. viator* included a total of 265 samples. The distribution data of *A. sinuosa* were extracted from Mestre et al. (2014). The study analyses the factors associated with the



**Fig. 1** Sampling locations for the analysis of the effect of salinity on the geographical distribution of the two study species: **a** *V. viator* and **b** *A. sinuosa*. White circles on the *V. viator* map **a** represent localities with absence of the species, and black circles indicate presence. The pie charts on the *A.*

*sinuosa* map **b** indicate the prevalence of this ostracod at the sampling locality (i.e. proportion of crayfish occupied by the symbiont). The red dot in the Balearic Islands represents two sampling localities in close proximity to each other

presence and abundance of this commensal ostracod at 26 localities in the Iberian Peninsula and Balearic Islands (a single sampling per locality; Fig. 1b).

Occurrence data of *V. viator* were analysed with a generalised linear mixed-effects model of binomial family, in order to control for the effects of repeated sampling from the same locality (GLMM; Zuur et al., 2009). Presence-absence records were used as the response variable, electrical conductivity of water from the sampling locality (mS/cm) as fixed-effects explanatory variable, and sampling locality as random-effects factor. The GLMM was carried out with the lme4 package v. 1.1.27 (Bates et al., 2015). For *A. sinuosa*, we used prevalence as the response variable, defined as the percentage of crayfish occupied by the symbiotic ostracod at a locality. The effects of salinity on the prevalence of *A. sinuosa* was also analysed with a generalised linear model of the binomial family. Because host size and abundance are known to influence *A. sinuosa* prevalence (Aguilar-Alberola et al., 2012; Mestre et al., 2014), we controlled their effects by including, as fixed effects, an index of crayfish abundance (crayfish caught per trap), and the mean crayfish weight (g) sampled at the locality. In

all GLMs (*V. viator* and *A. sinuosa*), explanatory variables were standardised.

#### Survival experiments

We assessed survival of adults of the two study species at different degrees of salinity under laboratory conditions, with the aim of estimating their salinity tolerance range. Experimental individuals were collected from the Albufera of Valencia N2000 site. Individuals of *V. viator* were captured at Masía de Santa Rita, south of the locality of El Saler (coordinates: 39.3747° N, 0.33253° W; conductivity = 2.4 mS/cm; salinity = 1.54 g/l). The sampling point for *A. sinuosa* was the Tancat de la Pipa, Valencia (coordinates: 39.36018° N, 0.34541° W; conductivity = 1.4 mS/cm; salinity = 0.7 g/l). Specimens of the crayfish *Procambarus clarkii* (Girard, 1852) were captured using bait traps and transferred to the laboratory in containers filled with source water. In the laboratory, symbiotic ostracods were isolated alive from crayfish following a removal protocol described by Mestre et al. (2011). That is, crayfish are immersed in a container filled with carbonated water for two minutes,

which causes the ostracods to detach from their hosts. The carbonated water is then filtered through a 100  $\mu\text{m}$  mesh to isolate the ostracods; shells of *A. sinuosa* adults measure 370–430  $\mu\text{m}$  in length and 180–250  $\mu\text{m}$  in height (Aguilar-Alberola et al., 2012). The mesh was immediately immersed in a container with mineral water (Cortes®, 0.5 mS/cm) to release live ostracods. Individuals were kept alive in the water container until the start of the experiment. This commercial water was chosen because it is rich in carbonates, as are most freshwaters in the area of study.

Four types of water with different degrees of salinity were prepared by dissolving different amounts of aquarium salt (Sera®; major ionic composition: 55.20%  $\text{Cl}^-$ , 30.77% Na, 7.72 %  $\text{SO}_4^{2-}$ , 3.68%  $\text{Mg}^{2+}$ , 1.18 %  $\text{Ca}^{2+}$ , 1.14%  $\text{K}^+$ ) in mineral water (Cortes®; dry residue: 0.2 g/l; ionic composition: 67.4%  $\text{HCO}_3^-$ , 4.04%  $\text{SO}_4^{2-}$ , 2.22%  $\text{Cl}^-$ , 21.9%  $\text{Ca}^{2+}$ , 1.97%  $\text{Mg}^{2+}$ , 0.25%  $\text{K}^+$ , 1.94%  $\text{Na}^+$ ), depending on the salinity to be achieved: (i) low (0.2 g/l; 0.5 mS/cm), (ii) intermediate-low (0.6 g/l; 1.2 mS/cm), (iii) intermediate-high (3.1 g/l; 5.7 mS/cm), and (iv) high (5.8 g/l; 10.2 mS/cm). Salinity ranges were selected based on empirical data available for the species. Subsequently, the bottles with the four different conductivity treatments were autoclaved in order to carry out the experiment under sterilised conditions. For each treatment, using a high magnification stereomicroscope (Leica MZ16), 24 adults of each species were selected. In the case of *A. sinuosa*, it was possible to isolate 12 males and 12 females due to a clear sexual dimorphism. Adult females have 390–430  $\mu\text{m}$  valves with a “convex” appearance; in contrast, adult males measure 360–390  $\mu\text{m}$  and have a characteristic copulatory apparatus usually visible through the transparent valves (Hart & Hart, 1974; Aguilar-Alberola et al., 2012). By contrast, the sexing of live individuals of *V. viator* proved to be more complicated due to their high mobility, and we decided to select adults at random, and identify the sex of individuals after the end of the experiment. To carry out individual sexing, dead individuals were preserved in 96% alcohol. A total of 96 experimental individuals per species were isolated. Multiwell plates were prepared with 24 wells for each treatment and species, each well with 2 ml of water from the respective treatment and a single individual.

The plates with the ostracods were kept in a culture chamber at a constant temperature of 20 °C for the

entire duration of the experiment, with a photoperiod of 12 h of light and 12 h of darkness. The condition of each individual was checked daily and recorded in a table. To do so, alive ostracods were handled with brushes and pipettes under a stereomicroscope for proper examination. A condition index with four values was used from least to most active: 0 = confirmed death, 1 = no movement, 2 = movement of a limb, 3 = movement of the whole body across space. Confirmed death was attributed only to cases where the individual had the valves clearly open, having some of the limbs out of the valves in an “unnatural” position, and without showing any type of movement when stimulated with a brush. Any events that might alter ostracod survival were recorded. For instance, during daily examinations, we observed some individuals of *A. sinuosa* floating in the surface layer of water in the well, trapped by the surface tension. The floating individuals were sunk by pushing them down with a paintbrush to the bottom of the well (thus breaking the surface tension). We also found cases of individual disappearances or deaths clearly not associated with the treatment (e.g. death by desiccation due to an individual being trapped stuck to the wall of the well, outside the water). Each dead individual was removed from the well, and preserved individually in a microtube with ethanol 96% for further checking and sexing. The experiment finished after all experimental individuals died.

We tested the effects of conductivity on adult survival applying the non-parametric method of Kaplan-Meier (Therneau & Grambsch, 2000; Kleinbaum & Klein, 2011). We incorporated censored data into the analysis, i.e. data indicating that an individual disappeared during handling, or that it died due to causes unrelated to the treatment (e.g. death by desiccation of an ostracod attached to the wall of the well). For each species, 4 Kaplan-Meier survival curves were estimated, one for each treatment. Differences in survival between treatments were tested via Mantel–Cox tests. The Kaplan-Meier analysis does not control for the effects of other variables that may interfere with salinity. However, it is typical for ostracods that males survive less than females (Cohen & Morin, 1990). Thus, Cox regression models were used to control for sex effects and assess their influence on the survival-salinity relationship (Therneau & Grambsch, 2000; Kleinbaum & Klein 2011). Cox regression allows multiple effects of several factors to be integrated



into a single model. The response variable in Cox regressions is the instantaneous potential for death to occur given that the individual survived to time  $t$ , i.e. the risk of dying (Kleinbaum & Klein, 2011). The explanatory variables were salinity (variable of interest) and sex (potential interfering variable). Models were compared including and not including sex. Finally, a likelihood ratio test was used to find the best model in explaining variation in adult survival. For the survival analyses, we used the *survival* package v. 3.2.11 for R (Therneau, 2021). All statistical analyses were done with R v. 4.1.0 (R Core Team, 2021).

#### Reanalysing occurrence data based on survival experiments

In the case of *V. viator* where we found no significant effects of salinity based on occurrence data, we considered the possibility that it could be due to the existence of a non-linear relationship. The fact that the GLMM models assume the same mean effect for the whole range of the predictor variable could lead to situations of a lack of an overall effect when non-linear effects are present (i.e. effects that vary across the range of the predictor). We tested the hypothesis of hidden non-linear effects by reanalysing the occurrence data as follows. First, experimental results were used to identify ranges of salinity where its effect on experimental survival is monotonic (i.e. either negative or positive). Second, subsets of occurrence data specifically covering the identified ranges were reanalysed separately to test whether the GLMM results showed range-specific effects consistent with those observed in the experiments. In addition, we also applied a generalised additive model (GAM) of

binomial family to check for a non-linear relationship between conductivity and probability of presence of *V. viator*. The GAM was implemented with the *mgcv* package v. 1.8.40 (Wood, 2011).

## Results

### Occurrence data analyses

The METACOM-SET project database records the presence of *V. viator* in only 4 out of 32 sampled localities, all of them located in littoral wetlands in southern Valencia (Fig. 1a). The mean conductivity among localities was 0.86 mS/cm (SD = 1.48; see also Table 1). The GLMM for *V. viator* with METACOM-SET data indicates that there is no significant effect of salinity on the presence of the ostracod at the sampled localities (Table 2).

The species *A. sinuosa* was present in 24 out of 26 sampling localities (Fig. 1b). The mean conductivity of the 26 sampling localities of *A. sinuosa* was 0.84 mS/cm (SD = 4.09). Mean host abundance was 1.83 crayfish caught per trap (SD = 1.68). The mean host weight per locality was 19.32 g (SD = 5.92). Iberian-Balearic populations of *A. sinuosa* showed generally very high prevalences, with 84% of crayfish harbouring ostracods per locality on average (SD = 30%). Most of the sampled crayfish populations had prevalences above 75%. Prevalence values in the Balearic Islands were lower, including one locality with ~ 50% infested crayfish and two other localities without *A. sinuosa* (Fig. 1a). A GLM incorporating the variables salinity, abundance and mean host weight showed a non-significant effect of mean crayfish weight ( $z = 0.42$ ;  $df = 22$ ;  $P = 0.13$ ). Therefore, we removed the

**Table 1** Descriptive statistics of the explanatory variables used for the analysis of the effect of salinity on the occurrence of *V. viator* and *A. sinuosa*

Species	Variable	Units	Min.	1st quartile	Median	3rd quartile	Max.
<i>V. viator</i>	Cond	mS/cm	0.06	0.19	0.31	0.57	7.92
<i>A. sinuosa</i>	Cond	mS/cm	0.03	0.45	0.83	2.25	19.4
	HAb	ind./trap	0.27	0.68	1.19	2.25	6.75
	HWeight	g	8.86	13.89	18.91	25.15	28.48

Cond is water conductivity; HAb is host abundance, i.e. abundance of crayfish *Procambarus clarkii* (individuals captured per trap); and HWeight is the mean crayfish weight at the sampling location

**Table 2** Results of generalised linear models to analyse the effect of salinity on the presence of *V. viator* and the prevalence of *A. sinuosa*.  $\beta$  represents the effect coefficient, SE isthe standard error of the effect, 95CI is the 95% confidence interval of  $\beta$ ,  $z$  is the significance test statistic of the effect, and  $P$  is the  $P$ -value of the test ( $H_0: \beta = 0$ ;  $H_1: \beta \neq 0$ )

Model	Variable	$\beta$	SE	95CI	$z$	$P$
<i>V. viator</i>	Cond	0.28	0.64	(−0.99, 1.55)	0.43	0.67
	HAb	4.13	0.99	(2.37, 6.30)	4.15	<0.001*
<i>A. sinuosa</i>	Cond	−1.66	0.32	(−2.36, −1.09)	−5.15	<0.001*
	HAb	4.13	0.99	(2.37, 6.30)	4.15	<0.001*

Cond is the water conductivity of the sampling point and HAb is the host abundance (see Table 1). All variables were standardised in the models to make the effects comparable

\* $P < 0.05$

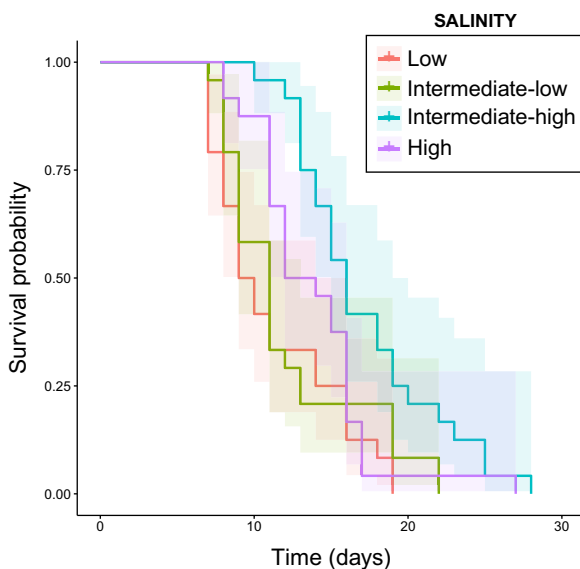
mean host weight from the model. A GLM without the weight showed a negative effect of conductivity, and a positive effect of host abundance. The effect size of host abundance was twice that of conductivity (Table 2).

### Survival experiments

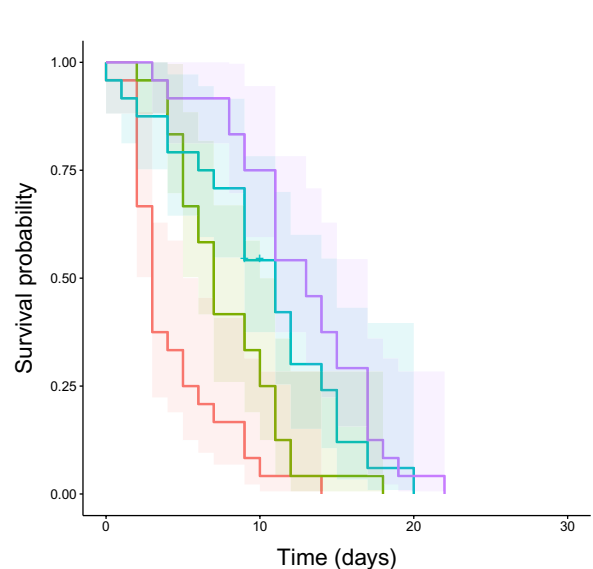
The Kaplan-Meier curves of *V. viator* for each salinity treatment had very similar shapes among them (Fig. 2a). All individuals of *V. viator* survived the first

7 days of the experiment, regardless of the treatment. Therefore, *V. viator* showed a very high survival at the beginning of the experiment, until day 7, after which survival started to drop abruptly. The survival drop began earlier in the two low salinity treatments, followed by the higher salinity treatment. The intermediate-high salinity treatment is the one that showed the most delayed drop in survival (Fig. 2a). The median survival of each treatment (Table 3) reflects the same survival relationship between treatments, although the confidence intervals of the medians overlap

### a) *Vizcainocypria viator*



### b) *Ankylocythere sinuosa*



**Fig. 2** Kaplan-Meier curves for *V. viator* (a) and *A. sinuosa* (b). Each species was subjected to 4 salinity treatments: low, intermediate-low, intermediate-high and high

**Table 3** Median survival time ( $T$ ) for *V. viator* and the 95% confidence interval (95CI)

Species	Salinity	$N$	$T$ (days)	95CI
<i>V. viator</i>	Low	24	9.5	(9, 14)
	Intermediate-low	24	11.0	(9, 13)
	Intermediate-high	24	16.0	(15, 19)
	High	24	13.0	(12, 16)
<i>A. sinuosa</i>	Low	24	3	(3, 5)
	Intermediate-low	24	7	(6, 10)
	Intermediate-high	24	11	(9, 15)
	High	24	13	(11, 17)

$N$  = number of individuals subjected to each treatment.  $T$  is the time for survival to attain 0.5 (50% of individuals remain alive)

between them (except for the intermediate-high salinity). The Mantel–Cox test applied to all Kaplan–Meier curves indicates significant differences in survival among treatments (Table 4). Treatment-specific pairwise tests show that the intermediate-high salinity

**Table 4** Mantel–Cox tests for comparison between Kaplan–Meier survival curves at different salinity levels for *V. viator* and *A. sinuosa*

Species	Test	$\chi^2$	$df$	$P$
<i>V. viator</i>	General	15.84	3	0.001*
	1 vs. 2	1.20	1	0.27
	1 vs. 3	13.43	1	<0.001*
	1 vs. 4	2.21	1	0.18
	2 vs. 3	10.03	1	0.002*
	2 vs. 4	0.90	1	0.34
	3 vs. 4	5.47	1	0.019*
	<i>A. sinuosa</i>	General	44.85	3
1 vs. 2		9.02	1	0.003*
1 vs. 3		17.19	1	<0.001*
1 vs. 4		32.33	1	<0.001*
2 vs. 3		4.23	1	0.040*
2 vs. 4		13.87	1	<0.001*
3 vs. 4		2.07	1	0.15

The general test compares the curves of all treatments. In the rest of the tests, treatments are compared pairwise. The treatment codes are 1 for low salinity, 2 for intermediate-low salinity, 3 for intermediate-high and 4 for high salinity

\* $P < 0.05$

differs from the other treatments, and there is no evidence that the other treatments differ from each other (Table 4).

In contrast to *V. viator*, mortality of *A. sinuosa* starts to be expressed earlier in the experiment (Fig. 2b). By the third day of the experiment, deaths of individuals had already occurred in all treatments. In addition, the *A. sinuosa* curves showed greater divergence from each other (especially between extreme treatments). The appearance of the survival curves suggests that, within the salinity range of the experiment, *A. sinuosa* improves its survival with increasing salinity. The median survival time for each treatment reflects this apparent pattern (Table 3). According to the overall Mantel–Cox test, *A. sinuosa* showed significant survival differences between treatments (Table 4). In addition, all but one of the pairwise treatment comparisons were significant. The only treatment pair comparison without significant differences was intermediate-high salinity with high salinity (Table 4).

Two Cox regression models were compared to assess the role of individual sex as an interfering factor on the salinity–survival relationship. The first model (Model 1) only considers conductivity as an explanatory variable. The second model (Model 2) incorporates sex as an additional explanatory variable. In the case of *V. viator*, likelihood ratio tests indicate that Model 1 has a better goodness-of-fit than a null model with no explanatory variables (Table 5). Therefore, salinity is relevant to the survival of *V. viator*. Furthermore, the inclusion of sex in Model 2 significantly increases goodness-of-fit, thus indicating

**Table 5** Comparison between Cox regression models to assess the role of sex in the salinity–survival relationship for *V. viator* and *A. sinuosa*

Species	Models	$\chi^2$	$df$	$P$
<i>V. viator</i>	1 vs. null	15.94	3	<0.001*
	1 vs. 2	8.39	1	0.004*
<i>A. sinuosa</i>	1 vs. null	36.89	3	<0.001*
	1 vs. 2	2.03	1	0.15

The models were compared using a likelihood ratio test. The null model does not include any explanatory variables, Model 1 considers only conductivity, and Model 2 also includes sex

\* $P < 0.05$



that sex is important in assessing the effect of salinity on survival of *V. viator*. According to Model 1, intermediate-high salinity produces an average survival improvement of 67% over the baseline low salinity treatment (Table 6). Model 2 shows that the risk of instantaneous death is 1.96 times higher in males than females. In addition, the presence of sex in the model slightly corrects the effect of salinity. In particular, the effect of improved survival at intermediate-high salinity observed in the first model becomes slightly smaller when we control for the effect of sex in the second model (compare hazard ratios of Model 1 and Model 2 of *V. viator* in Table 6).

Regarding *A. sinuosa*, Model 1 also shows higher goodness-of-fit than the null (Table 5), in agreement with the results of the Kaplan-Meier curves, in the same way that occurs in *V. viator*. However, unlike *V. viator*, Model 2 of *A. sinuosa* does not differ significantly from Model 1 in its goodness-of-fit (Table 5). Therefore, sex does not influence the survival of *A. sinuosa* adults under the experimental conditions of this study. Regarding the effect of salinity on the

survival of *A. sinuosa*, all treatments different from the base treatment (low salinity) improve the survival of individuals (regardless of sex). The higher the salinity of the treatment, the greater the effect compared to the base treatment. The greatest effect occurs in the high salinity treatment, with an average increase in survival of 85% over the low salinity base treatment (see *A. sinuosa* Model 1 in Table 6).

#### Reanalysing occurrence data based on survival experiments

The lack of a significant positive effect of salinity on the field presence of *V. viator* could be due to the existence of a non-linear relationship, as shown by the experimental data obtained. That is, the effect of salinity is positive at low-intermediate salinities, and becomes negative at high salinities. To test the hypothesis of non-linear effects, the data were filtered by removing locations with conductivities falling in the range of negative effect according to the experimental data (i.e. > 4 mS/cm). The result was

**Table 6** Results of Cox regression models to assess the effects of salinity and sex on adult survival of *V. viator* and *A. sinuosa*

Model	Factor level	$\beta$	SE	$z$	$P$	HR	95CI
<i>V. viator</i>							
Model 1	ils	-0.25	-0.86	0.29	0.39	0.78	(0.44, 1.38)
	ihs	-1.12	-3.69	0.30	<0.001*	0.33	(0.18, 0.59)
	hs	-0.51	-1.72	0.30	0.09	0.60	(0.34, 1.06)
Model 2	ils	-0.01	-0.02	0.31	0.98	0.99	(0.55, 1.81)
	his	-1.00	-3.28	0.31	0.001*	0.37	(0.20, 0.66)
	hs	-0.34	-1.11	0.30	0.27	0.71	(0.39, 1.29)
	male	0.67	2.92	0.23	0.004*	1.96	(1.25, 3.08)
<i>A. sinuosa</i>							
Model 1	ils	-0.92	-3.09	0.30	0.002*	0.40	(0.22, 0.72)
	ihs	-1.54	-4.83	0.32	<0.001*	0.21	(0.11, 0.40)
	hs	-1.93	-6.00	0.32	<0.001*	0.15	(0.08, 0.27)
Model 2	ils	-0.90	-3.02	0.30	0.003*	0.41	(0.23, 0.73)
	ihs	-1.61	-4.95	0.33	<0.001*	0.20	(0.11, 0.38)
	hs	-1.96	-6.06	0.32	<0.001*	0.14	(0.07, 0.27)
	male	0.31	1.43	0.22	0.15	1.37	(0.89, 2.10)

Model 1 includes a single factor as explanatory variable: salinity. Model 2 includes two factors: salinity and sex of the individual. The salinity factor has 4 levels: low, intermediate-low (ils), intermediate-high(ihs) and high (hs) salinity. The sex factor has two levels: female and male. The base levels of the factors in the models are low salinity and female sex. The effects of each level are with respect to the base level.  $\beta$  represents the effect coefficient, SE is the standard error of  $\beta$ ,  $z$  is the significance test statistic of the effect, and  $P$  is the  $P$ -value of the test ( $H_0: \beta=0$  or  $HR=1$ ;  $H_1: \beta \neq 0$  or  $HR \neq 1$ )

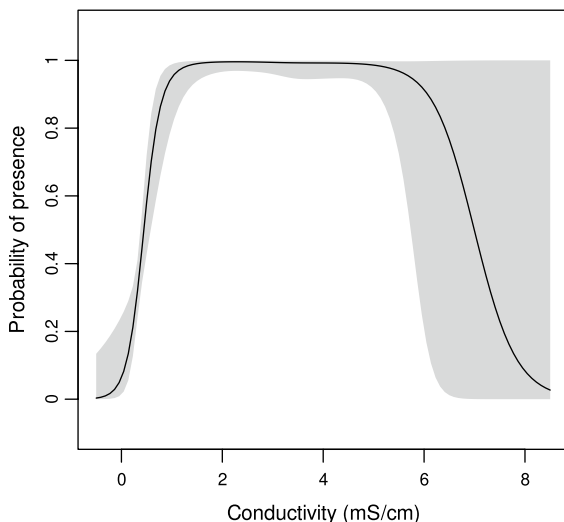
HR hazard ratio, 95CI 95% confidence interval of the HR

\* $P < 0.05$

as expected: a positive effect of conductivity on the presence of *V. viator* ( $\beta = 2.78$ ;  $SE = 0.52$ ;  $z = 5.33$ ;  $P < 0.001$ ). On the contrary, when removing locations with conductivities  $< 4$  mS/cm, the mean effect was negative though not significant ( $\beta = -0.50$ ;  $SE = 1.64$ ;  $z = -0.30$ ;  $P = 0.76$ ). Furthermore, the GAM analysis confirmed this non-linear relationship (Fig. 3). The GAM predictions showed high uncertainty at the upper range of conductivity (i.e. above 6 mS/cm) due to the scarcity of data covering this range (only 5 out of 265 records had a conductivity higher than 6 mS/cm). Nevertheless, the decrease in the probability of presence of *V. viator* at high salinities predicted by the GAM (Fig. 3) was supported by the negative impact of a high salinity on adult survival (Fig. 2a, Table 4).

## Discussion

Based on occurrence data analyses alone, there is no evidence from GLMMs that salinity influences the presence of *V. viator* in the eastern Iberian Peninsula. However, the results obtained from the survival experiments show that there are significant differences in adult survival. The intermediate-high salinity



**Fig. 3** Non-linear relationship between salinity and probability of presence of *V. viator*, based on a generalised additive model (GAM) of binomial family. The black line is the fitting curve, whereas the shadows represent the confidence bands (i.e. two standard errors below and above the fitting curve)

treatment (3.1 g/l; 5.65 mS/cm) showed a higher survival than the rest of tested salinities. This suggests that the optimum salinity of the population sampled in Tancat de la Pipa is found at salinities above 0.6 g/l (1.2 mS/cm) and below 5.8 g/l (10.2 mS/cm). The salinity of the source locality of the experimental individuals falls within the estimated optimal range (1.54 g/l). The median conductivity of the 32 sampling sites is 0.31 mS/cm. In contrast, the minimum conductivity of sites with presence of *V. viator* is 0.62 mS/cm thus suggesting that *V. viator* is absent at locations with low conductivity in the range of studied sites.

Reanalysing the occurrence data based on experimental results, we found a previously hidden, non-linear relationship. That is, the effect of salinity on the presence of *V. viator* is positive at low-intermediate salinities, and becomes negative at high salinities (Fig. 3). Our results highlight the importance of assessing potential shortcomings derived from the linearity assumption of GLMs when analysing occurrence data. The combination of analysis of occurrence data with data from survival experiments supports the hypothesis that the distribution of *V. viator* is limited by the survival of adults at very low salinities (below 0.6 mS/cm) where they do not occur. Nevertheless, the results of the experiment also show that very high salinities (above 10 mS/cm) have a negative impact on adult survival. But, as we have seen, a negative effect of salinity on the distribution of *V. viator* at the high salinity range is not so evident from the field dataset. This is consistent with the typical habitat of taxonomically related species, i.e. rice fields, which tend to have intermediate-high salinities (Savatenalinton, 2017).

In this study it was found that adult females of *V. viator* have a higher survival rate than adult males. This may be related to the fact that ostracod males tend to have lower tolerance ranges and shorter lifespan than females (Cohen & Morin, 1990). The higher survival of female individuals would explain the female-biased sex ratio in many ostracod populations (Havel et al., 1990). The sex bias in survival has been attributed to genetic causes (Chaplin et al., 1994). One possible cause of the shorter lifespan of male individuals is the investment in searching for females, i.e. the costs of male sexual behaviour (Cohen & Morin, 1990). By contrast, females of some species remain immobile on the substrate waiting for males,

thus expending less energy (e.g. Danielopol et al., 2002). However, the expectation of higher male mortality related to their higher mobility remains to be tested in further survival and behavioural experiments and for a wider variety of taxa. The results of this work highlight the importance of incorporating sex as a critical factor for future studies focused on the survival analysis of *V. viator* and other podocopid ostracods.

The analysis of *A. sinuosa* occurrence data indicates a negative effect of salinity on symbiont prevalence, though the effect size is small compared to the positive effect of crayfish abundance (Table 2). Results are consistent with the important role of host abundance in the population dynamics of horizontally transmitted symbionts such as *A. sinuosa* (Mestre et al., 2020). In contrast, the survival experiment suggests a positive effect of salinity throughout the range tested in the experiment. This apparent contradiction may have several explanations. On the one hand, it may be that the observed effect of salt content is caused by a failure to consider some important variables that covary with conductivity in the populations at the sampling locations (Bolker, 2008). Mestre et al. (2014) conducted a more comprehensive analysis of the prevalence and abundance of *A. sinuosa*, including a larger number of variables, such as crayfish moult status, water physicochemical variables and climatic variables. In their analysis, conductivity was not selected as a significant variable to explain the prevalence of the symbiont. However, in the same analysis, conductivity was selected for ostracod abundance (with a negative effect). Another possible explanation is that, unlike *V. viator*, the range of conductivities in the experiment (0.5–10.21 mS/cm) did not cover the full range of salinities that were sampled for presence of *A. sinuosa* (0.03–19.4 mS/cm). In any case, the experimental results of the present study indicate that the optimum salinity of *A. sinuosa* is above 10.21 mS/cm. In future studies, a more precise estimation of this optimum could be made by extending the upper limit of the salinity range used in our experiment to one closer to the upper limit of the salinity range of the localities. On the other hand, the negative effect of conductivity on the prevalence of *A. sinuosa* might be associated with a vital parameter other than adult survival outside the host (e.g. juvenile survival, reproduction rate, etc.) Another possible explanation is that salinity indirectly affects

the ostracod via the host. For example, high salinities may produce metabolic changes in *Procambarus clarkii* (Bissattini et al., 2015), and these changes could reduce the activity of the crayfish, thus lowering the transmission rates of the symbiont, causing a negative effect on its prevalence. Finally, the experimental results show that adult males and females of *A. sinuosa* do not differ in their survival and, therefore, sex does not interfere with the salinity-survival relationship of this symbiotic ostracod.

Our survival experiments indicate that *A. sinuosa* tolerates better high salinities than *V. viator*. The habitat of close ancestors most likely explains these contrasting differences. The ostracod *A. sinuosa* belongs to a family, the Entocytheridae (Cytheroidea), that includes extant genera living in marine environments (*Hartiella* and *Microsyssitria*; Hart & Hart, 1974). Indeed, according to the fossil record, repeated transitions from marine to non-marine habitats have occurred along the Cenozoic and Mesozoic within the Cytheroidea. By contrast, *V. viator* is a member of Cypridoidea, a superfamily without extant species inhabiting marine environments, and without known fossil evidence of marine-to-freshwater transitions, though it has related superfamilies with marine species, i.e. Macrocypridoidea and Pontocypridoidea (Horne, 2003). This evidence supports the hypothesis that *V. viator* might have lost the osmoregulatory abilities to bear high salinities present in its ancient marine ancestors. A few extant cypridoideans are known to tolerate high salinities, including e.g. *Heterocypris salina* (Brady, 1868), *Heterocypris barbara* (Gauthier & Brehm, 1928), *Candelacypris aragonica* (Brehm & Margalef, 1949), *Sarscypridopsis aculeata* (Costa, 1847) or *Arctocypris mareotica* (Fischer, 1855) (see Ganning, 1971; Baltanás et al., 1990; Gusakov et al., 2021), but we are not aware of any halotolerant ostracod species in the Cyclocyprididae, the family to which *V. viator* belongs.

Despite the need to incorporate physiological experiments to better define the niche of species and improve predictions on their response to environmental change (Kotta et al., 2019), experimental data on nonmarine ostracod tolerance to salinity changes is very scarce. This is unexpected, considering the long tradition of the use of ostracods as paleoenvironmental indicators (Delorme, 1969; Carbonel et al., 1988), which has been apparently based mostly on field data. Furthermore, published data on ostracod salinity

tolerance (Ganning, 1971; Martens, 1985; Santamaria et al., 1992; Gandolfi et al., 2001; Wang et al., 2021) commonly show inconsistencies with field data; ostracods are usually found to have wider tolerance ranges under laboratory conditions compared with field studies. This is not surprising considering that negative biotic interactions such as interspecific competition and natural enemies, which have been classically considered as key drivers of species distributions (e.g., Staniczenko et al., 2018), are absent in laboratory experiments. Our experimental results also suggested a wider tolerance to salinity gradients in the studied species compared to occurrence data in the field. However, in our study cases we should consider not only potential effects of interspecific competition or natural enemies explaining these differences, but also the strong dependency of *A. sinuosa* on crayfish, and the possibility that *V. viator* has not had time enough to disperse to occupy all its potential distribution in the area. Other potential explanations for the observed discrepancies are different ionic compositions, oxygen contents or food availability between field and experimental water, the presence of pollutants in the field water, or the fact that our experiments only considered adult survival, thus disregarding potential limiting factors specifically affecting immature stages. Furthermore, in the case of *A. sinuosa*, because experimental individuals were detached from their hosts, we measured adult survival outside the host, disregarding the effects of the host micro-environment. Our results point to a need for more integrative approaches to the study of species-environment relationships (Jiménez-Valverde et al., 2011) that incorporate experimental estimates of the abiotic niche (Holt, 2009), and account for the influence of strong biotic interactions such as host availability for symbiotic species (Mestre et al., 2013), and dispersal limitation for exotic species in earlier invasion stages. As a final remark, we envisage that the expected future increase in anthropogenic salinisation of inland waters in semiarid and arid regions (Williams, 2001; Cañedo-Argüelles, 2020) will facilitate the spread of exotic halotolerant species like those investigated in this study, as observed in other invasive crustaceans (Cuthbert et al., 2020).

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**Data availability** Occurrence data were obtained from published data (see ‘Materials and methods’). The dataset of survival experiments is available in Supporting Information.

#### Declarations

**Conflict of interest** We have no conflicts of interest to declare.

**Informed consent** We performed survival experiments with two exotic ostracod species (*Vizcainocypria viator* and *Ankylocythere sinuosa*) following the standards of animal welfare.

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