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3 Portuguese native *Artemia parthenogenetica* and invasive *Artemia*
4 *franciscana* reproductive parameters, under different abiotic conditions.

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16 Abstract

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18 There are only two known populations of native *Artemia* in Portugal: one in the
19 Rio Maior saline, the other in the Aveiro salines complex, both of the diploid *Artemia*
20 *parthenogenetica* species. All other Portuguese hypersaline environments where
21 *Artemia* can be found have been invaded by *Artemia franciscana*, which has eradicated
22 the native strains. Given the actual widespread interest in the conservation of native
23 *Artemia* biodiversity, the survival of the two Portuguese' native *Artemia* strains and of
24 the invasive *A. franciscana*, when exposed to variations of several abiotic factors, was
25 recently studied, and the differences in the survival of the Portuguese *Artemia* species
26 were evident. To complement that previous study, this work evaluates the reproductive

performance of the two native strains and of the invasive species, when exposed to the same abiotic variations. After both studies, the *A. parthenogenetica* from Rio Maior seems to be very well adapted to its specific biotope characteristics, which, together with the inland saline localisation, favours its resistance to invasion. On the other hand, the *A. parthenogenetica* from Aveiro proved to perform much worse than its invasive competitor, in the conditions tested. Its permanence in its biotope is a still unexplained phenomenon. The only two explanations that we currently glimpse for this fact are that either *A. franciscana* has by chance not been introduced there, or a chemical barrier related to the pollution has been preventing invasion. Further studies are needed to discern the true reasons, and they have to take into account relations between local conditions, such as environmental problems, and the specific biological traits of the local *Artemia* strains.

Keywords: *Artemia*, biological traits, invasive species, abiotic conditions, salines, Portugal.

Introduction

Artemia is a cosmopolitan genus (Pacios and Muñoz, 2010). Nine reproductively isolated species, with sexual or parthenogenetic reproduction (Browne and Bowen, 1991) have been identified, four of which with natural populations on the Iberian Peninsula (Amat et al., 1995; Amat et al., 2007; Pacios and Muñoz, 2010). There are, however, only two known populations of native *Artemia* in Portugal: one in the Rio Maior saline (39 ° 21'47 "N 8 ° 56'33" W), the other in the Aveiro saline complex (40°38'37" N, 8°39'57" W), both of the diploid *Artemia parthenogenetica* species (Amat et al., 2007; Pinto et al., 2013a). All other Portuguese hypersaline environments where

Artemia can be found have been invaded by *Artemia franciscana* (Amat et al., 2007, Pinto et al., 2013a), which has eradicated the native strains.

Artemia franciscana is a native species from the American continent (Amat et al., 2005), but can currently be found on all continents where *Artemia* has been described, evidencing its large invasive power (Ruebhart et al., 2008). Several studies (e.g. Ruebhart et al., 2008) describe characteristics of *A. franciscana* that favour development and spread of this invasive species in comparison to other *Artemia* species. *A. franciscana* is very euryhaline and eurythermal, maintaining reproductive success at a variety of temperatures and salinities (Browne and Wanigasekera, 2000). Amat et al. (2007), compared the sexual fitness of several *Artemia* species from different places of the Mediterranean basin, under standard conditions, and found that *A. franciscana* had the best reproductive performance.

Given the actual widespread interest in the conservation of native *Artemia* biodiversity, Pinto et al. (2013b) recently studied the survival of the two Portuguese native *Artemia* strains and of the invasive *A. franciscana*, when exposed to variations of several abiotic factors: salinity, temperature, light and quantity of available food. They found significant differences in the survival of the Portuguese *Artemia* species, showing that native *Artemia* strains vary in terms of resistance to abiotical changes. Distinct physiological responses of different *Artemia* populations belonging to the same species are common in this genus (Browne and Bowen, 1991; Browne, 1992). These variations, which are possibly genetic, suggest local adaptations of the populations, in response to different selective pressures experienced in the most varied hypersaline environments they inhabit (Persoone and Sorgeloos, 1980; Vanhaecke et al., 1984). Pinto et al. (2013b) suggested possible local adaptations of the Portuguese native *Artemia* species to specific characteristics of the studied biotopes (the salines of Aveiro, located in a

large lagoon, and the inland saline of Rio Maior), and identified local factors which potentially limit the invasive ability of *A. franciscana*. However, to fully understand the invasive capacity of *A. franciscana* in these environments and the resilience of the native *Artemia* strains which resist invasion, next to survival, reproductive features and the dynamics of these three populations need to be assessed. This work complements Pinto et al. (2013b), studying the reproductive performance of the two native strains and of the invasive species when exposed to abiotic variations in salinity, temperature, light and food availability. Pre-reproductive and reproductive periods, as well as the type and quality of reproduction are assessed, as these factors decisively influence the biological fitness and lifetime of each of these species (Allan, 1976; Barata et al., 1995, 1996a,b; Browne et al., 1984, 1988, 1991), as well as the permanence or elimination of native strains in these hypersaline environments (Amat et al., 2007).

Material and methods

Biological material

Adult diploid *A. parthenogenetica* were collected at two sites: the Troncalhada saline (40°38'40"N, 8°39'46"W) located in the Ria de Aveiro lagoon, an artisanal, solar saline covering an area of 4.2 ha; and the saline of Rio Maior (39°21'50"N, 8°56'38"W), an inland saline supplied by pumped up brine from naturally dissolved rock salt (more information about these salines can be found in Pinto et al., 2013b) (Fig. 1). *A. franciscana* were hatched from a commercial brand of *Artemia* cysts (Ocean Nutrition™, Great Salt Lake). The animals were kept in the laboratory, separated per population, and allowed to acclimatise to a salinity of 70 ppt and a temperature of 24°C.

The first 24 females of each *Artemia* strain to reach sexual maturity (or less, when fewer animals reached sexual maturity during the experiment, which happened for

some extreme experimental conditions) were transferred immediately and individually (for *A. parthenogenetica*) or together with a male (for *A. franciscana*) to 50 ml Falcons tubes, maintaining the acclimatization culture conditions. Females were considered to be sexually mature after developing their ovisac and males when their antenna and penis were clearly observable.

Experimental setup

We considered 70 ppt salinity, 24°C water temperature, 12:12 h L:D photoperiod and 300000 cells ml⁻¹ *Tetraselmis suecica* food supply as standard conditions. In each experiment one of these parameters was varied to assess its effect on reproduction; the remaining parameters were kept constant. Treatment conditions were: salinities of 70 ppt, 110 ppt and 150 ppt (prepared using natural sea water and Tropic Marin Sea Salt® and confirmed with a refractometer); temperatures of 24°C, 29°C and 34°C ±1°C (maintained keeping the falcons in water baths, with temperatures regularly checked with a thermometer); photoperiods of 12:12h L:D, constant light and constant darkness; and three levels of food supply, with ±300000 cells ml⁻¹, ±150000 cells ml⁻¹ and ±37500 cells ml⁻¹ of *T. suecica* respectively (densities were obtained through dilution, counting *T. suecica* cells in a Neubauer chamber). Notice that supplied food concentrations were independent of the number of individuals present in each replicate (one female for parthenogenetic populations and a couple for the bisexual species), but the medium was renewed every second day.

Each treatment condition was applied to up to 24 replicates (depending on the number of mature females obtained). For each replicate, the experiment finished with the death of the parthenogenetic individual or the *A. franciscana* female. If a male *A.*

franciscana died before its replicate's female, the male was replaced by another that had been kept under the same treatment conditions.

Every second day, the animals were observed to determine the following life history features: pre-reproductive, reproductive and post-reproductive periods (in days); life span (days); broods per female; inter-brood interval (days); total offspring; offspring per brood and female; ovoviviparous offspring percentage and quality; and oviparous offspring percentage quality. Cyst quality was evaluated by its chorion appearance and floating capacity only; good external appearance and floating capacity in the salinities tested were considered indicators of good reproductive viability. The time between broods was assessed considering only females that produced at least 3 broods during the experiment (seen as females with a consistent breeding period).

2.2 Data analysis

Means and SD of life periods and offspring data were plotted and compared for the different *Artemia* strains and culture conditions. Given that many treatment results failed the Shapiro-Wilk test for normality (with $p < 0.05$) (Royston, 1982), also when log transformed, differences between *Artemia* sources and between treatment effects were assessed through a distribution-free analysis of variance, using the Kruskal-Wallis test to determine if any treatment group was different from the others, and post-hoc pairwise Wilcoxon rank sum tests, corrected for multiple testing (with p-value adjustment by the Holm method; Holm 1979), to see which groups differed (Hollander and Wolfe, 1973).

Multivariate analyses were used to compare *Artemia* from different sources. A correlation-based Principal Component Analysis (PCA; the DCA gradient lengths were all < 3) of the life table and reproductive parameters was carried out for each experimental treatment. The distinction between *Artemia* strains was further tested

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158 through an Analysis of Similarity (ANOSIM), following a 2D Non-Metric
159 Multidimensional Scaling (NMDS) based on standardized and normalized Euclidian
160 distances. Multivariate analyses did not consider time between broods, to avoid
161 eliminating females that produced less than three broods from the data.

162 PCA were performed using CANOCO 4.5 (TerBraak and Smilauer, 1998),
163 NMDS and ANOSIM using Primer 5 (Clarke and Warwick, 2001). All other statistical
164 analyses were done in R (R Dev. Core Team, 2009).

165 166 **Results**

167 168 *Survival and maturation*

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170 Not all of the experimental conditions lead to the survival (Pinto et al., 2013b)
171 and maturity of 24 females (Table 1). For instance, at 34°C temperature no animals of
172 any of the studied *Artemia* strains reached maturity. And at 29°C, only 13 individuals
173 from Rio Maior reached maturity and only one produced more than 2 broods. High
174 salinities proved to be deadly for *A. parthenogenetica* from Aveiro (only one individual
175 matured at 110 ppt, none at 150 ppt) and *A. franciscana* (no animal matured at 150 ppt).
176 *A. parthenogenetica* from Rio Maior, on the other hand, was more tolerant to high
177 salinity. Food shortage affected mainly *Artemia* from Aveiro. The photoperiods studied
178 hardly affected survival and maturation.

179 180 *Life time and reproductive periods*

181 Mean values (and SD) of the Life time and reproductive periods are presented in
182 Figure 2. The Wilcoxon rank sum test results comparing *Artemia* strains are presented
183 in Table 2, and those comparing treatment levels for each strain in Table 3.

Under standard conditions, *A. parthenogenetica* from Rio Maior had the longest mean pre-reproductive period (PRP) of all tested species; the shortest was obtained for *A. franciscana*. Both *A. franciscana* and *A. parthenogenetica* from Rio Maior showed the longer mean reproductive periods (RP) than *Artemia* from Aveiro, which presented a relative long PRP, but short RP and total life span (LS) (Fig. 2).

At 110 ppt salinity, the mean PRP of *A. franciscana* was significantly shorter than that of *Artemia* from Rio Maior, and the RP was bigger than at 70 ppt for all species, although this difference was only significant for the *A. franciscana*. Too few (1) animals of *A. parthenogenetica* from Aveiro survived to allow comparison. At 150 ppt only *Artemia* from Rio Maior was able to reproduce.

At 29°C temperature, *A. parthenogenetica* from Aveiro showed significantly longer PRP and (consequently) LS than the other strains and that at 24°C. Both *A. parthenogenetica* from Rio Maior and *A. franciscana* reduced their RP and LS at 29°C, in comparison to the 24°C treatment. *Artemia* from Rio Maior had the shortest RP. At 29°C *A. franciscana* displayed shorter pre and post reproductive periods than the strain from Aveiro.

Halving the food supply strongly affected *A. parthenogenetica* from Aveiro, lengthening its PRP and shortening (though not significantly) its RP. This strain did not survive extreme food shortage (37500 cells ml⁻¹ of *T. suecica*). Differences between the less affected *Artemia* from Rio Maior and *A. franciscana* were non-significant for the halved food supply, but significant under extreme food shortage, when the RP of *Artemia* from Rio Maior became very short.

Continuous light caused shorter PRP and LS for *A. franciscana*, in comparison to the other strains. Continuous darkness mainly decreased the RP and LS of *A. parthenogenetica* from Rio Maior.

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210 ***Broods and offspring quantity***

211 Mean values (and SD) of brood and offspring numbers are presented in Figure 3.

212 The Wilcoxon rank sum test results comparing *Artemia* strains are presented in Table 2,
213 and those comparing treatment levels for each strain in Table 3.

214 Under standard conditions, there were significantly less broods per female
215 (BPF), offspring per female (OPF) and offspring per brood (OPB) for *A.*
216 *parthenogenetica* from Aveiro than for the other two strains. *A. franciscana* had
217 furthermore more OPB and a shorter time between broods (TBB) than *Artemia* from
218 Rio Maior. At 110 ppt, *A. franciscana* produced significantly more OPF and OPB than
219 *Artemia* from Rio Maior, and more BPF and OPF than under standard conditions. At
220 150 ppt only *A. parthenogenetica* from Rio Maior was able to reproduce. An increase in
221 temperature to 29°C strongly reduced the number of BPF and OPF in *A. franciscana*
222 and *Artemia* from Rio Maior. *A. parthenogenetica* from Rio Maior had the lowest
223 reproduction, though most differences between strains were not significant. Halving the
224 food supply reduced OPF and OPB in *A. franciscana* and *Artemia* from Aveiro, but did
225 not negatively affect *Artemia* from Rio Maior (except for the OPB). *Artemia* from
226 Aveiro produced significantly less BPF, OPF and OPB than the other two strains. Under
227 extreme food shortage, only *A. franciscana* produced few but still more BPF and OPF
228 than *Artemia* from Rio Maior. Under both continuous light and continuous darkness,
229 *Artemia* from Rio Maior produced less BPF and OPF. *A. franciscana* preferred
230 continuous light to continuous darkness, whereas *Artemia* from Aveiro showed little
231 photoperiod effects.

232 There were few significant differences in time between broods (TBB),
233 comparing *Artemia* strains or treatment levels. Under standard conditions and at 110 ppt

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234 salinity, *A. franciscana* had shorter TBB than *Artemia* from Rio Maior, but both strains
235 did not differ in TBB from the Aveiro strain. Under continuous light *A. franciscana* had
236 shorter TBB than the other strains.

237 238 ***Offspring type and quality***

239 Offspring type and quality are quantified in Figure 4, as mean percentages per
240 *Artemia* strain and experimental condition. Mean values (and SD), as well as Wilcoxon
241 rank sum test results comparing *Artemia* strains in terms of offspring quantity and
242 quality are presented in Table 4.

243 Under standard conditions, the offspring from *A. parthenogenetica* from Rio
244 Maior and Aveiro was about half ovoviviparous, but offspring quantity and quality was
245 much worse for the Aveiro strain (Fig. 4, Table 4). *A. franciscana* reproduced mainly
246 through good quality oviparous offspring. An increase in salinity (to 110 ppt),
247 temperature (29°C) or light (24h) led to predominantly ovoviviparous reproduction and
248 less offspring quality for the Aveiro strain, while food shortage caused more oviparous
249 offspring. *Artemia* from Rio Maior, on the other hand, reacted with predominantly
250 oviparous reproduction to 110 ppt salinity and continuous light, but with ovoviviparous
251 reproduction under food shortage and at 29°C (with many dead and abortive embryos).
252 At 150 ppt only *Artemia* from Rio Maior was able to reproduce, mostly with good
253 quality offspring. *A. franciscana* produced more cysts than the other strains at 29°C and
254 under food shortage, but with rather bad quality offspring when food shortage was
255 extreme. Compared to standard conditions, continuous light or darkness and increased
256 temperature (except for *A. franciscana*) caused losses in offspring quality.

257 258 ***Multivariate analysis***

Considering base conditions the first axis of the PCA separates samples according to offspring quantity and the second axis according to offspring quality (Fig. 5). *Artemia* from Aveiro are characterised by less offspring and broods, whereas *Artemia* from Rio Maior and *A. franciscana* samples are separated on the second axis, according to their preference for oviparous and ovoviviparous reproduction, respectively.

At 110 ppt salinity only one *Artemia* from Aveiro matured. Again *Artemia* from Rio Maior and *A. franciscana* samples are separated on the second axis, according to their main reproduction mode, but also due to the longer pre-reproductive periods and less OPB observed for *Artemia* from Rio Maior.

High temperature lead to a well defined cluster of *Artemia* from Rio Maior, on the left side of the first axis, with less offspring, broods and ovoviviparous reproduction. *Artemia* from Aveiro and *A. franciscana* are separated on the second PCA axis, characterised by less oviparous reproduction and longer PRP and post-reproductive periods in samples from Aveiro.

Halving the food supply showed similar patterns as the base condition: a first axis separating samples according to offspring quantity and a second one according to offspring quality, with *Artemia* from Aveiro characterised by less offspring and broods, and *Artemia* from Rio Maior and *A. franciscana* separated, according to their preference for oviparous and ovoviviparous reproduction, respectively. Samples from Aveiro show furthermore long PRP. Extreme food shortage separates *Artemia* from Rio Maior and *A. franciscana* on the first axis, with less offspring and broods, less oviparous offspring and longer PRP for samples from Rio Maior.

Continuous light condition separated *Artemia* from Rio Maior on the first axis, with samples characterised by less offspring and broods, and less ovoviviparous

offspring, and on the second axis with samples characterised by less oviparity. Complete darkness also separated *Artemia* from Rio Maior on the first axis due to less offspring and broods, and due to longer PRP. Under these conditions, *Artemia* from Aveiro and *A. franciscana* were mainly separated on the second axis, characterized by the preference of oviparous reproduction in the Aveiro samples.

Analysis of similarity (ANOSIM) after 2D NMDS showed significant ($p<0.05$) discrimination between *Artemia* sources, except for the very overlapping samples of *Artemia* from Rio Maior and *A. franciscana* kept at 110 ppt salinity, and often little separation between samples from different sources ($R<0.250$). Reasonable separation was obtained for the Rio Maior samples in continuous light ($R>0.500$) and food shortage conditions ($R>0.400$), and between the Rio Maior and Aveiro samples kept in complete darkness ($R>0.400$).

Discussion

The results of the present study, together with those obtained previously by Pinto et al. (2013b), provide a broad understanding of the different responses of the two studied Portuguese native parthenogenetic *Artemia* strains and the invasive *A. franciscana* to environmental conditions. They also provide clues to species invasiveness or resistance to invasion and guidelines for the best abiotic conditions for each of these *Artemia* species.

The native parthenogenetic strains from Aveiro and Rio Maior showed very different reproductive performance, in quantity and in quality, confirming the variability in diploid *Artemia* strains (Pinto et al., 2013b; Browne, 1992). *A. franciscana* showed overall better survival (Pinto et al., 2013b) and reproductive abilities and, therefore potential competitive advantage over *A. parthenogenetica* from Aveiro. This advantage

was less evident compared to the Rio Maior strain; this strain even survived and reproduced better than *A. franciscana* at high salinities (150 ppt) and with halved food supply. However, available food density was the same for the single parthenogenetic *Artemia* and the couple of *A. franciscana* in each experiment (although the medium was renewed every second day), which favoured the Rio Maior strain.

Previous studies have confirmed the eurythermal characteristic of *A. franciscana* (Wear and Haslett, 1986; Browne, 1988; Browne et al., 1991; Browne and Wanigasekera, 2000). *A. franciscana* showed much higher survival at 29°C than the native Portuguese parthenogenetic strains, and even some survival at 34°C (Pinto et al., 2013b). In terms of reproductive ability however, the present study shows that *A. franciscana* prefers a lower temperature. This species showed larger reproductive periods and oviparity output and quality at 24°C than at 29°C, and animals died without reproducing at 34°C. In comparison to the parthenogenetic strains, *A. franciscana* showed a small advantage at 29°C, presenting smaller pre-reproductive periods and more abundant good cysts. Even under base conditions (24°C, 70 ppt, abundant food and 12:12 L:D photoperiod), *A. franciscana* presented overall better reproductive success than *A. parthenogenetica* from Aveiro and similar success to *A. parthenogenetica* from Rio Maior.

A. franciscana showed also better adaption to intermediate/high salinities than the Portuguese parthenogenetic strains, in terms of both survival (Pinto et al., 2013b), and offspring quantity, although it had no clear advantage in terms of offspring composition and quality. *A. franciscana* was the only strain showing better reproductive figures at 110 ppt than at 70 ppt, whereas *A. parthenogenetica* from Rio Maior performed better at 150 ppt than the other two strains, producing viable offspring at this high salinity.

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334 *A. parthenogenetica* from Rio Maior also performed best in the halved food
335 supply experiment. Although *A. franciscana* and *A. parthenogenetica* from Rio Maior
336 showed both a good adaptation to lack of food, there was a small advantage of the
337 parthenogenetic strain, which had better quality ovoviviparous and oviparous offspring
338 than *A. franciscana* (notice that, as mentioned, food density was not the same per
339 individual, which may have biased this result). This resistance to food shortage had
340 already been demonstrated in terms of survival (Pinto et al., 2013b). Under extreme
341 food shortage (an eighth of the base food density) *A. franciscana* and *A.*
342 *parthenogenetica* from Rio Maior still reproduced, but with great difficulty. *A.*
343 *parthenogenetica* from Aveiro was the most sensitive to food shortage reproducing
344 badly at halved food and not even surviving extreme food shortage.

345 In relation to the photoperiods studied, continuous light and continuous darkness
346 affected reproductive success of *Artemia* from Rio Maior negatively, *A. franciscana*
347 preferred continuous light to continuous darkness, whereas *Artemia* from Aveiro
348 showed little photoperiod effects. Although these results, which are mostly in agreement
349 with those obtained in previous survival experiments, are difficult to relate to natural
350 conditions, where continuous light or darkness never occur, variations in the
351 reproductive behaviour of these species in changing photoperiods might be important
352 for the invasive capacity of *A. franciscana*.

353 So, what has (so far) prevented the introduction *A. franciscana* in the two
354 studied Portuguese hypersaline biotopes? The Rio Maior saline has unique
355 characteristics that reduce the possibility of an accidental introduction of the invader.
356 Firstly, its geographical inland location, which lays far from the main bird migration
357 routes and is far from fish farming facilities and urban areas with aquaria (Amat et al.,
358 2007). Other characteristics that may help reduce the invader introduction are its source

of saltwater, pumped up from a rock salt mine, with an output salinity of 150 ppt and a low abundance and diversity of *Artemia* food organisms (the tanks are made of cement and cleaned at the end of every salt season production, showing little algae concentration during the salt production season). Finally, the rather deep water tanks used for water storage, which are unusual for salines. The results here presented suggest that the local *A. parthenogenetica* is better adapted to the particular conditions of this saline than *A. franciscana*. *A. parthenogenetica* from Rio Maior was the only strain that survived and reproduced at 150 ppt. It had better reproductive results at 24°C and worse at 29°C than its invasive competitor, which may be advantageous in the deep, less warmed up tanks. *Artemia* from Rio Maior also outperformed *A. franciscana* when food supply was halved. But we also found features that should enhance invasiveness of *A. franciscana* in the Rio Maior biotope: *A. franciscana* outcompetes the local parthenogenetic *Artemia* at very low food concentrations, at higher temperatures and in complete darkness, which may constitute an advantage in the deep tanks of this saline with little light incidence.

For the native population from the Aveiro salines complex the situation is completely different, both in terms of biotope characteristics and of the weak reproductive capacity of this strain in comparison to *A. franciscana*. The Aveiro saline complex is fed by water from the Ria de Aveiro lagoon, with up to 35 ppt salinity. Water tanks are shallow with natural sediment bottoms. Salinity increases gradually from the inlet channel to the crystallisers, where 300 ppt can be reached (Vieira, 1989). Local conditions may explain why *A. franciscana* outcompeted the local *A. parthenogenetica* in all experimental conditions. For instance, the weak performance of *Artemia* from Aveiro at low food concentrations may be due to its adaptation to the abundant and diversified food species found in the Aveiro saline water (Vieira and Bio,

2011). Hence, this saline must be seriously threatened by invasion, especially given that there are records of *A. franciscana* in a saline of the same complex, not too distant from the saline here studied, and given that the lagoon and salines are inhabited by numerous bird species that could disseminate the invasive *Artemia* strain (Amat et al., 2007). The fact that, in spite of its poor survival, biological fitness and lifespan, *A. parthenogenetica* persists in the saline may be due to two explanations. First, although very unlikely for the reasons previously exposed, *A. franciscana* has not yet reached that saline, which would mean that its introduction should urgently be prevented to avoid elimination of the native *Artemia* strain. Second, *A. franciscana* already appeared in that biotope but was not able to establish itself there. This second option could be explained by the existence of a chemical barrier, as suggested by Pinto et al. (2013b), related to contaminants in the polluted Ria de Aveiro lagoon water, such as heavy metals or pesticides (e.g. Martins et al., 2010). Future studies should assess the effect of the chemicals present in this particular saline on *Artemia* survival and reproduction, as local chemical biotope characteristics may be of crucial importance for the maintenance of native *Artemia* strains.

Conclusions

A broad assessment of strain-specific traits is needed to understand competition, resilience and resistance of native *Artemia* strains in relation to the invasive *A. franciscana*. *A. parthenogenetica* from Rio Maior seems to be very well adapted to its specific biotope characteristics, which, together with its inland localisation, favours its resistance to invasion. It is nevertheless important to take measures to avoid the introduction of *A. franciscana*, which is probably capable of displacing the local *Artemia* strain in biological terms.

A. parthenogenetica from Aveiro, on the other hand, proved to perform much worse than its invasive competitor, in the conditions tested. Its permanence in its biotope is still an unexplained phenomenon. The only two explanations that we currently glimpse for this fact are that either *A. franciscana* has by chance not been introduced there, or a chemical barrier related to the pollution has been preventing invasion. Further studies are essential to discern the true reasons.

Future studies are needed to continue to analyse the specific characteristics of the European hypersaline biotopes, taking into account local conditions such as environmental problems, and relate them to the specific biological traits of the local *Artemia* strains we aim to preserve, especially against exotic invasive species.

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1 **Table 1.** Total number of mature females (obtained from the initial 30) and number of females producing
2 more than two broods obtained for the different treatment conditions. The first treatment constitutes the
3 base treatment. Treatments used to make comparisons per treatment variable are marked in bold.

Treat- ment	Salinity	Temper- ature ^a	Light	Food supply	<i>A. parthenogenetica</i> females					
					Rio Maior		Aveiro		<i>A. franciscana</i>	
					total	>2 broods	total	>2 broods	total	>2 broods
1	70	24	12	300000	24	20	22	11	23	21
2	110	24	12	300000	12	9	1 ^b	1 ^b	24	23
3	150	24	12	300000	18	9	0 ^b	0 ^b	0 ^b	0
4	70	29	12	300000	13	1 ^b	18	11	22	9
5	70	24	12	150000	23	16	23	4	24	20
6	70	24	12	37500	12	0 ^b	0 ^b	0 ^b	14	3
7	70	24	0	300000	24	3	23	18	21	19
8	70	24	24	300000	24	4	23	11	24	12

^a No animals reached maturity at 34°C

^b Too few samples to allow statistical analysis

Table 2. Pairwise Wilcoxon rank sum test results for the comparison of lifetable parameters between *Artemia* from different sources (AV: *A.parthenogenetica* from Aveiro, RM: *A.parthenogenetica* from Rio Maior, FR: *A. franciscana*, c./ml: cells ml⁻¹ of *Tetraselmis* food supply), considering the different treatments, using the Holm (1979) adjustment for multiple comparisons; —: not enough data for testing, ns: non-significant, *: $\alpha=0.05$, **: $\alpha=0.01$, ***: $\alpha=0.001$. Only females producing at least three broods were considered for the calculation of the time between broods.

Treatment	Source	Pre-reproductive	Reproductive	Post-reproductive	Lifespan	Broods female ⁻¹	Time between broods	Offspring female ⁻¹	Offspring brood ⁻¹	ovoviparous	Life embryos	Dead embryos	Abortive embryos	oviparous	Good cysts	Bad cysts
Base	AV-RM	**	***	ns	***	***	ns	***	***	**	*	ns	ns	**	**	ns
	AV-FR	***	***	ns	*	***	ns	***	***	ns	ns	ns	ns	***	***	*
	RM-FR	***	ns	**	*	ns	**	ns	**	*	*	ns	ns	**	*	*
110 ppt	AV-RM	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	AV-FR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	RM-FR	***	ns	ns	ns	ns	*	*	**	ns	ns	ns	**	ns	ns	ns
150 ppt	AV-RM	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	AV-FR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	RM-FR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
29°C	AV-RM	***	*	ns	***	*	ns	*	ns	ns	*	ns	ns	ns	ns	ns
	AV-FR	***	ns	*	***	ns	ns	ns	*	ns	ns	***	ns	ns	***	ns
	RM-FR	***	*	ns	ns	ns	ns	*	*	ns	ns	ns	ns	*	***	ns
150000 c./ml	AV-RM	***	***	***	ns	***	ns	***	***	***	***	ns	ns	*	**	ns
	AV-FR	***	***	ns	ns	***	ns	***	***	ns	ns	ns	ns	***	***	ns
	RM-FR	ns	ns	ns	ns	ns	ns	ns	ns	***	***	ns	**	***	*	ns
37500 c./ml	AV-RM	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	AV-FR	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	RM-FR	***	***	*	ns	***	—	***	ns	ns	***	—	*	***	**	***
24h	AV-RM	ns	ns	*	**	*	ns	ns	**	***	***	**	***	***	***	***
	AV-FR	***	ns	ns	**	ns	*	ns	**	*	ns	ns	ns	*	**	ns
	RM-FR	***	ns	ns	**	*	*	**	ns	***	***	**	**	***	***	***
0h	AV-RM	***	***	ns	*	***	ns	**	ns	***	***	ns	ns	***	**	**
	AV-FR	**	ns	ns	ns	*	ns	***	***	***	***	**	***	ns	ns	ns
	RM-FR	***	***	ns	*	***	ns	***	***	***	***	*	***	*	ns	ns

Table 3. Pairwise Wilcoxon rank sum test results for the comparison of lifetable parameters between treatment levels, considering *Artemia* from different sources (AV: *A.parthenogenetica* from Aveiro, RM: *A.parthenogenetica* from Rio Maior, FR: *A. franciscana*, c./ml: cells ml⁻¹ of *Tetraselmis* food supply), using the Holm (1979) adjustment for multiple comparisons; —: not enough data for testing, ns: non-significant, *: $\alpha=0.05$, **: $\alpha=0.01$, ***: $\alpha=0.001$. Only females producing at least three broods were considered for the calculation of the time between broods.

Source Parameter		Pre-reproductive	Reproductive	Post-reproductive	Lifespan	Broodsfemale ⁻¹	Time betweenbroods	Offspringfemale ⁻¹	Offspringbrood ⁻¹	ovoviviparous	Lifeembryos	Deadembryos	Abortiveembryos	oviparous	Goodcysts	Badcysts
AV	70-110ppt	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
	24-29°C	***	ns	*	***	ns	ns	ns	*	ns	ns	**	ns	ns	**	ns
	300000-150000c./ml	***	ns	*	**	ns	ns	*	**	***	***	ns	ns	ns	ns	ns
	12-24 h	**	ns	ns	*	ns	ns	ns	ns	*	*	ns	*	ns	ns	ns
	12-0 h	**	*	ns	***	ns	ns	ns	ns	***	***	ns	ns	***	**	**
	24-0 h	ns	ns	ns	*	ns	ns	ns	ns	***	***	ns	***	***	***	***
RM	70-110ppt	***	ns	ns	ns	ns	**	ns	ns	*	*	ns	ns	ns	ns	*
	70-150ppt	**	*	ns	ns	**	ns	***	***	*	*	ns	*	***	***	ns
	110-150ppt	ns	ns	ns	ns	ns	*	*	***	ns	ns	ns	ns	*	*	**
	24-29°C	ns	***	ns	***	***	ns	***	***	***	***	ns	ns	***	***	ns
	300000-150000 c./ml	***	ns	ns	ns	ns	—	ns	*	ns	ns	ns	ns	ns	ns	ns
	300000-37500 c./ml	***	***	ns	***	***	—	***	***	***	***	**	ns	***	***	**
	150000-37500 c./ml	***	***	ns	ns	***	—	***	***	***	**	*	*	***	***	**
	12-24 h	***	***	***	***	***	ns	***	ns	***	***	**	ns	ns	ns	***
	12-0 h	***	***	ns	*	***	ns	***	ns	**	***	ns	ns	*	*	ns
	24-0 h	***	ns	ns	***	ns	ns	ns	ns	**	**	ns	ns	*	ns	***
FR	70-110ppt	***	***	**	***	***	***	***	ns	***	**	ns	***	ns	ns	ns
	24-29°C	ns	***	ns	**	***	ns	***	***	ns	ns	ns	ns	***	***	*
	300000-150000 c./ml	***	*	ns	ns	*	ns	***	***	ns	ns	ns	ns	*	*	ns
	300000-37500 c./ml	***	**	***	***	***	ns	***	***	ns	ns	ns	*	***	***	ns
	150000-37500 c./ml	***	**	***	***	**	ns	***	***	ns	ns	ns	**	***	***	ns
	12-24 h	**	***	ns	**	***	ns	***	ns	**	ns	ns	**	***	***	***
	12-0 h	***	ns	ns	*	ns	ns	ns	ns	**	ns	ns	***	*	**	ns
	24-0 h	ns	***	ns	***	***	ns	***	ns	ns	ns	ns	*	***	***	*

Table 4. Mean offspring quantities (and SD) for the different *Artemia* strains (AV: *A. parthenogenetica* from Aveiro, RM: *A. parthenogenetica* from Rio Maior, AF: *A. franciscana*), depending on treatment conditions; emb.: embryos. Different superscript letters indicate significant differences between *Artemia* source according to the multiple Wilcoxon rank sum tests; * means that only one female did reproduce, so that no statistical testing was possible.

Treat.	Source	Ovoviviparous	Life embryos	Dead emb.	Abortive emb.	Oviparous	Good cysts	Bad cysts
Base	AV	80.6 (80.7) ^a	67.5 (66.9) ^a	6.3 (21.5) ^a	6.8 (20.9) ^a	92.2 (167.2) ^a	49.3 (69.8) ^a	42.9 (120.3) ^a
	RM	252.0 (232.1) ^b	245.6 (228.4) ^b	4.4 (9.4) ^a	2.0 (5.5) ^a	225.8 (360.1) ^b	201.1 (351.1) ^b	24.8 (51.6) ^a
	FR	162.8 (279.2) ^a	158.3 (275.7) ^a	3.6 (10.5) ^a	0.9 (3.0) ^a	484.3 (402.8) ^c	387.5 (375.0) ^c	96.7 (103.4) ^b
110 Ppt	AV	321.0 (0.0)*	311.0 (0.0)*	4.0 (0.0)*	6.0 (0.0)*	11.0 (0.0)*	0.0 (0.0)*	11.0 (0.0)*
	RM	96.0 (40.6) ^a	90.1 (40.9) ^a	1.5 (1.4) ^a	4.4 (6.3) ^a	507.7 (513.1) ^a	302.4 (302.1) ^a	205.3 (240.5) ^a
	FR	605.7 (617.3) ^a	397.5 (397.9) ^a	1.9 (2.8) ^a	206.4 (300.5) ^b	671.4 (556.8) ^a	492.0 (472.4) ^a	179.3 (240.4) ^a
150 Ppt	AV							
	RM	92.7 (51.1)	83.6 (49.4)	1.2 (2.4)	7.9 (9.5)	38.4 (68.6)	24.8 (39.5)	13.6 (31.3)
	FR							
29°C	AV	109.4 (105.1) ^a	98.7 (96.6) ^a	2.9 (3.8) ^a	7.8 (14.2) ^a	22.2 (21.1) ^{ab}	0.2 (0.7) ^a	22.1 (20.9) ^a
	RM	35.9 (21.0) ^a	23.8 (17.6) ^b	2.5 (3.4) ^{ab}	9.7 (19.8) ^a	6.8 (9.9) ^b	0.0 (0.0) ^a	6.8 (9.9) ^a
	FR	123.4 (181.1) ^a	112.1 (171.2) ^{ab}	0.9 (3.2) ^b	10.4 (25.7) ^a	121.8 (152.5) ^a	85.3 (112) ^b	36.5 (67.3) ^a
150000 c./ml	AV	18.8 (39.5) ^a	13.3 (30.1) ^a	3.8 (11.3) ^a	1.7 (5.4) ^{ab}	46.4 (39.0) ^a	26.0 (33.7) ^a	20.4 (20.0) ^a
	RM	379.1 (232.1) ^b	371.5 (233.2) ^b	1.1 (1.8) ^a	6.5 (12.1) ^b	100.8 (79.5) ^b	80.4 (69.5) ^b	20.3 (31.7) ^a
	FR	29.4 (59.7) ^a	28.3 (57.9) ^a	0.5 (2.0) ^a	0.5 (2.1) ^a	253.3 (174.6) ^c	193.3 (193.9) ^c	60.0 (66.3) ^a
37500 c./ml	AV							
	RM	13.8 (9.3) ^a	13.7 (9.1) ^a	0.0 (0.0)	0.2 (0.6) ^a	0.0 (0.0) ^a	0.0 (0.0) ^a	0.0 (0.0) ^a
	FR	17.1 (16.3) ^a	4.8 (13.0) ^b	0.0 (0.0)	12.4 (15.0) ^b	26.9 (39.5) ^b	12.9 (25.4) ^b	14.0 (15.5) ^b
24h	AV	166.6 (149.5) ^a	144.2 (142.3) ^a	2.7 (5.7) ^a	19.7 (30.0) ^a	19.7 (23.8) ^a	7.6 (18.4) ^a	12.1 (14.8) ^a
	RM	6.9 (13.7) ^b	5.8 (12.8) ^b	0.2 (0.4) ^b	0.9 (2.8) ^b	129.8 (76.9) ^b	57.7 (67.6) ^b	72.1 (42.7) ^b
	FR	275.7 (213.2) ^c	213.4 (201.6) ^a	15.4 (38.1) ^a	46.9 (61.4) ^a	16.9 (38.0) ^c	0.0 (0.0) ^c	16.9 (38.0) ^a
0h	AV	3.1 (12.7) ^a	0.7 (3.3) ^a	1.0 (3.3) ^a	1.4 (6.2) ^a	261.5 (153.1) ^a	108.7 (79.5) ^a	152.8 (152.1) ^a
	RM	69.6 (78.6) ^b	53.4 (70.6) ^b	1.7 (3.6) ^a	14.5 (33.2) ^a	64.3 (61.9) ^b	43.5 (46.8) ^b	20.8 (32.6) ^b
	FR	403.4 (308.6) ^c	275.4 (244.7) ^c	17.9 (39.0) ^b	110.1 (109.8) ^b	236.1 (227.0) ^a	122.2 (131.1) ^{ab}	113.9 (156.9) ^{ab}

Table 5. Analysis of similarity results for the life table parameters of the different *Artemia* sources, depending on treatment conditions (AV: *A. parthenogenetica* from Aveiro, RM: *A. parthenogenetica* from Rio Maior, FR: *A. franciscana*, c./ml: cells ml⁻¹ of *Tetraselmis* food supply); ANOSIM *R* and respective *p*-values are given (—: not enough data for testing).

Treatment	2D NMDS stress	Global		AV-RM		AV-FR		RM-FR	
		<i>R</i>	<i>p</i>	<i>R</i>	<i>P</i>	<i>R</i>	<i>p</i>	<i>R</i>	<i>p</i>
Base	0.12	0.233	0.001	0.175	0.001	0.311	0.001	0.233	0.001
110ppt	0.10	—	—	—	—	—	—	0.092	0.092
29°C	0.16	0.228	0.001	0.199	0.005	0.176	0.004	0.364	0.001
150000 c./ml	0.17	0.400	0.001	0.426	0.001	0.335	0.001	0.476	0.001
37500 c./ml	0.10	—	—	—	—	—	—	0.456	0.001
24h light	0.14	0.393	0.001	0.530	0.001	0.063	0.026	0.574	0.001
0h light	0.13	0.351	0.001	0.337	0.001	0.424	0.001	0.228	0.001

Figure 1. Location of the *A. parthenogenetica* sampling sites.

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Salinity

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Temperature

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Food supply (*Tetraselmis* cells ml⁻¹)

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Light

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■ pre-reproductive ■ reproductive □ post-reproductive

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Figure 2. Mean values (+SD) of pre-reproductive, reproductive and post-reproductive periods, depending on *Artemia* source and culture conditions; FR: *A. franciscana*, AV: *A. parthenogenetica* from Aveiro, RM: *A. parthenogenetica* from Rio Maior. Notice that there is only 1 observation for AV at 110 ppt.

Figure 3. Mean values and SD of brood and offspring parameters depending on *Artemia* source and culture conditions; AV: *A. parthenogenetica* from Aveiro, RM: *A. parthenogenetica* from Rio Maior, FR: *A. franciscana*. The first set of bars in each plot refers to the base treatment condition. Only females producing at least three broods were considered for the calculation of the time between broods.

Figure 4. Mean offspring composition, depending on *Artemia* source and culture conditions; AV: *A. parthenogenetica* from Aveiro, RM: *A. parthenogenetica* from Rio Maior, FR: *A. franciscana*. Only the offspring from females producing at least three broods was considered.

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Figure 5. First two axes of the correlation-based PCAs of *Artemia* life table parameters, depending on treatment conditions. Samples are identified according to the *Artemia* source (AV: *A. parthenogenetica* from Aveiro, RM: *A. parthenogenetica* from Rio Maior, FR: *A. franciscana*, emb.: embryos, abort.: abortive) and percentages of variance explained by the axes are given.

Figure 5. (Continuation).

Figure 5. (Continuation).