Journal of Experimental Marine Biology and Ecology 465 (2015) 142–152

Contents lists available at ScienceDirect

Journal of Experimental Marine Biology and Ecology

journal homepage: www.elsevier.com/locate/jembe

# Life-history traits, abiotic environment and coexistence: The case of two cryptic rotifer species

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ARTICLE INFO

*Article history:*
Received 10 November 2014
Received in revised form 28 January 2015
Accepted 29 January 2015
Available online 14 February 2015

*Keywords:*
*Brachionus*
Diapause
Niche differentiation
Trade-off

ABSTRACT

Trade-offs are important in life-history evolution and coexistence of competitors. However, how alternative life-history optima might promote competitor coexistence has received little attention. The rotifers *Brachionus plicatilis* and *Brachionus manjavacas* are two cryptic species with a considerable ecological overlap in relation to biotic factors. These species often co-occur in temporal brackish ponds characterised by environmental fluctuations. In these rotifers, sexual reproduction results in diapausing stages, which are able to survive through adverse conditions but constrain current population growth. In the present work, the differential responses of both cryptic rotifer species to varying salinities and temperatures in terms of population growth, sexual reproduction and diapause patterns (i.e., investment, viability and hatchability of diapausing eggs) were investigated to assess their possible roles in species coexistence. *B. plicatilis* and *B. manjavacas* showed differential responses to salinity and temperature and differed consistently in traits involved in diapause. The niche overlap between *B. plicatilis* and *B. manjavacas* decreased dramatically when the temperature and salinity response niche axes were added to a number of previously studied biotic niche axes. The results suggest that *B. plicatilis* exploits ephemeral and favourable conditions (low salinity) to invest in diapause, which would accelerate its exclusion from the water column but promote its long-term persistence. The findings show that cryptic species may evolve different compromises among life-history traits, allowing ecological divergence, and that ecological divergence may be uncoupled from morphological divergence for some factors in the abiotic environment.

## 1. Introduction

How competing species co-occur is a fundamental question in ecology. Co-occurrence of competitors in the same habitat may be transient, i.e. some species are slowly being driven to exclusion by the interactions with other species (Leibold and McPeek, 2006). However, species also may co-occur in the same habitat because each species is protected from exclusion, so that they show stable coexistence, even if their population densities fluctuate and are not at equilibrium (Adler et al., 2007; Chesson, 2000). Trade-offs among competitor capabilities are a necessary, but not a sufficient condition for their stable coexistence (Chesson, 2000; McPeek, 2014; Siepielski and McPeek, 2010). A trade-off occurs whenever a trait that confers an advantage when performing one function is linked to a disadvantage in performing another function (Stearns, 1992). Trade-offs can result in niche differentiation if neither species presents higher values of all relevant fitness components; the advantages that one species may present over others are offset by compensating disadvantages; for instance, being an efficient consumer of a kind of resources compromises being an efficient consumer of other resources. In the absence of trade-offs, one of the species could achieve higher performance than their competitor in all features, and then becomes the superior competitor. Additionally, stable coexistence is favoured by fitness equivalence among competitors (Chesson, 2000). If competitors have similar fitness, a small stabilizing niche difference allows stable coexistence (Adler et al., 2007). Therefore, the analysis of trade-offs and niche differentiation of competitors are needed steps in the study of stable coexistence, and can provide cues on what other conditions need to be investigated in order to demonstrate that a stable coexistence mechanism is acting. There are several potential trade-offs that can promote coexistence between species, such as trade-offs between growth capacity and low-resource tolerance (Angert et al., 2009), between competitive ability and predator susceptibility (Wellborn, 2002) or dispersal ability (Lei and Hanski, 1998), or between the efficiencies of using different resources (Tilman, 1982).

Relevance of trade-off analysis in ecology and evolution is not limited to the study of competition, as this analysis is crucial in the evolutionary theory of life history. Trade-offs involving reproduction shape life histories, and are expected to evolve in response to a suit of environmental features. Traits such as age at maturity, clutch size and the period of parental care are thought to evolve as a result of trade-offs between fecundity and survival or somatic growth (Stearns, 1992). These adaptive changes in life history traits can have implications on niche differentiation. However, with the exception of classical conceptual framework

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http://dx.doi.org/10.1016/j.jembe.2015.01.016
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of r- and K-selection, the connection between interspecific competition and those trade-offs on which life history theory focuses on is relatively poorly understood.

The effects of life-history trade-offs on competition are particularly interesting in the case of cryptic species (i.e., closely related species with high morphological similarity); as life-history traits may evolve uncoupled from morphology, they may promote niche differentiation and they even open the possibility of coexistence mechanisms not based on niche differentiation (Montero-Pau and Serra, 2011; Zhang et al., 2004). Additionally, life-history analysis is useful to understand why a competitor becomes dominant and how the species respond to the competitive dynamics. Coexistence of cryptic species is a challenging and puzzling phenomenon (Leibold and McPeek, 2006) as they are expected to present high ecological overlap (i.e., niche conservatism) (Violle et al., 2011) given their lack of morphological differences and phylogenetic relatedness.

In the present work, the response of two cyclically parthenogenetic cryptic species to their physical environment was investigated. The model organisms are two rotifer species — *Brachionus plicatilis* and *Brachionus manjavacas* — belonging to the cryptic *B. plicatilis* complex. This study differs from prior studies performed with other species of this complex in one important respect: *B. manjavacas* and *B. plicatilis* cannot be identified on a morphological basis (Campillo et al., 2005; Fontaneto et al., 2007), whereas other species of the complex show differences in shape and size (Ciros-Pérez et al., 2001b). Interestingly, in this last case niche differentiation has been demonstrated in both biotic (Ciros-Pérez et al., 2001a, 2004) and abiotic factors (Gómez et al., 1997).

Rotifers are one of the major zooplanktonic groups found in continental waters and represent a key component of the trophic webs of these habitats. *B. plicatilis* and *B. manjavacas* show high ecological overlap for biotic niche axes (Gabaldón et al., 2013). The two species co-occur in brackish ponds across the Iberian Peninsula (Gómez et al., 2002, 2007), likely showing stable dynamics (Montero-Pau et al., 2011). In temperate latitudes, the ponds where they inhabit are unsuitable for periods of varying predictability (Ortells et al., 2000), either because the pond dries or because seasonal conditions result in their exclusion from the water column. In these rotifers, re-colonisation of the water column takes place via the hatching of diapausing eggs stored in the sediment (Gilbert, 1974). *Brachionus* species, like cladocerans, aphids and others rotifers, are cyclical parthenogens (De Meester et al., 2004). Asexual diploid females hatch from diapausing eggs, and after an initial phase of clonal propagation, sexual reproduction is induced by a density-dependent infochemical produced by the rotifers themselves (Carmona et al., 1993; Kubanek and Snell, 2008; Stelzer and Snell, 2003), although asexual reproduction does not completely cease. In the sexual phase, sexual females produce haploid eggs that develop into males if unfertilised or into diploid diapausing female embryos, referred to as diapausing or resting eggs, if fertilised. Diapausing eggs are embryos in an arrested stage of development that are able to survive through adverse environmental conditions, such as drought and high or low temperature for several years or decades.

Thus, diapause is a key feature for the survival of these organisms, and encompasses different life history trade-offs. First, diapausing egg production involves diversion of resources from current, asexual proliferation to resting stages and future growth (e.g., Serra and King, 1999). Diapause is costly in these animals because it involves the two-fold cost of sex (Maynard Smith, 1971), diapausing eggs require more resources than subitaneous, asexual eggs (Alekseev et al., 2007; Clark et al., 2012; Gilbert and Schröder, 2004), and because diapausing eggs imply an obvious generation time increase. Thus, if an organism starts producing diapausing stages too early, before the end of the suitable period for growing, it will miss opportunities for its descendants to proliferate, while if too late, it will incur the risk of dying before producing any diapausing stages. Second, diapausing egg survival, diapause duration, and hatching success are dependent on the allocation of resources into the diapausing egg, hence compromising the number of diapausing eggs produced or the timing – with more or less resources in the environment – when these eggs are produced. Third, diapause cessation implies risks for the hatchlings due to unpredictable suitability of the water column, but opens opportunities for fast proliferation. Thus, how to pattern the diapausing egg hatching implies a trade-off. Interestingly, both diapausing egg production (e.g. sex initiation without cessation of asexual proliferation in rotifers (Fussmann et al., 2007)) and diapause termination are expected to be spread over time as bet-hedging strategy in unpredictably fluctuating environments (Cohen and Levin, 1987; Ellner, 1997).

Diapausing eggs and processes involved in diapause are relevant to intraspecific competition in different ways. On the one hand, competition dynamics in temporarily active populations could be better assessed focusing on diapausing egg production, since exclusion during the period of activity does not necessarily mean long-term exclusion. On the other hand, diapausing eggs are protected against competition, and such as stage is needed if competing species are differentially favoured during different periods in a fluctuating environment (Chesson, 2000). Finally, investment in diapause decreases the current rate of population proliferation and may affect the population dynamics of competing species during their growing season (Montero-Pau and Serra, 2011). Thus, a complex interplay occurs because diapause, being a response to abiotic or biotic, predictable or unpredictable environmental fluctuations, has consequences for the competitive dynamics and long-term competitor coexistence.

It has been suggested that coexistence of *B. plicatilis* and *B. manjavacas* is possible partly due to salinity fluctuations (Montero-Pau et al., 2011). The brackish ponds on the Iberian Peninsula where these cryptic species co-occur are characterised by environmental fluctuations (Comín et al., 1992). In the case of *B. plicatilis* and *B. manjavacas*, salinity is the only ecological factor known to differentially affect these species: *B. manjavacas* grows better at higher salinities (Montero-Pau et al., 2011). However, previous work has not thoroughly investigated how cryptic species with virtually identical biotic niches respond to major abiotic factors. Specifically, it is unknown, whether differential response to salinity and trade-offs resulting from a key life-cycle stage — namely, diapause — occurs, which is most relevant in understanding how the long-term co-occurrence of these species is maintained.

The objective in the present work was to test if *B. plicatilis* and *B. manjavacas* differentiate their niches in relation to two major abiotic factors: salinity and temperature. The focus was on their effect on current growth rates, sexual reproduction and diapause patterns. Three experiments were performed to explore differences in (1) population growth rates and sexual reproduction ratio under different salinities and temperatures, (2) hatching proportion and hatching pattern of diapausing eggs produced and hatched under three salinities, and (3) degradation pattern of diapausing eggs produced under the same three salinities. The aim was to assess how these two important abiotic variables affect niche differentiation of *B. plicatilis* and *B. manjavacas* in ways that may allow for the rotifer species to co-occur.

## 2. Materials and methods

### 2.1. Rotifer isolation, species identification and culture conditions

The experiments were carried out with *B. plicatilis* and *B. manjavacas* clones founded from hatchlings from diapausing eggs. Because rotifers can grow asexually, clonal lines can be established starting from a single female. Diapausing eggs were isolated from sediment samples collected in June 2010 with a Van Veen grab (Eijelkamp Agrisearch Equipment) from the upper sediment layer of Salobrejo Lake (Eastern Spain: 38° 54.765′ N, 1° 28.275′ W). Samples were taken in different points of the lake and thoroughly mixed. Sediment was stored in darkness at 4 °C during three months before diapausing egg isolation and hatching induction. Hatchlings were identified at species level by PCR-RFLP (for isolation and identification details, see Gabaldón et al., 2013). For each rotifer species, 25 clonal lines were established. The clones were maintained in stock cultures at 10 g/L salinity, at 20 °C and weekly fed the unicellular microalga *Tetraselmis suecica* (see Gabaldón et al., 2013 for details).

The pre-experimental and experimental rotifer culture media were the same as described for the stock cultures, but salinity was fixed at the indicated experimental values (see below). In the third experiment, in which rotifers were grown under several different salinity conditions, inert *T. suecica* was used as food. Using inert algae avoids the problem of growing *T. suecica* at salinities outside of its optimum and ensures a constant quality of the food provided in the different salinity treatments. Inert algae have been used to grow *Brachionus* efficiently (Lubzens et al., 1995; Yamasaki et al., 1989). To obtain inert algae, cultures of *T. suecica* were grown at 27.5 g/L salinity and 20 °C, which are intermediate conditions within the range of experimental conditions, and then were concentrated by centrifugation for 11 min at 1500 r.c.f. (relative centrifugal force) and maintained at −80 °C until use.

### 2.2. Effect of salinity on the hatching patterns of diapausing eggs

Differences between *B. plicatilis* and *B. manjavacas* in the hatching success of diapausing egg were tested in relation to salinity. The hatching of diapausing eggs produced at three different salinities (salinity for diapausing egg formation: 8, 16 and 24 g/L) and induced to hatch at the same three salinities (salinity for diapausing egg hatching) was recorded for the two species (18 experimental combinations: 3 egg-producing salinities × 3 egg-hatching salinities × 2 species).

Obtaining sufficient diapausing eggs for the experiments required the culture of rotifers at a high density in larger volumes than used for the stock cultures. For this purpose, the clonal rotifer stock cultures were gradually increased in volume. First, each stock culture was transferred to 250 mL of culture medium with 12 g/L salinity and grown at 20 °C, under constant illumination and agitation in an orbital incubator shaker at low speed (50 rpm). A salinity of 12 g/L was selected because this was an intermediate value among the three experimental salinities. After 7 days, six pre-experimental multiclonal populations (2 species × 3 salinities for egg formation) were established by combining females from the 25 stock clones. About 10,000–15,000 individuals from each clone were transferred to 2 L of fresh culture medium with salinities of 8, 16 and 24 g/L. After three days of acclimation, the pre-experimental populations were transferred to 5 L of culture medium to establish experimental populations under the corresponding conditions. As diapausing eggs tend to sink, to avoid accidental transfer of diapausing eggs, only the upper part of the culture was transferred. The experimental cultures were allowed to grow and produce diapausing eggs for 10 days. Then, the diapausing eggs accumulated at the bottom of the cultures were isolated. This collection of eggs was split to perform two experiments: the experiment described in this subsection and the experiment described in the next subsection.

In the first experiment, the eggs were kept at the same salinity as during egg formation, in the dark and at 4 °C for one month to ensure the completion of the required period of egg dormancy (Hagiwara and Hino, 1989). After this period, diapausing eggs were individually isolated in 96-multiwell dishes (Nunc$^{TM}$) and induced to hatch in water with salinities of 8, 16 or 24 g/L at 25 °C under constant illumination. For each of the 18 experimental combinations (2 species × 3 salinities for egg formation × 3 salinities for egg hatching), 40–60 eggs were assayed (total: 960 eggs). Diapausing eggs were checked every 24 h, and hatchings were recorded for 17 days.

The effects of the species, salinity for diapausing egg formation and salinity for diapausing egg hatching on the proportion of diapausing eggs that hatched were analysed using a generalised linear model (GLM) with binomial errors and a logit link function. The goodness of fit of the model was assessed with a Chi-square test. These analyses were carried out using SPSS statistical software (version 19.0, IBM Corp R, 2010). The effects of the species, salinity for egg formation and salinity for egg hatching on the timing of diapausing egg hatching were tested using a robust three-way ANOVA, performed with R statistical software, v. 2.12.1 (R Development Core Team, 2010), using the functions of the WRS package (Wilcox, 2010). Robust ANOVA was used due to the lack of homogeneity of variances.

### 2.3. Effect of salinity on diapausing egg deterioration

The deterioration of the diapausing *B. plicatilis* and *B. manjavacas* eggs produced at three different salinities (8, 16 and 24 g/L) was monitored for one year. Diapausing eggs were exposed to outdoor temperature conditions to mimic the conditions that the eggs experience in lake sediments.

Diapausing eggs from the two species produced at three salinities and collected as described in the previous subsection were used. For each experimental combination (2 species × 3 salinities for egg formation), 384 eggs were individually transferred to 96-multiwell dishes (NuncTM) containing 200 μL of 30 g/L saline water and maintained in wet chambers outdoors in the dark. High salinity and darkness were used to prevent egg hatching (Pourriot and Snell, 1983). The diapausing eggs were checked weekly for the first month, then monthly during the next 5 months and, finally, in the 9th and 12th months (11 observations in total). As light is a cue that induces hatching (Hagiwara et al., 1985) special care was taken during monitoring to minimise the exposure of the eggs to light. During each assessment, the diapausing eggs were classified according to their deterioration status, based on the proportion of the egg occupied by the embryo: Type I if the proportion occupied by the embryo is more than 100%; Type II if the proportion is more than 75% but less than 100%; Type III if the proportion is between 50 and 75%; Type IV if the proportion is between 25 and 50%; and Type V if the proportion is less than 25% (García-Roger et al., 2005). The relative contribution of each egg type to hatchlings found by García-Roger et al. (2005) was: Type I = 78.5%, Type II = 20.4%, Type III = 1.1%, and Type IV and V = 0.0%. In order to have a reasonable number of observations per category, in the subsequent statistical analyses these five egg types were grouped into viable (Type I and II) and degraded (Type III, IV and V).

The effects of the species and the salinities on the degradation status of the diapausing eggs were tested using a generalised linear model (GLM) with a multinomial distribution and logit link function. The goodness of fit of the model was assessed with a Chi-square test. The survival functions ($l_x$) for diapausing eggs were estimated for each rotifer species and salinity for egg formation in order to analyse the time to diapausing egg degradation. Here, survival means that the egg conserved the Type I or II status (i.e., viable eggs). Diapausing eggs that remain without degrading at the end of the experiment were treated as censored data. Despite the high salinity and dark conditions imposed on eggs, it was observed that some diapausing eggs hatched during the experiment (see the Results section), so these data were also considered censored. To compare survival curves between the (1) species and (2) the salinity treatments within each species, two non-parametric tests (log-rank and Breslow tests) were performed. The log-rank test assigns equal importance to all observations, while the Breslow test gives more weight to the initial part of the survival curve (Cox and Oakes, 1984). Statistical analyses were carried out using SPSS statistical software (version 19.0,IBM Corp R, 2010).

To test whether the hatching and degradation rates were related, Pearson's correlation coefficients between the two variables were calculated for each species. Additionally, the effects of the rotifer species and hatching rate on the degradation rate of the diapausing eggs were tested via ANCOVA. These statistical analyses were carried out using R statistical software, v. 2.12.1 (R Development Core Team, 2010).

### 2.4. Effects of salinity and temperature on growth rates

The combined effects of seven different salinities (5, 10, 20, 30, 40, 50 and 60 g/L) and three temperatures (15, 20 and 25 °C) on both the intrinsic population growth rate and sexual investment (i.e., the proportion of females reproducing sexually) in the *B. plicatilis* and *B. manjavacas* populations were studied. Pre-experimental multiclonal populations were established by combining females from the 25 stock clones in 250 mL flasks (approx. 1 female $mL^{-1}$ per clone). In the first step, these populations were grown for one week under intermediate conditions of salinity (27.5 g/L) and temperature (20 °C), with constant illumination to obtain a high rotifer density. In the second step, these multiclonal populations were used to initiate pre-experimental cultures at the experimental salinity and temperature conditions by transferring 200 rotifers that were randomly selected from the multiclonal populations to 100 mL flasks (42 cultures: 2 species × 3 temperatures × 7 salinities). These pre-experimental cultures were fed inert algae *T. suecica* to a density of 250,000 cells/mL every three days. The rotifers were allowed to grow for 10 days (approx. 3 generations) for acclimatisation. Then, experimental cultures were initiated by randomly transferring 20 females from each pre-experimental culture to Petri dishes with 50 mL of medium containing 100,000 cells/mL of inert algae (same salinity and temperature as in the pre-experimental conditions). Three replicate Petri dishes were included in the experiment (126 cultures: 2 rotifer species × 3 temperatures × 7 salinities × 3 replicates). Pre-experimental and experimental multiclonal cultures fed with inert microalgae were maintained under constant agitation in an orbital shaker (50 rpm) in the dark to avoid algal degradation. Both pre-experimental and experimental food concentrations were chosen to be above the daily clearance rate of rotifers during the period of culture, so food was not a limiting factor.

After 4 days of growth, the cultures were fixed with 0.3% Lugol solution, and rotifers were counted. The following data were recorded: (1) the number of non-ovigerous females; (2) the number of ovigerous females, classified based on the types of eggs they were carrying (Carmona et al., 1995) as asexual, unfertilised sexual or fertilised sexual; (3) the number of eggs (asexual, unfertilised sexual and fertilised sexual) both carried and detached; and (4) the number of males. Using these counts, the observed growth rate ($r_{obs}$) and sexual reproduction ratio (i.e. the fraction of sexual females to total females produced) at different salinities and temperatures were calculated. Population growth rate was calculated as $r_{obs} = \ln (N_t / N_0) / t$, where $N_t$ and $N_0$ are the number of females at the beginning and after 4 days of incubation, respectively, and $t$ is the time in days. The sexual ratio was calculated as the proportion of ovigerous females that were sexual.

Additionally, the potential growth rate ($r_{pot}$) was calculated (Montero-Pau et al., 2014). The $r_{pot}$ is defined as the growth rate that a population would have if all of its females were reproducing asexually. $r_{pot}$ is a performance measure that brings to a single metric the investment in current population growth and in sex and diapause. This makes the efficiency of converting resources into offspring and survival possible to compare (Montero-Pau et al., 2014), instead of the current proliferation rate. The relevance is that it neutralizes the effect of the diapausing investment strategy, which might have evolved by a suit of factors not related to the physiological efficiency of a genotype in the abiotic conditions of interest.

A robust three-way ANOVA was carried out to analyse the effects of the temperatures, salinities and rotifer species on $r_{pot}$. To test whether the investment in sex, i.e., the sexual reproduction ratio, responded to population density differently depending on species, an ANCOVA was performed independently for each experimental temperature. Both analyses were performed with R statistical software, v. 2.12.1 (R Development Core Team, 2010), using the functions of the WRS package (Wilcox, 2010) for the robust three-way ANOVA.

### 2.5. Niche overlap

Interspecific abiotic niche overlap was estimated based on the studied life-history traits using the analytical approach of Geange et al. (2011). This method allows the consideration of multiple niche axes, each of which is characterised by a different type of data, and computes a unified niche overlap analysis. The hatching success, degradation ratio and $r_{pot}$ datasets were used to calculate the abiotic niche overlap between *B. plicatilis* and *B. manjavacas* along the 42 axes detailed in Fig. 5. Following Geange et al. (2011), niche overlap indexes (*NO*) were calculated for each dimension, and a single unified niche overlap index was then obtained by averaging the niche overlap along each axis. *NO* ranges from 0 (disjoint niches) to 1 (complete niche overlap). For the unified niche overlap index, data on eight biotic axes from a previous study by Gabaldón et al. (2013) related to clearance rates, susceptibility to predation and ability to withstand starvation were included.

To assess the statistical niche differences between species, null model permutation tests were performed to test whether the niche overlap along each axis and the mean niche overlap were significantly lower than expected by chance (Geange et al., 2011; Gotelli and Graves, 1996). To correct for multiple comparisons, a sequential Bonferroni adjustment (Quinn and Keough, 2005) was performed. The niche overlap calculations and associated null model tests were performed using R version 2.12.1 (R Development Core Team, 2010) with the source code provided as supporting information in Geange et al. (2011).

## 3. Results

### 3.1. Effect of salinity on the hatching patterns of diapausing eggs

Diapausing *B. manjavacas* eggs showed a relatively synchronous pattern of hatching, with most of the hatchings occurring in the first 6 days of the experiment (Fig. 1). In contrast, the diapausing eggs of *B. plicatilis* continued hatching for up to 12–17 days of incubation. For both rotifer species, the synchrony of the hatching pattern increased with an increase in the salinity for egg formation. Mean time to hatching for diapausing eggs of both species (Table 1) showed statistically significant differences (Table 2), the values being 4.08 days for *B. plicatilis* and 1.6 days for *B. manjavacas*. Both salinity for egg formation and salinity for egg hatching were statistically significant as well as the interaction of species with both salinity for egg formation and salinity for egg hatching. A consistent trend was that hatching was delayed with an increased salinity for diapausing egg hatching, with the exception of eggs formed at the highest salinity, which showed a rather flat response (Table 1). High salinities during egg formation significantly decreased the time to hatching in *B. plicatilis.* Interactions between salinity for egg formation and salinity for egg hatching and the triple interaction were not statistically significant.

GLM analysis showed that the hatching proportion (Table 1) was significantly affected by the salinity for diapausing egg formation and the salinity for hatching, as well as by species × salinity for egg formation and species × salinity for egg hatching interactions (Table 2). However, statistical differences were not found between species, and both the interaction of salinity for egg formation × salinity for egg hatching and the triple interaction were not significant. Globally, the proportion of hatching of both species was higher at lower salinities for hatching. However, the salinity for diapausing egg formation had a species-specific effect. *B. plicatilis* presented a higher hatching proportion at higher salinity for egg formation, except when salinity for egg hatching was 8 g/L, in which case the hatching proportion at 24 g/L for egg formation was lower than at 16 g/L. Instead, *B. manjavacas* presented its highest hatching proportion at the lowest tested salinity for egg formation.

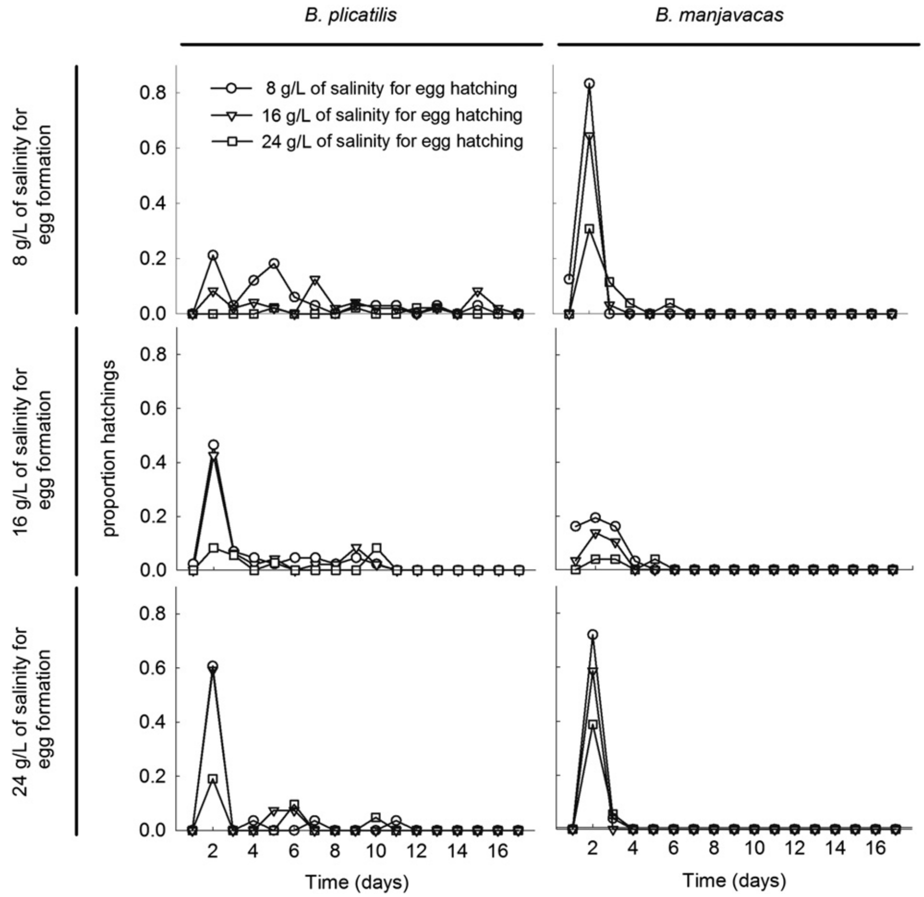

**Fig. 1.** The hatching dynamics of the diapausing eggs of *B. plicatilis* and *B. manjavacas* formed and hatched at 8, 16 and 24 g/L of salinity.

### 3.2. Effect of salinity on diapausing egg deterioration

Diapausing *B. manjavacas* eggs began to degrade during the first week of incubation, earlier than those of *B. plicatilis*, which did not show a notable deterioration until the 180 days of incubation (Fig. 2). Once deterioration began, the diapausing eggs of both species began to degrade at a high rate. According to the GLM analysis, the deterioration response is dependent upon the interaction between the species and salinity for diapausing egg formation ($P < 0.001$). The survival curves of diapausing *B. plicatilis* and *B. manjavacas* eggs formed under different salinity conditions differed remarkably between the species, and the log-rank and Breslow tests showed this difference to be statistically significant ($P < 0.001$). Both non-parametric tests performed independently for each species also revealed that salinity for egg formation affected the survival schedule of diapausing eggs ($P = 0.011$ and 0.010 for *B. plicatilis* and $P = 0.001$ and 0.007 for *B. manjavacas*; log-rank and Breslow test, respectively).

Although diapausing eggs were incubated in the dark to prevent the induction of hatching, 520 of the 2295 eggs included in these experiments hatched during the time course of the experiment (one year). No hatching of *B. plicatilis* eggs occurred within the first two months of incubation, with most of the hatchings for this species occurring after 180 days. In contrast, diapausing *B. manjavacas* eggs only hatched in the first 90 days of the experiment. For *B. plicatilis* the mean time to hatching (245, 261 and 241 days for 8, 16 and 24 g/L, respectively) was nearly coincident with the mean deterioration time (260, 257 and 259 days for 8, 16 and 24 g/L, respectively). This coincidence was not as clear for *B. manjavacas* (mean times to hatching were 146, 82 and 69 days and the mean deterioration times were 89, 89 and 124 days for 8, 16 and 24 g/L, respectively). For both rotifer

**Table 1**
The mean time to hatching (days) and proportion of hatching (%) of diapausing eggs of *B. plicatilis* and *B. manjavacas* formed and hatched under 8, 16 and 24 g/L of salinity.

| | | Salinity for diapausing egg hatching (g/L) | | | | | |
|---|---|---|---|---|---|---|---|
| | | 8 | | 16 | | 24 | |
| Salinity for diapausing egg formation (g/L) | Species | Mean time to hatching (days) | Hatching proportion (%) | Mean time to hatching (days) | Hatching proportion (%) | Mean time to hatching (days) | Hatching proportion (%) |
| 8 | *B. plicatilis* | 4.85 | 78.79 | 7.38 | 52.08 | 9.25 | 8.89 |
| | *B. manjavacas* | 1.37 | 95.83 | 1.55 | 67.74 | 2.19 | 50.00 |
| 16 | *B. plicatilis* | 3.07 | 81.82 | 3.26 | 70.21 | 4.72 | 27.03 |
| | *B. manjavacas* | 1.62 | 54.84 | 1.75 | 30.00 | 2.83 | 12.00 |
| 24 | *B. plicatilis* | 2.30 | 71.43 | 2.20 | 74.07 | 3.79 | 33.33 |
| | *B. manjavacas* | 1.55 | 76.00 | 1.50 | 58.62 | 1.63 | 44.44 |

**Table 2**
*P*-values after three-way robust ANOVA on the mean time to hatching (days) and after GLM analysis on the proportion of hatching (%) of diapausing eggs of *B. plicatilis* and *B. manjavacas* formed and hatched under 8, 16 and 24 g/L of salinity.

| | Mean time to hatching | Hatching proportion |
|---|---|---|
| Species | <0.001 | 0.794 |
| Salinity for diapausing egg formation | <0.001 | 0.004 |
| Salinity for diapausing egg hatching | 0.002 | <0.001 |
| Species × salinity for diapausing egg formation | 0.001 | <0.001 |
| Species × salinity for diapausing egg hatching | 0.022 | 0.027 |
| Salinity for diapausing egg formation × salinity for diapausing egg hatching | 0.14 | 0.169 |
| Species × salinity for diapausing egg formation × salinity for diapausing egg hatching | 0.1 | 0.989 |

**Table 3**
*P*-values after ANCOVA on degradation rate of diapausing egg of *B. plicatilis* and *B. manjavacas* produced under 8, 16 and 24 g/L of salinity and using egg hatching rate as a covariate. Data were collected during one year of monitoring.

| | *P*-value |
|---|---|
| Species | 0.001 |
| Hatching rate | <0.001 |
| Species × hatching rate | 0.49 |

species, there was a correlation between the daily hatching rate (hatchings per egg per day) and deterioration rate (eggs deteriorated per day). Pearson's correlation coefficients were 0.89 and 0.64 for *B. plicatilis* and *B. manjavacas*, respectively ($df = 28$, $P < 0.0001$ for both species). According to the ANCOVA with the hatching rate as a covariate, the deterioration rate was dependent on the species, and the correlation between the hatching and deterioration rates was confirmed, although this analysis did not detect a significant effect of interaction with the species factor on this correlation (Table 3).

### 3.3. Effect of salinity and temperature on growth rates

The observed growth rates ($r_{obs}$) and sexual reproduction ratios of the *B. plicatilis* and *B. manjavacas* differed when populations grow under the different experimental salinity and temperature combinations (Fig. 3). Within the tested ranges, temperature had a positive effect, and salinity tended to have a negative effect on $r_{obs}$. At 15 °C, the $r_{obs}$ values for both species decreased approximately linearly with salinity, while at the other tested temperatures, $r_{obs}$ tended to peak at an intermediate salinity, but in the low part of the tested range. Regardless of the global effects of these abiotic conditions, *B. manjavacas* always exhibited slightly higher growth rates than *B. plicatilis*. The mean sexual reproduction ratios were between 0 and 0.13 for both rotifers. In the range of 10–20 g/L, where the two species showed very close $r_{obs}$ values, *B. plicatilis* exhibited higher sexual reproduction ratios than *B. manjavacas* at 20 and 25 °C. *B. plicatilis* consistently exhibited a higher $r_{pot}$ than *B. manjavacas* within this salinity and temperature

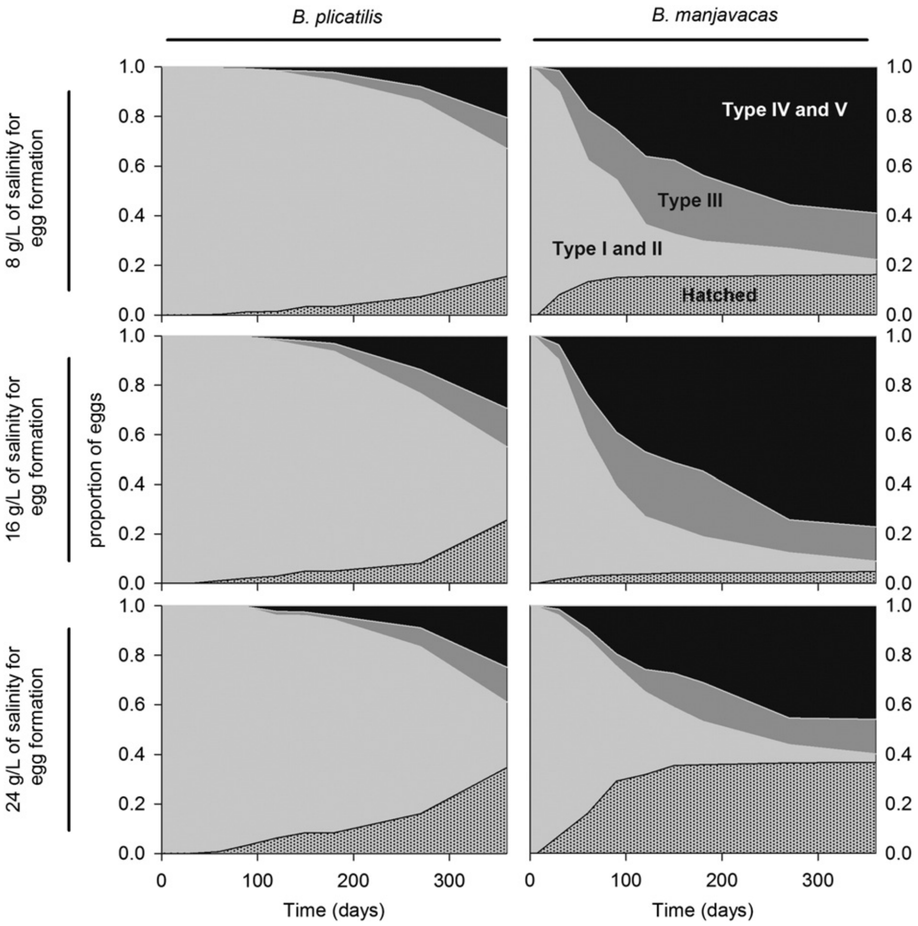

**Fig. 2.** Cumulative proportions of the different diapausing egg status of *B. plicatilis* and *B. manjavacas* along one year of exposure to natural conditions. Unhatched eggs were classified into five types (I–V) according to their degradation following the embryo-size criterion (García-Roger et al., 2005). The relative contribution of each egg type to hatchlings found by García-Roger et al. (2005) was: Type I = 78.5%, Type II = 20.4%, Type III = 1.1%, and Type IV and V = 0.0%.

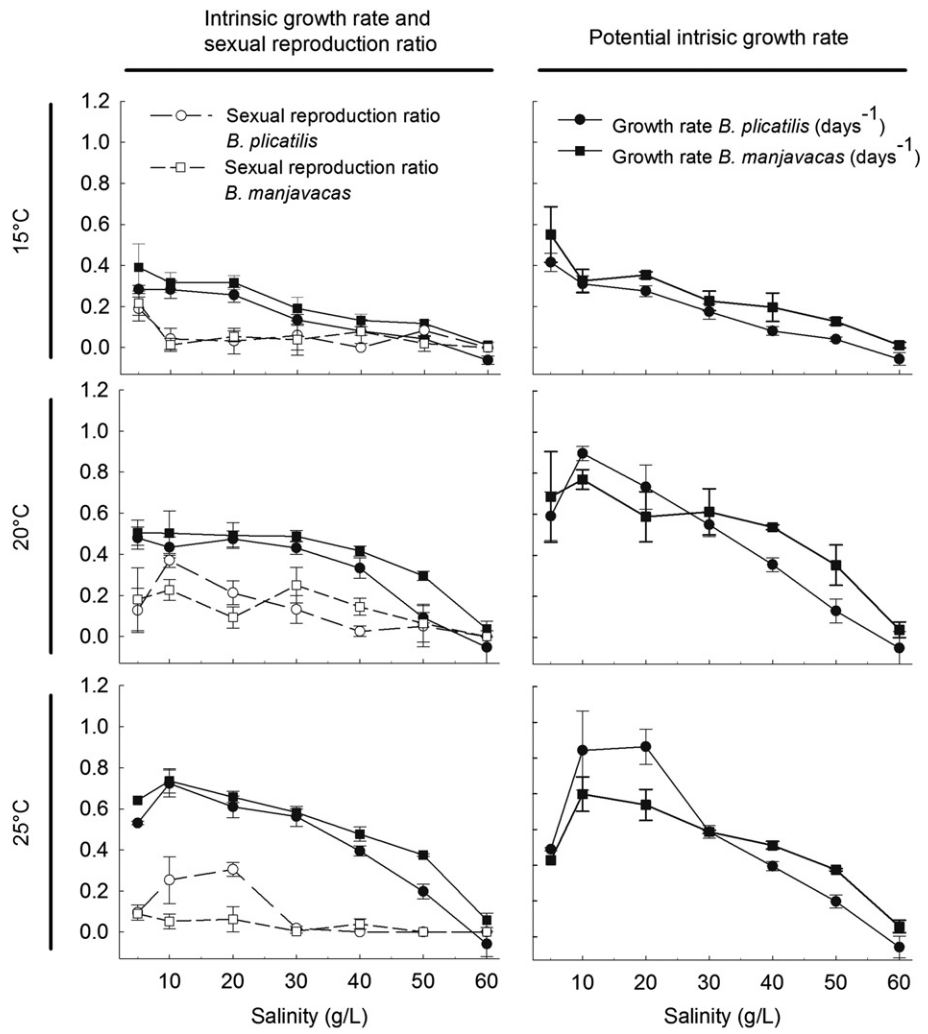

**Fig. 3.** Growth rates and sexual reproduction ratios of the *B. plicatilis* and *B. manjavacas* populations grown under seven salinities (5, 10, 20, 30, 40, 50 and 60 g/L) and three temperatures (15, 20 and 25 °C). Left panels: observed intrinsic growth rate and sexual reproduction ratio. Right panels: potential intrinsic growth rates. Vertical bars are the lower and upper 95% confidence intervals.

intervals (Fig. 3), and this difference became greater as the temperature increased. Robust ANOVA showed that the species, temperature and salinity as well as their double and triple interactions had an effect on $r_{pot}$ (all $P < 0.02$).

A positive relationship between sexual reproduction ratio and population densities of both rotifer species was observed, with *B. plicatilis* tending to present higher sexual reproduction ratios than *B. manjavacas* (Fig. 4). According to the ANCOVAs for the sexual reproduction ratios applied separately to each temperature (covariate: population density), the sexual reproduction ratio exhibited the expected significant relationship with population density (Table 4). At 15 and 20 °C, the species had no significant effect on the sexual reproduction ratio or the relationship between sexual reproduction and population density. In contrast, at 25 °C, *B. plicatilis* exhibited a significantly higher sexual reproduction ratio than *B. manjavacas,* with the former species showing a significantly stronger relationship between the sexual reproduction ratio and population density.

### 3.4. Niche overlap

The niches of *B. plicatilis* and *B. manjavacas* were found to be different (mean niche overlap = 0.51, *P*-value < 0.001) according to the niche overlap indexes (*NO*) between both species for abiotic axes studied in the present work and the biotic axes studied in Gabaldón et al., 2013 (Fig. 5). After sequential Bonferroni correction, the highest *NO* values were those associated with the hatching proportion axes, except for the hatching proportion of diapausing eggs formed at 8 g/L and hatched at 24 g/L and those both formed and hatched at a concentration of 16 g/L. Additionally, the *NO* values showed that *B. plicatilis* and *B. manjavacas* had similar niches regarding the axes for diapausing egg hatching timing, except for the three axes corresponding to the hatching timing of eggs formed at 8 g/L. The *NO* values from the diapausing egg degradation axes showed niche differentiation between the two species. For axes associated with $r_{pot}$, the *NO* values varied and were dependent on temperature: at higher temperatures, more of the niche axes showed low overlap between species.

## 4. Discussion

Co-occurrence of cryptic species is a common phenomenon (Bickford et al., 2007), and the rotifers *B. plicatilis* and *B. manjavacas* are a good example (Gómez et al., 2007). In these highly similar rotifers, the niche differentiation based on biotic factors (predation and resources) is low (Gabaldón et al., 2013), and their morphological similarity therefore translates into ecological similarity for these niche axes. Thus, the physical environmental factors addressed in the present study (temperature and salinity) were good candidates for playing a role in niche differentiation. In fact, differentiation between *B. plicatilis* and *B. manjavacas* based on salinity has been previously suggested by experimental and field observations (Montero-Pau et al., 2011).

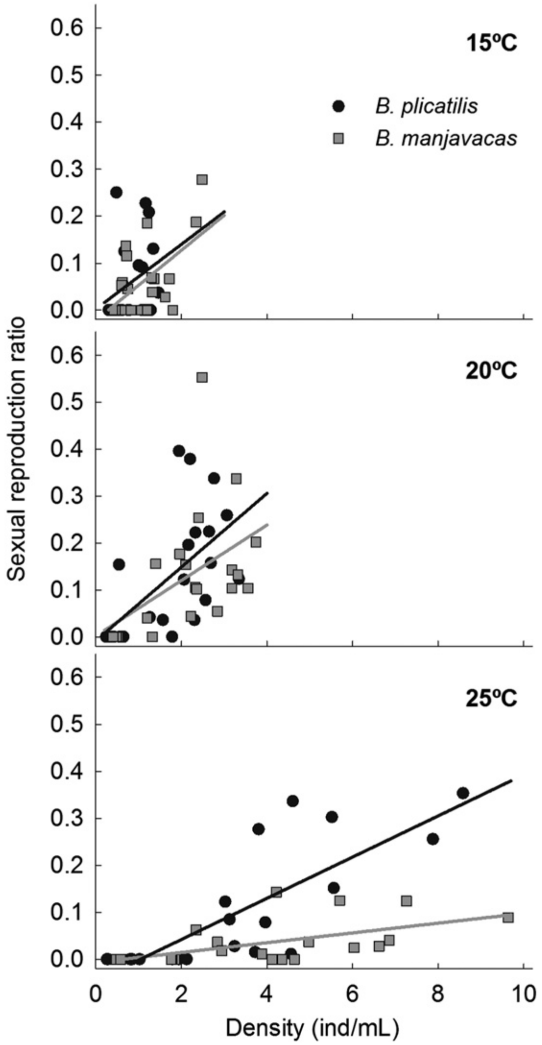

**Fig. 4.** The relationship between the sexual reproduction ratio and the population densities of *B. plicatilis* and *B. manjavacas* rotifer species, separately for the three experimental temperatures (data for the different salinities are not distinguished). Lines are linear regressions relating sexual reproduction ratios to population densities for each species and temperature.

The fluctuations of temperature and salinity in the natural habitats where these species co-occur are significant (Comín et al., 1992), and each species might therefore have the opportunity to be competitively dominant in a different period. In this work, the genotypes of *B. plicatilis* and *B. manjavacas* used were from the same pond to avoid a possible confounding effect due to local adaptation (Campillo et al., 2010). It was observed that these populations responded differentially to salinity and temperature in terms of several fitness components. Moreover, they consistently differed regarding life-history traits involved in diapause, from the investment in sex to the hatching of diapausing eggs. Differentiations of this kind have been invoked as mechanisms allowing stable species coexistence (Chesson, 2000; Montero-Pau and Serra, 2011). They are expected to work as stabilizing processes on the competition of these species, which on the other hand, show similar fitness, so that even weak stabilization might be sufficient to allow stable coexistence if it is actually occurring in the wild.

Some of the observed patterns are shared by the two species to some extent. First, our results show that the diapausing eggs of these species undergo significant levels of short-term deterioration. For instance, 37.6–94.2% of the eggs deteriorated in less than one year. The high ratio of deteriorated diapausing eggs detected in our experiment for these species is in agreement with the values found in the sediment egg bank of their habitat, Salobrejo Lake, where 88% of the eggs were identified as deteriorated (E.M. Garcia-Roger, personal communication). The clones studied here inhabit shallow ponds, which may dry out, so that our experimental conditions mimic the natural conditions at least of some dry years. This means that survival through an adverse period, from a growing season to a future time, might require a large investment in the production of diapausing eggs and, hence, in sexual reproduction. The correlation between hatching and deterioration rates was clear for *B. plicatilis* and suggests that deterioration is more likely to occur during embryonic development once diapausing egg hatching is induced and embryonic developmental arrest ends. The disruption of diapause could be associated with an increase in egg coat permeability, as observed in other taxonomic groups (e.g., Perry et al., 1983), which might make the embryo more vulnerable. Moreover, diapausing eggs are the result of sexual reproduction, and during the development of the embryo, new gene combinations are expressed for the first time in the individual. Thus, possible deleterious effects of these combinations would occur at this time. Inbreeding depression has been found to be relatively stronger in terms of diapausing egg viability (Tortajada et al., 2009), which determines the hatching of diapausing eggs, the first step of the asexual proliferation phase in the rotifer lifecycle. Our data also indicate how costly sexual reproduction can be, as sex can reduce the finite growth rate (i.e., exp ($r$)) of clonal proliferation by up to 38.2%.

Second, significant differences in diapausing egg quality (degradability and hatchability) related to salinity conditions during diapausing egg production were found. In rotifers, the sexual phase of the life cycle, which is the phase involved in diapausing egg production, can be more sensitive to environmental change than the asexual phase (Snell and Carmona, 1995; Snell et al., 1998). In agreement with this observation, our findings emphasise that the life-history traits involved in diapause are the most relevant to understanding the success of populations and their long-term persistence.

Third, regardless of the two studied species that have been reported to be active in the water column at salinities above 45 g/L salinity (Montero-Pau et al., 2011), salinity has an adverse effect on the growth of these animals, except close to the mesohaline range. The growth rate, investment in sex and diapausing egg hatching decline with salinity in the range explored in the present study. One caveat regarding hatching is that salinity might act as a cue rather than a constraining physical condition. If, as frequently observed (Gómez et al., 1995; Montero-Pau et al., 2011), salinity tends to increase during the growing season, low salinity might promote exit from diapause because it indicates that a suitable season has begun (e.g., heavy rainfall has refilled the lake), even if the optimal salinity occurring in that season is not the initial salinity. Nevertheless, the negative effect of salinity on performance is not surprising. Similar to many other invertebrates dwelling in saline lakes, both studied rotifer species are likely osmoregulators (Lowe et al., 2005), which entails metabolic costs. In fact, saline inland lakes, even being 47% of all lakes (Wetzel, 2001), show low species richness, which is typical in adverse conditions.

In contrast to these shared tendencies, the cryptic species *B. plicatilis* and *B. manjavacas* showed remarkable differences in their life-history traits related to diapause. First, *B. plicatilis* tended to show a greater propensity for sexual reproduction, although its higher investment in

**Table 4**
*P*-values of ANCOVAs on sexual reproduction ratio of *B. plicatilis* and *B. manjavacas* growing under 15, 20 and 25 °C using population density as a covariate.

| | Temperature (°C) | | |
|---|---|---|---|
| | 15 | 20 | 25 |
| Species | 0.913 | 0.967 | 0.002 |
| Population density | 0.003 | <0.001 | <0.001 |
| Species × population density | 0.912 | 0.598 | <0.001 |

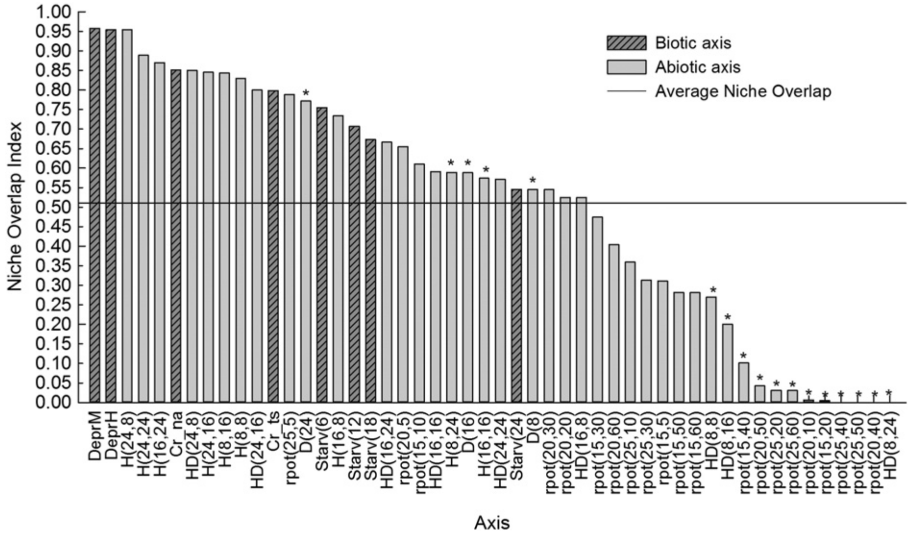

**Fig. 5.** Niche overlap indexes (*NO*) between *B. plicatilis* and *B. manjavacas.* From this study: *H*(*I,J*) is based on the hatching fate of each single diapausing egg (binary data), and *I* the salinity for egg formation and *J* the salinity for egg hatching; *HD*(*I,J*) is based on the hatching day of each single diapausing egg (categorical data; *I* and *J* as above); *D*(*I*) is based on the degradation status of each diapausing egg after one year of maintenance (categorical data; *I* as above); $r_{pot}(I,J)$ is based on the potential intrinsic growth rate at temperature *I* and salinity *J* (continuous data). From (Gabaldón et al., 2013): Cr_ts and Cr_na are based on the clearance rate for *T. suecica* and *Nannochloris atomus*, respectively (continuous data); DeprH are DeprM are based on the susceptibility to predation by copepod females and by copepod males, respectively (binary data); Starv(*I*) is the potential intrinsic growth rate after starvation, and *I* the hours of starvation (continuous data). Axis with statistically different niches, as identified by null model tests ($P < 0.001$), after the Bonferroni correction are indicated by asterisks.

sexual reproduction in response to population density was only statistically significant at 25 °C. As suggested by the differences between potential and actual growth rates, this pattern likely implies incurring in a cost by decreasing asexual proliferation. Such a high investment is expectable to evolve in response to environment uncertainty (Fussmann et al., 2003; Serra and King, 1999) or with growing seasons that are short due to, for example, early deterioration of the physical environment or competitive exclusion. Second, the females of this species produce diapausing eggs that show lower degradation rates. Diapausing eggs are more costly than subitaneous eggs (Alekseev et al., 2007; Clark et al., 2012; Gilbert and Schröder, 2004). If this extra cost is related to keeping viability, then it is expectable for more resistant diapausing eggs to be associated with fewer egg productions or to be produced at better environmental conditions, likely occurring early in the growing season. As the latter causes decreased environment exploitation, both cases imply a cost. Third, the diapausing eggs of *B. plicatilis* present a hatching pattern that is extended in time. This last feature has been proposed to be an opportunistic strategy associated with unpredictable environments (Pourriot and Snell, 1983). It is reasonable to infer that if eggs have a large time window in which to hatch, then this strategy will require diapausing eggs to be viable for long periods with low degradation rates. All these features fit in what is expectable for an opportunistic species.

In another study on *B. plicatilis* and *B. manjavacas*, Montero-Pau et al. (2011) observed a subtle differential effect of salinity on population growth and differences in the temporal distribution of these species in the water column, this temporal distribution being differentially associated with changes in salinity. Consequently, these authors suggested that salinity is a factor allowing these species to differentiate their niches. Accordingly, our robust ANOVA results showed that the potential growth rate of *B. plicatilis* was found to be more negatively affected by salinity than that of *B. manjavacas* (species × salinity interaction statistically significant) and that temperature significantly interacts with salinity in determining the growth rate. Additionally, high salinity conditions during diapausing egg formation appear to have a greater effect on the egg-hatching pattern of *B. plicatilis* than on that of *B. manjavacas*. In agreement with this observation, a dramatic decrease of mean niche overlap between these species was found when temperature and salinity were added to a number of biotic factors as axes for computing the *NO* index (from $NO = 0.78$ to $NO = 0.51$, (Gabaldón et al., 2013)). However, on the basis of the observed growth rates, there is no range of temperature and salinity combinations under which *B. plicatilis* grows faster than *B. manjavacas*, although these rates were very similar at the lowest tested salinity. Nevertheless, at the two highest temperatures tested, the higher performance of *B. plicatilis* at low salinity is clear when the investment in sex is taken into account and the comparison between species is based on the potential growth rates ($r_{pot}$). In other words, if neither species invests in sex, then *B. plicatilis* will exhibit higher clonal proliferation rates than *B. manjavacas* at low salinities, whereas the opposite pattern will occur at high salinities.

Our results show that the trade-off between diapause and current population growth is relevant in these species. *B. plicatilis* consistently showed a slightly lower observed growth rate ($r_{obs}$) than *B. manjavacas* at low salinity. Thus, the prediction is that, in the absence of sex and under a hypothetical scenario of constant low salinity, *B. plicatilis,* which has a higher performance (higher $r_{pot}$), would be able to exclude *B. manjavacas*. Notice that a difference in growth rate of 0.1 $\text{days}^{-1}$ would result in a two-fold population density difference between species in a week. By contrast, when the demographic cost of the higher investment in sex of *B. plicatilis* is considered, the expectation is that *B. manjavacas* would have an opportunity to increase according to the mechanism modelled in Montero-Pau and Serra (2011). Nevertheless, these assumptions are unlikely; the populations studied here inhabit in a highly variable environment, and they do always invest in sex. The inland pond where they were isolated, similar to many other ponds in the same region of the Iberian Peninsula, floods and dries out relatively regularly, hence determining the hydroperiod (length of the inundation phase) and causing variable salinity. Frequently, rainfall occurs in the region at only a few times, seldom in Summer, and in large amounts (Domínguez-Castro et al., 2008). In dry years, the salinity is high for long periods, such as the period studied by Montero-Pau et al. (2011), and especially during the hot season due to evaporation, while in years that are relatively wet, a period of low, but increasing salinity from the cold to the hot season, is expected to occur. This period of low salinity offers a window of opportunity for *B. plicatilis*, given its higher performance at low salinities. However, as suggested by our findings *B. plicatilis*, rather than proliferating at higher rates than *B. manjavacas* would use its higher performance during the low salinity periods to produce diapausing eggs, which would then be stored in the sediment.

These eggs appear to be suited to resist long diapause periods and to bet-hedge their hatching as a way to explore and take advantage of suitable low-salinity periods, an adaptive strategy if periods of low salinity will be scarce. In summary, *B. plicatilis* would represent an in-time fugitive species in this type of ecological system, and in the trade-off between leaving diapause synchronically (i.e., trusting the cues for habitat suitability), or extending diapausing egg hatching at a lower rate over the growing season, the latter was selected for.

Given the strong similarity between *B. plicatilis* and *B. manjavacas*, it is expected that they share major biological features in a greater extent than co-generic species. Life-history divergence has been previously documented within genus (Reznick et al., 2000). It is likely that the evolution of the resource allocation patterns shaping life histories is not strongly internally constrained because such evolution implies a diversion of resources into different sinks, rather than an increase of the resources acquired or the acquisition of a new function. The crucial idea here is that all relevant fitness components cannot be maximised simultaneously and that increasing one component results in a trade-off with other components. Thus, different species with very similar biology may evolve different compromises among life-history traits if they match with different niches. Hence, a special attention should be paid to life histories when studying the ecological divergence of closely related species.

According to phylogeographic studies, *B. plicatilis* and *B. manjavacas* have co-occurred in this region of the Iberian Peninsula since the last glaciation, 2.5 million years ago (Gómez et al., 2000, 2007), and paleolimonological data suggest that these species can co-occur in the same pond for at least decades (Montero-Pau et al., 2011). As these two rotifers show very similar performances, long-lasting transient exclusion of the inferior competitor or random walks cannot be ruled out. However, stable coexistence based on niche temporal differentiation is a plausible explanation. If so, this coexistence might be based on time fluctuations or in spatial variation, although phylogeographic studies suggest that effective migration between ponds is rather low (De Meester et al., 2002; Gómez et al., 2007). A storage effect (Chesson, 2000) might be functioning because (1) the physical environment experienced by these species fluctuates;(2) these species are specialised for this environment; and (3) the variance of the diapausing egg densities in the sediment layers shows the expected signatures if the rare species is relatively free of competition, i.e. higher variance in the diapausing egg recruitment of the rare species (Montero-Pau et al., 2011). Moreover, density-dependent investment in sex, rather than mediating coexistence in the water-column (Montero-Pau and Serra, 2011), appears to function as a way to effectively exploit short, unpredictable, beneficial periods. This would reinforce the storage effect by producing a stage of the lifecycle free of competition, i.e., the diapausing egg stage. Accumulative evidence makes this hypothesis a strong candidate to explain the long-lasting co-occurrence of these very similar species, and thus worthy of further investigation. This study has shown that salinity and temperature do decrease dramatically niche overlap, but other unstudied factors may work similarly.

Classical theories of niche differentiation have stressed the correlation between the niche and morphology (e.g. Fenchel, 1975). However, the consistent discovery of cryptic species challenges this classical view, creating an opportunity for the development of new stable coexistence mechanisms and for the use of these species as model organisms to test the limits of the ecological theory. It would be interesting to investigate if cryptic species are prone to differentiate their niches on abiotic factors, rather than on biotic ones, more related to resources acquisition and anti-predator defence, and evolving different life history options for traded-off traits. Cryptic species provide a perfect benchmark for identifying adaptations that might offer opportunities for niche differentiation associated with low morphological divergence and for studying the effects of life history traits on ecological features, without other confounding effects.

## Acknowledgements

This work was funded by a grant from the former Spanish Ministerio de Ciencia e Innovación co-financed with European Union FEDER funds (CGL2009-07364). C.G. was supported by a fellowship from the Spanish Ministerio de Ciencia e Innovación (BES2010-036384). We thank the anonymous referee for the careful reading of our manuscript and the supportive and valuable comments **[SS]**.

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