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Deterioration patterns in diapausing egg banks of *Brachionus* (Müller, 1786) rotifer species

Eduardo M. García-Roger*, María José Carmona, Manuel Serra

Institut Cavanilles de Biodiversitat i Biologia Evolutiva, Universitat de València, A.O. 22085, València 46071, Spain

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Abstract

Rotifers are cyclical parthenogens that produce sexual diapausing eggs at some stage in their life cycle. These eggs are encysted embryos that remain viable for extended periods in lake and pond sediments, thus acting as an egg bank with many ecological and evolutionary consequences. Despite its importance to rotifer evolution, there are no studies on resting egg deterioration and associated processes in natural environments. In this study, more than 4000 diapausing eggs of species from the *Brachionus plicatilis* complex, which includes several closely related cryptic species, were collected from different sediment depths in 15 ponds in eastern Spain and were classified according to three features thought to be related to their viability: shell integrity, embryo size, and embryo colour. A positive association was found between embryo size and hatching success in those eggs having an intact shell. Diapausing eggs that showed good shell integrity and no more than a 25% reduction of multinuclear embryo maximum size were classified as healthy and 98.9% hatched. Darkening of diapausing egg embryo was an indicator of viability loss. A decreasing frequency of healthy-looking diapausing eggs was observed with increasing sediment depth, although some exceptions were found.

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Keywords: Deterioration; Diapausing eggs; Rotifers; Viability

1. Introduction

Zooplankton populations of continental waters usually inhabit temporary and seasonally unsuitable habitats and hence produce diapausing eggs as adaptations to cope with the variation in their

environments. Most planktonic rotifers are cyclic parthenogens (see, e.g., Birky and Gilbert, 1971; Wallace and Snell, 1991), combining asexual reproduction in the absence of males (asexual phase) with occasional bouts of sexual reproduction (sexual phase). The results of sexual reproduction are cysts, the so-called resting or diapausing eggs, containing multinucleate diapausing embryos encased in a three-layered shell that protects them from external stressors, like desiccation and temperature extremes (Wurdak et al., 1978; Clément and Wurdak, 1991).

* Corresponding author. Tel.: +34 963543664; fax: +34 963543670.

E-mail address: eduardo.garcia@uv.es (E.M. García-Roger).

Development of the embryo is known to be arrested before the end of the mitotic phase of embryogenesis (Gilbert, 1983). Nutrient reserves (i.e., glycogen and lipid droplets) are also known to be contained in the diapausing embryo (Wurdak et al., 1978).

After production, diapausing eggs sink to the bottom and can remain viable in lake and pond sediments for several decades (Marcus et al., 1994; Kotani et al., 2001). When favourable conditions return, those eggs that have completed an obligatory period of diapause complete their development. However, not all of them hatch to reestablish the active population. A fraction remains viable and accumulates in the sediment forming resting egg banks, similar to the seed banks of plants.

Some ecological and evolutionary consequences of diapausing egg banks are (Templeton and Levin, 1979; Fryer, 1996; Hairston, 1996, 1998; Ortells et al., 2000) (1) diapausing eggs allow long-term population survival through unpredictable and unfavourable environmental conditions (e.g., series of bad years) by reducing local extinction risk; (2) they act as a genetic reservoir slowing down natural selection responses by the recruitment of old genotypes that may not be present in the current population; (3) they may act as a dispersal device (e.g., wind or animal-mediated); and (4) they contribute to community diversity maintenance through the “storage effect” [for example, temporal recruitment fluctuations allow the coexistence of competitors that otherwise would not coexist (Cáceres, 1997)].

The *Brachionus plicatilis* complex includes an undetermined number of closely related cyclical parthenogen rotifer species which are known to produce egg banks in coastal lagoons and endorheic ponds in Spain (Gómez et al., 1995, 2000; Gómez and Snell, 1996; Serra et al., 1998; Ortells et al., 2000).

Diapausing eggs of the rotifer species complex *B. plicatilis* are able to resist many adverse conditions, but they are not invincible. A decrease in rotifer diapausing egg viability with time has been reported (e.g., Pourriot et al., 1980; Balompapueng et al., 1997a,b). Studies on other zooplankton invertebrates have shown that only diapausing eggs in the upper (younger, in the absence of sediment mixing) sediment layers contribute to hatchlings (Herzig, 1985; Cáceres and Hairston, 1998). Most of the literature on zooplankton diapausing eggs in nature have focused

on recording data of maximum depth of occurrence in the sediment and survival times (Moritz, 1987; Marcus et al., 1994; Hairston et al., 1995; Katajisto, 1996; Weider et al., 1997), although hatching could be minimal from resting eggs a few centimeters below the surface because of deterioration processes.

Despite its importance for long-term population survival and evolution, knowledge of degenerative changes of diapausing eggs in the field and their relationship to sediment depth and viability in rotifers is nonexistent. Environmental factors affecting hatching proportion of diapausing eggs have been studied in *B. plicatilis* (Minkoff et al., 1983; Hagiwara et al., 1985, 1995; Hagiwara and Hino, 1989) and related species (*B. rotundiformis*, Hagiwara et al., 1989; *B. rubens*, Pourriot et al., 1980). However, they are limited to laboratory clones and address the effect of environmental factors of the water column (e.g., salinity, temperature, and food), while sediment conditions (e.g., anoxia, hydrogen sulphide concentration, sediment compaction, and depth and age of the sediment), which may affect diapausing egg viability, have not been yet studied.

The aims of this paper are (1) to analyse the morphology in view of the aspect of diapausing egg related to deterioration changes in natural conditions and (2) to study the viability associated with each deterioration stage described in relation to sediment depth. We hypothesize that diapausing egg aspect can be used to predict viability. A third goal is to describe the vertical distribution pattern of these degenerative changes in several ponds in eastern Spain. We analyze the relationship between the observed vertical pattern and both depth and drying pattern of the sampled ponds.

2. Methods

2.1. Collecting sites

Diapausing eggs of the *B. plicatilis* species complex were collected from the sediments of 15 ponds located in eastern Spain (Fig. 1), where a diapausing egg bank was previously detected (Ortells et al., 2000).

These ponds represent different habitat conditions as they differ in size (from 7×10^{-5} km² in Poza Norte

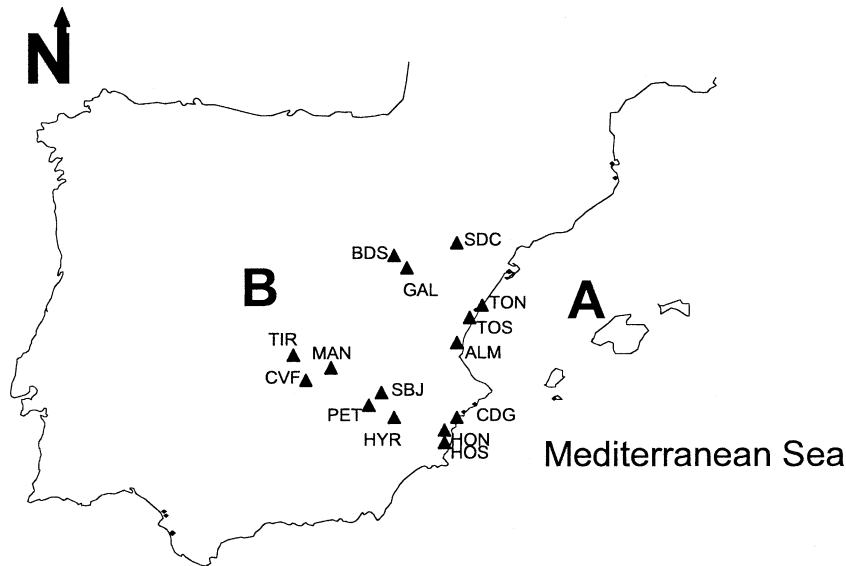


Fig. 1. Map of Spain showing the location of the 15 collecting sites. A: Coastal area: ALM, laguna de Almenara (shortcut: Almenara); CDG, Clot de Galvany; HON, Charca Norte, “El Hondo de Elche” Natural Park (shortcut: Hondo Norte); HOS, Charca Sur, “El Hondo de Elche” Natural Park (shortcut: Hondo Sur); TON, Poza Norte, Cabanes–Torreblanca Marsh; TOS, Poza Sur, Cabanes–Torreblanca Marsh. B: Inland: BDS, Balsa de Santed; CVF, Laguna del Camino de Villafranca (shortcut: Camino Villafranca); GAL, Laguna de Gallocanta (shortcut: Gallocanta); HYR, Hoya Rasa; MAN, Laguna de Manjavacas (shortcut: Manjavacas); PET, Laguna de Pétrola (shortcut: Pétrola); SDC, Salada de Chiprana; SBJ, Laguna del Salobrejo (shortcut: Salobrejo); TIR, Laguna de Tírez (shortcut: Tírez).

in Cabanes–Torreblanca Marsh to 13.3 km² in Gallocanta), depth (from 0.1 m in Hoya Rasa to more than 5 m of average depth in Salada de Chiprana), salinity (from nearly freshwater ponds, like Almenara to hypersaline, to more than 150 g l⁻¹ in Tírez), and drying pattern. Ponds with short and long but unpredictable hydroperiods as well as seasonal and permanent ponds were sampled in this study.

2.2. Sediment sampling

Sediment samples were taken with a piston core sampler (diameter: 63×57 mm, length: 60 cm; Eijkkamp Agrisearch Equipment). Three replicate samples were taken from three randomly chosen sampling stations in each pond during summer 2001 when most of the ponds studied were dry or exhibited low water levels. This timing was chosen to minimize the effect of diapausing eggs hatching which could give an unrepresentative sample of the bank.

Each core sample was divided into 2-cm-width slices until a maximum depth of 10 cm by means of a hydropneumatic discharge system that allowed an accurate partitioning. Slices were individually placed

in 100-ml plastic vessels and kept closed at 4 °C in the dark. Storage was for at least 2 months in order to ensure the completion of diapause in recently produced diapausing eggs and to obtain higher hatching rates as suggested by Hagiwara and Hino (1989).

2.3. Diapausing egg extraction

In order to isolate *B. plicatilis* species complex diapausing eggs from the collected sediments, we followed the sugar flotation technique described by Gómez and Carvalho (2000) with small modifications. This technique has been successfully utilized to collect zooplankton diapausing eggs by other authors (Onbé, 1978; Snell et al., 1983; Marcus, 1989; Katajisto, 1996; Næss, 1996; Ortells et al., 2000). Due to the fact that *B. plicatilis* inhabits high osmotic potential environments, the viability of diapausing eggs is thought to be unaffected by sucrose flotation technique. Sediment fractions of about 10 g (wet weight) were taken from the central area of each sediment slice in order to avoid any contamination by diapausing eggs from upper layers. This amount of sediment was resuspended in 1.75

mol l⁻¹ sucrose solution by homogenizing it as much as possible with a spatula. Resuspended sediment samples were then placed into 45-ml centrifuge tubes, filled with the sugar solution, and centrifuged at 12.6 ×g for 5 min. After centrifugation, the low-density material remaining in suspension (diapausing eggs were expected to be neutrally buoyant) was washed thoroughly through a 30-µm nylon mesh in order to remove finer sediment components and sucrose remains. The 30-µm retained material was transferred to a plankton counting chamber for *Brachionus* diapausing egg identification and isolation. When samples contained significant quantities of larger material, such as rootstalks, leaves, or insect parts, a prefiltering 200-µm device was used to remove this material. The contents of the 200-µm sieve were also checked for diapausing eggs under a stereomicroscope.

In some ponds, diapausing eggs belonging to other brachionid species (e.g., *Brachionus calyciflorus*, *Brachionus quadridentatus*) were also observed. These eggs were morphologically distinct from *B. plicatilis* eggs, and their taxonomical status confirmed after isolation and hatching.

The sugar flotation extraction is not 100% efficient as diapausing eggs were isolated after several repeated extractions on the same sample, but with diminishing efficiency. In order to increase sample sizes and to calculate reliable hatching fractions, the flotation process was repeated 2–4 times on each sample until no more eggs were extracted.

2.4. Diapausing egg classification and hatching procedure

Each individual diapausing egg was scored for three features thought to be related to viability: (1) shell integrity, as diapausing egg layers are believed to protect the embryo from adverse conditions, including environmental stress and mechanical injury during diapause, (2) embryo size (relative to maximum egg volume), as it is expected to be reduced due to deterioration and/or related to embryo reserve level, and (3) embryo colour, as bright and lighter colour is observed in freshly laid diapausing eggs in laboratory cultures. Only diapausing eggs that showed shell integrity and embryonic content were recognized as potentially viable diapausing eggs;

these eggs were expected to hatch and used to estimate viability. Embryo size was scored once the egg, and so the embryo, was rehydrated. Regarding embryo colour, three egg colour types were distinguished: orange, brown, and grey. Note that the egg colour was recorded before diapausing eggs were induced to hatch. Thus, unlike Minkoff et al.'s (1983) egg colour classification, ours is not related to developmental stage of the embryo after hatching induction.

Diapausing eggs with shell integrity were individually placed into 96-multiwell dishes (Nunc™) containing 200 µl of 6 g l⁻¹ artificial seawater (Instant Ocean®; Aquarium Systems) and incubated at 25 °C under constant illumination (150–170 µmol quanta m⁻² s⁻¹) up to 14 days. This experimental salinity has been proven to be suitable to induce diapausing egg hatching in the populations studied (Gómez et al., 2002). As some diapausing eggs did not hatch and continued to look healthy after this incubation period, individual eggs were transferred to a fresh culture medium at 2 g l⁻¹ salinity and incubated for 7 days more. Water was renewed every 2 days in order to prevent fungal infection. Cultures were checked for hatchlings at 12-h intervals.

Generalization of Fisher's Exact Test for independence for a R×C cross-table was performed by intensive calculation using R v.1.7.1 statistical software [R Development Core Team 2003 (Ihaka and Gentleman, 1996)] on each pond sampled in order to evaluate statistically whether there was any association between sediment depths and egg deterioration types. Inasmuch as data did not meet the assumptions for ANOVA, a nonparametric Kruskal–Wallis rank sum test was applied to determine if the time of hatching was affected by the diapausing egg type. Only data on diapausing egg types with sample sizes higher than 50 hatchlings were used in this analysis.

3. Results

3.1. Diapausing egg type and its relationship to viability

Diapausing eggs were classified into 18 different types (Fig. 2) according to shell integrity, embryo


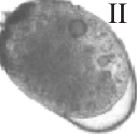



Type	Description	Embryo colour	Hatching rate (n)
 I	Double external layer (S1) and second shell layer (S2) both intact. Multinuclear embryo fills completely S2-delimited space.	Orange	0.631 (339)
		Brown	0.511 (407)
		Grey	0.029 (34)
 II	Multinuclear embryo fills more than 75%, but less than 100%, of S2 layer-delimited space.	Orange	0.556 (99)
		Brown	0.201 (274)
		Grey	0.000 (50)
 III	Multinuclear embryo fills about 50-75% of S2 layer-delimited space.	Orange	0.026 (114)
		Brown	0.007 (424)
		Grey	0.000 (57)
 IV	Multinuclear embryo fills about 25-50% of S2 layer-delimited space.	Orange	0.000 (203)
		Brown	0.000 (708)
		Grey	0.000 (107)
 V	Multinuclear embryo fills less than 25% of S2 layer-delimited space.	Orange	0.000 (179)
		Brown	0.000 (1178)
		Grey	0.000 (148)
Damaged RE	No damage is observed in the double external (S1). At least one of the following features is observed: (1) S2 layer is not observed. (2) Embryo traces are not observed.		
Hatched RE	Double external (S1) layer open <i>via operculum</i> .		
Broken RE	Double external (S1) layer shows fractures or discontinuities other than opened <i>operculum</i>		

Fig. 2. Morphological classification of *B. plicatilis* diapausing eggs, and its relationship to hatching rate. Egg morphology terminology is according to Wurdak et al. (1978). Embryo colour was recorded before incubation for hatching. Criteria for nonviable diapausing eggs typification are also indicated.

size, and embryo colour. Regarding shell integrity, a group of diapausing eggs, including three types (damaged, hatched, and broken diapausing eggs), was not expected to hatch and was not further analyzed. Thus, only the group of diapausing eggs that showed shell integrity and five different levels of embryo size were recognized as potentially viable diapausing eggs.

A total of 4321 diapausing eggs showing shell integrity were grouped according to two classification factors: embryo size and colour, independently

of sediment depth, sampling station, and pond. Results of hatching success for each type are shown in Fig. 2.

According to the embryo size classification criterion, we have identified five (I–V) major types of potentially viable diapausing eggs belonging to the *B. plicatilis* species complex. Globally, only a small fraction (12.5%) of the eggs hatched after the complete incubation period, and only one of those hatchlings occurred when diapausing eggs were transferred from 6 to 2 g l⁻¹ salinity. However,

differences were found for each diapausing egg type contribution to total hatchlings. Most of the hatchlings (78.5%) were obtained from type I diapausing eggs. Decreasing percentages over total hatchlings were obtained for subsequent categories. Hatchlings from type II diapausing eggs represented 20.4%, and hatchlings from type III represented 1.1%. No hatchlings were obtained from types IV and V. Hatching fraction of type I eggs was more than twice that for type II diapausing eggs, and type III hatching fraction was very low. Therefore, hatching success dramatically decreased with embryo size.

Type I and II diapausing eggs, those showing multinuclear embryo filling more than 75% of maximum embryo volume (see description in Fig. 2), can be considered as healthy-looking diapausing eggs due to their appearance, hatching fraction (Fig. 2), and contribution to total hatching success. Diapausing eggs belonging to type III represented an intermediate deterioration stage as they had a multinuclear embryo filling about 50–75% of maximum embryo volume and produced occasional hatchlings.

Type IV and V diapausing eggs have been considered nonviable diapausing eggs.

Hatching fractions were also calculated taking into account embryo colour (see Fig. 2). In those diapausing egg types where hatchlings were found (types I–III), orange eggs always showed higher hatching fractions than brown and grey types (Fig. 2). Only 1 of 396 grey diapausing eggs hatched after the incubation period.

Hatching timing analysis was restricted to those diapausing egg categories that showed higher hatching rates. Fig. 3 shows the timing of hatching for orange and brown diapausing eggs belonging to types I and II in the first 96 h of incubation. Note that, independent of embryo colour, type I diapausing eggs needed a longer time to reach 50% hatching than eggs belonging to type II. Furthermore, type II diapausing eggs showed a synchronous hatching pattern, reaching saturation after 72 h of incubation. Meanwhile, in type I (both orange- and brown-coloured) diapausing eggs, a few delayed hatchlings were observed until 228 and 216 h after the start of incubation, respectively. These differences on the

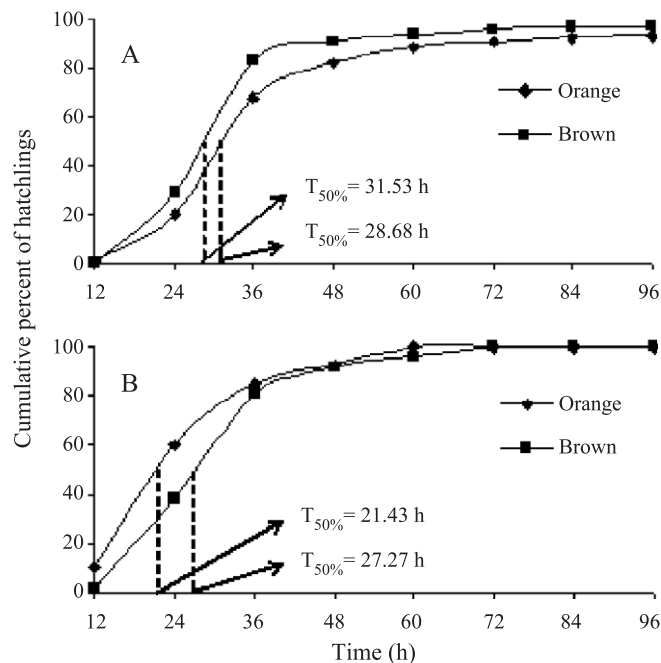


Fig. 3. Cumulative percent of *B. plicatilis* species complex hatchlings from type I (A) and type II (B) orange- and brown-coloured diapausing eggs after 96 h of incubation. Time when 50% of hatchlings was observed ($T_{50\%}$) is also included.

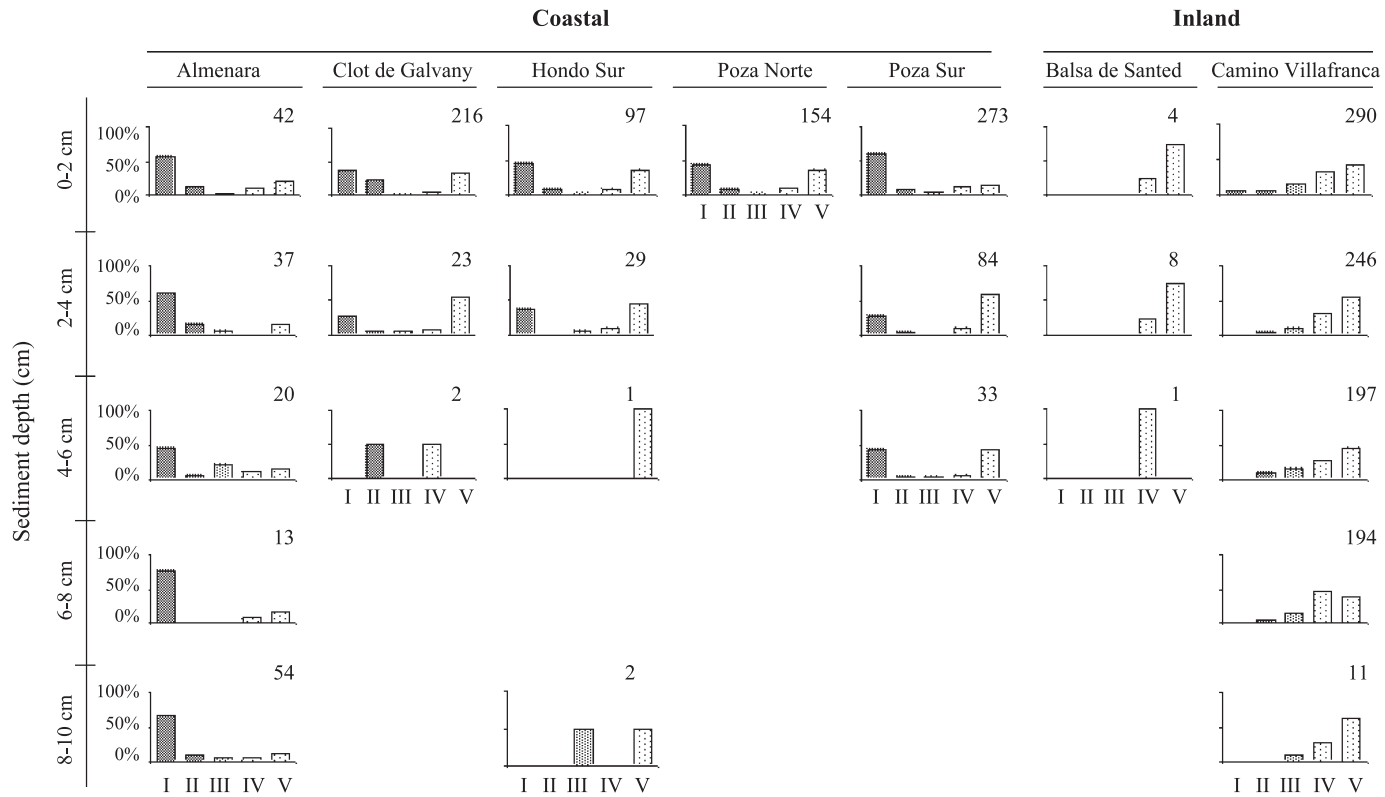


Fig. 4. Diapausing egg (type I–V) proportions at different sediment depths in each pond studied. Numbers indicate sample sizes. Colour decreasing pattern in the bars shows the relationship between diapausing egg morphological types and viability.



Fig. 4 (continued).

timing of hatching between egg types were statistically significant (Kruskal–Wallis $\chi^2=38.6541$, $df=3$, $P \ll 0.001$).

3.2. Diapausing egg type distribution with depth

The vertical distribution of the proportion of the five (I–V) major diapausing egg types in the upper 10-cm sediment samples of the different ponds is shown in Fig. 4. Data from different sampling stations in each pond were pooled due to low sample sizes. Ponds were classified as coastal or inland. Types I–V diapausing eggs were found in all but one pond, Hondo Norte, where only broken eggs and empty shells appeared in the samples. With this exception, coastal ponds showed higher proportions of healthy-looking diapausing eggs than those inland in almost all sediment depths.

As it can be seen in Fig. 4, in most ponds, the proportion of healthy-looking diapausing eggs decreased with sediment depth. A statistical test for independence showed that the proportion of diapausing egg types depended on sediment depth in all but four ponds (Almenara, Poza Sur, Hondo Sur, and Salobrejo) (Table 1).

4. Discussion

We have presented the first study on field-collected rotifer diapausing eggs relating appearance to physiological status (e.g., viability). Information on the physiological status of diapausing eggs is needed because physiological and biochemical processes affect survival, longevity, and the potential for future recruitment (Marcus and Boero, 1998; Tunnacliffe and Lapinski, 2003), which in turn have crucial ecological and evolutionary consequences. Most studies on rotifer diapausing egg viability have been performed on undamaged healthy-looking diapausing eggs obtained in the laboratory (Pourriot et al., 1980; Minkoff et al., 1983; Hagiwara and Hino, 1989, 1990; Hagiwara et al., 1989, 1995). Much less information is available on the ability of rotifer diapausing eggs to survive in natural environments for extended time periods (Marcus et al., 1994; Kotani et al., 2001).

Our data have shown that a negative relationship exists between embryo size reduction and hatching success. This indicates the expected association between diapausing egg degrading pattern and viability loss. Almost all hatchlings (98.9%) were obtained from healthy-looking (type I and II) diapaus-

Table 1
Fisher's Exact Test for independence on diapausing egg type and depth range contingency tables in each pond studied

Pond	Criteria		Fisher's Exact Test <i>P</i>
	Depth ranges (cm)	Types	
Almenara	0–2, 2–4, 4–6, 6–8, 8–10	(I+II), III, IV, V	0.324
Clot de Galvany	0–2, 2–4, 4–6, 6–8, 8–10	I, II, III, IV, V	0.018*
Hondo Sur	0–2, 2–4, 4–6, 8–10	I, II, III, IV, V	0.159
Poza Norte ^a			
Poza Sur	0–2, 2–4, 4–6	(I+II), III, IV, V	0.000**
Balsa de Santed	0–2, 2–4, 4–6	I, II, III, IV, V	0.452
Camino de Villafranca	0–2, 2–4, 4–6, 6–10	(I+II), III, (IV+V)	0.000**
Gallocanta	0–2, (2–6), (6–10)	(I+II), III, (IV+V)	0.000**
Hoya Rasa	0–2, (2–6), (6–10)	(I+II), III, (IV+V)	0.000**
Manjavacas	0–2, 2–4	I, II, III, IV, V	0.000**
Pétrola	0–2, 2–4, 4–6, 6–8	(I+II), III, (IV+V)	0.023*
Salada de Chiprana	0–2, 2–6, 6–10	(I+II), III, (IV+V)	0.000**
Salobrejo	0–2, 2–4, 4–6, 6–8, 8–10	I, II, III, IV, V	0.220
Tírez	0–2, 2–4, 4–6, 6–8, 8–10	I, II, III, IV, V	0.000**

Note that classes in parenthesis in rows (depth ranges) and columns (types) of some ponds were grouped because of computational requirements. *P* is the probability assumed to independence between diapausing egg type and sediment depth.

^a No independence test was performed on Poza Norte because depth criterium is a constant.

* Significant at $P < 0.05$ without Dunn–Šidák correction.

** Significant at $P < 0.004$ with Dunn–Šidák correction (Sokal and Rohlf, 1995).

ing eggs, but a few hatchlings were also obtained from diapausing eggs with 25–50% reduction in embryo size (type III). Although these hatchlings represented a small percentage of total hatchlings, hatching of type III eggs suggests that even a large loss of embryo mass may not affect survival. Expansion of some diapausing eggs with more than 50% of embryo size reduction (type IV–V) was also observed (personal observation), but no hatchlings were obtained after 21-day incubation period.

Embryo colour is another feature we found related to diapause egg viability. Orange eggs showed higher hatching fractions than brown eggs. This pattern holds independently of embryo size reduction (type I–III). The hatching fraction of grey diapausing eggs was negligible. This result is in agreement with Minkoff et al. (1983) who concluded that grey diapausing eggs can be considered dead eggs. Darkening and loss of brightness of the embryo have been interpreted as diapausing egg deterioration symptoms. In a previous study, Hagiwara and Hino (1990) also observed a reduction in hatchability from 91% to 60%, associated with darkening of the diapausing eggs produced in laboratory conditions by a clone of *B. plicatilis*. These authors suggested that colour differences in diapausing eggs may be related to diet; for instance, it has been shown that diapausing eggs accumulate carotenoids (Gilchrist and Green, 1962). Besides diet, carotenoid content may be affected by exposure time and conditions, and eggs would exhibit a less bright coloration, reflecting deterioration processes. As suggested by Hagiwara and Hino (1990), low carotenoid content would cause low hatching rates.

Our data show significant differences for the timing of hatching related to egg type. A faster and more synchronous hatching pattern was observed in type II diapausing eggs. This is surprising because a slower hatching response could be expected as diapausing egg deterioration increases. There are no data available to explain why faster hatching occurs in more deteriorated eggs. We hypothesize that egg permeability may increase with deterioration, and responses to environmental signals for hatching could be accelerated in those eggs that, although slightly deteriorated, are still able to hatch.

We have noticed that not all healthy-looking diapausing eggs hatch after incubation. Preservation, light, salinity, and temperature conditions used in this

study have proved to be efficient in previous hatching experiments on *B. plicatilis* (Minkoff et al., 1983; Hagiwara et al., 1985, 1989; Hagiwara and Hino, 1989; Gómez et al., 2002). However, it is difficult to ascertain whether eggs that do not hatch have lost their viability or whether hatching conditions were not adequate to induce hatching because of genetic differences among the diapausing egg population (King, 1972; Gilbert, 1974).

Our data show that the frequency of healthy-looking diapausing eggs changes with sediment depth in most of the ponds studied. Fisher's Exact Test showed the existence of link between egg type frequency and sediment depth in 9 of the 15 ponds studied. In these ponds, healthy-looking diapausing eggs are associated with upper sediment layers, and their frequency diminishes with sediment depth. It has been suggested that viable diapausing eggs should be less frequent with increasing sediment depth due to aging (deeper sediment layers are assumed to be older in the absence of sediment mixing). At the moment, we lack data on the age of the studied sediment layers as sedimentation rates are only available for Gallo-canta (Rodó et al., 2002). Average sedimentation rate reported for this lake is 0.31 ± 0.04 cm yr⁻¹, which means that deeper sediment slices may be 32 years old. Sediment dating of the studied ponds will be helpful to estimate age of the eggs, and it will be afforded in the near future.

Exposure to adverse environmental factors thought to cause mortality (e.g., low oxygen levels, increasing H₂S concentrations) could also explain increasing of proportion of deteriorated eggs with depth as it occurs in diapausing stages of copepods and cladocerans (Uye, 1980; Uye et al., 1984; Carvalho and Wolf, 1989). It is also possible that shell integrity and so embryo isolation from environment may be affected by some other extreme environmental conditions (i.e., compaction pressure while buried in the sediment, predation by nematodes or tardigrades that typically puncture cells to extract their contents). Shell deterioration could allow the colonization by bacteria, which would cause a reduction in embryo size.

While buried in the sediments, diapausing eggs are probably exposed to anoxia or low oxygen levels, two conditions that have been related to low diapausing egg viability (Uye et al., 1984; Lutz et al., 1994). Diapausing egg senescence might also occur if eggs

are not ametabolic. If so, the end products of metabolism would accumulate, and stored energy become exhausted. In other zooplankton resting stages, metabolic rates have been found to be dependent on oxygen conditions (Andrew, 1993; Clegg, 1997; Hand, 1998). To date, there is no report on metabolic rates of rotifer diapausing eggs, although the presence of lytic vacuoles in diapausing embryos in *Brachionus* has been related to metabolic activity (Wurdak et al., 1978). Our observation that eggs with smaller embryo size (relative to egg volume) showed lower viability is compatible with the idea that diapausing eggs are metabolically active and that this is a senescence factor.

Two of the fifteen ponds studied had to be excluded from our statistical analysis as potentially viable diapausing eggs were not found (Hondo Norte pond) or were restricted to the first layer studied (Poza Norte). In 4 of the 13 remaining ponds studied, the independence hypothesis of egg type with depth was not rejected. This fact could be explained in terms of several processes. Because it seems unlikely that sediment mixing occurs, we think that natural storage conditions may vary among sediment depths; this could explain the presence of non-healthy-looking diapausing eggs in top (young) sediments as upper layers may be forced to extreme conditions (desiccation, high temperature, etc.). However, alternative explanations arise when studying particular cases. For example, two of these ponds, Almenara and Salobrejo, are two of the deepest ponds studied (Almenara, Salobrejo, and Chiprana have a maximum depth higher than 3 m, whereas, in the other 12 ponds, it never reaches 1 m). It has been suggested that different hatching patterns may be expected in shallow and deep lakes (Gilbert and Schröder, 2004). Light exposure, high temperature, and oxygenation in shallow lakes may promote higher hatching rates than in deep lake sediments. If so, deteriorated diapausing eggs, unable to hatch, would tend to accumulate in the sediment of shallow ponds, while unhatched eggs would accumulate in deeper ponds and lakes. This process could explain the presence of healthy-looking diapausing eggs deep in the sediments of some of our deepest ponds.

Our results show that coastal ponds have higher proportions of healthy-looking diapausing eggs that

those inland. This suggests that inland pond conditions are worse for diapausing egg survival. Higher temperatures may be reached in inland ponds, and salty crust formations and a higher frequency of dry episodes might accelerate diapausing egg deterioration processes. Moreover, in the studied region, coastal pond margins exhibit richer vegetation than those in inland ponds. This fact might lead to a higher contribution of organic matter to sediments in coastal environments and thus to a higher ability for water retention and diapausing egg conservation.

Our study on the viability of diapausing eggs has implications for evolutionary aspects of the rotifer life cycle. The diapausing egg deterioration process is seen as a complex of physiological changes that occur in time, thus causing damage to vital functions or systems and resulting in viability loss and effective reduction of diapausing egg banks. Understanding the processes underlying viability loss and identifying those potentially viable eggs are critical to accurately estimate the diapausing egg bank size that can work as a source for possible future population recruitments. This is, in turn, important for long-term rotifer population survival. Deterioration processes cause viability loss, but other factors related to maintenance and repair cost could be involved. Hatchlings obtained from repaired diapausing eggs may be less fecund than hatchlings from healthy-looking diapausing eggs. A study, including life cycle measurements of hatchlings from diapausing eggs of different types, is needed to elucidate this relationship.

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