The role of diapausing eggs in zooplankton biodiversity and dispersal

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The role of diapausing eggs in zooplankton biodiversity and dispersal

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Memoria presentada por Emilio José Moreno Linares para optar al Grado de Doctor con Mención Internacional en Ciencias Biológicas por la Universidad de Granada. Esta memoria ha sido realizada bajo la dirección de Dr. José María Conde Porcuna y Dra. Carmen Pérez Martínez, Doctores en Ciencias Biológicas por la Universidad de Granada.

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A mi familia

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Resumen

Las masas de agua dulce continentales albergan una amplia diversidad de invertebrados acuáticos, entre los que se encuentran los tres grandes taxones que conforman en grupo zooplancton: cladóceros, copépodos y rotíferos. Las charcas y lagunas situadas en zonas Mediterráneas se encuentran sometidas a un elevado grado de temporalidad e impredecibilidad ambiental que repercute en cambios estacionales e interanuales en las comunidades de zooplancton. Bajo estas circunstancias muchas especies de zooplancton durante su ciclo de vida pueden entrar en fase de diapausa cuando las condiciones ambientales son adversas, como adultos o mediante la producción de huevos diapáusicos, permanecer viables durante largos periodos de tiempo y finalizar el periodo de diapausa cuando las condiciones vuelven a ser favorables en la columna de agua, permitiéndoles la recuperación de la población activa. Sin embargo, no todos los huevos diapáusicos eclosionan en el siguiente periodo de crecimiento (ante los estímulos que les indican la vuelta de las condiciones favorables), dejando una pequeña fracción de huevos diapáusicos en el sedimento que se van acumulando tras cada ciclo dando lugar a un banco de huevos diapáusicos. Los huevos diapáusicos presentan una serie de características especiales como son mecánica y resistencia a la desecación, que les permite resistir enterrados largos periodos de tiempo. Además esa serie de características facilita su transporte a través del aire por el viento o al tránsito por la ingestión por animales. La producción de huevos diapáusicos no sólo les permite dispersarse en el tiempo y hacer frente a eventos desfavorables, sino que también les permite dispersarse espacialmente, y en el caso de hacerse efectiva, colonizar nuevos hábitats. Para evaluar la eficiencia de la dispersión de los huevos diapáusicos del zooplancton, es importante señalar que la dispersión puede estar limitada por la viabilidad de los huevos diapáusicos. Además, la identificación de las señales apropiadas para provocar la eclosión de los huevos diapáusicos es de suma importancia para evaluar su viabilidad. Por tanto, es fundamental el estudio y caracterización de los bancos de huevos diapáusicos, así como conocer su capacidad de supervivencia y dispersión.

Se ha comprobado que la caracterización de los bancos de huevos diapáusicos como herramienta para conocer la biodiversidad de sistemas acuáticos ha sido muy efectiva. Principalmente, porque el banco de huevos diapáusicos actuaría como reservorio de la diversidad de especies y poblaciones de un sistema acuático. Sin embargo, la identificación taxonómica a partir de los huevos diapáusicos es muy limitada, debido principalmente a la amplia variedad de morfologías y ornamentaciones que presentan entre las especies de los mismos grupos. Una alternativa han sido los experimentos de eclosión, que han permitido la caracterización del banco de huevos diapáusicos a través de identificación de los individuos eclosionados. Pero presenta limitaciones como que los factores que inducen la eclosión varían entre especies, e incluso entre individuos de la misma especie, y que no todos los huevos diapáusicos eclosionan al haber completado su ciclo de diapausa. Una alternativa es el uso de técnicas moleculares como el DNA barcoding que posibilita la correspondencia de los huevos diapáusicos haciendo innecesario el procedimiento de incubación del sedimento. En el Capítulo 1 de la presente tesis caracterizamos el banco de huevos diapáusicos de rotíferos de las lagunas Dulce y Santa Olalla localizadas en el Parque Nacional de Doñana, y *Tinaja* en el Parque Natural de Ruidera mediante la técnica de

DNA barcoding. Además, se creó una lista de referencia de rotíferos adultos de la columna de agua usando la misma técnica, y que serviría de base de referencia para las correspondencias con los huevos diapáusicos. En las tres muestras de sedimento correspondientes a cada laguna analizadas se consiguió un alto número de correspondencias facilitando la identificación de los huevos diapáusicos en la mayoría de los casos a nivel de especie. El uso de herramientas de análisis de secuencias que permiten la delimitación de especies permitió la detección de potenciales especies crípticas en nuestras muestras. La disponibilidad de bases de datos de ADN como GenBank facilita la comparación con las secuencias con las que estemos trabajando. Por tanto, la aplicación del DNA barcoding como herramienta integral para el estudio de la biodiversidad de las especies de zooplancton reduce el esfuerzo de muestreo y permite cuantificar toda la biodiversidad con la identificación de potenciales especies crípticas.

Evaluar y cuantificar los distintos vectores de dispersión de los huevos diapáusicos del zooplancton puede ayudarnos a entender la dinámica y composición de las poblaciones de los sistemas acuáticos. Los huevos diapáusicos pueden dispersarse mediante vectores abióticos como el viento, la lluvia o los flujos de agua, o a través de vectores bióticos como las aves acuáticas o animales que frecuenten las lagunas o charcas en las que se encuentren los huevos diapáusicos. En el Capítulo 2 evaluamos y cuantificamos la dispersión por el viento de propágulos diapáusicos de dos localizaciones con características limonológicas y orográficas diferentes, Doñana y Ruidera. Hasta el momento no se ha tenido en cuenta la dispersión por el viento desde el punto de vista de la deposición o sedimentación pasiva, discriminando entre lo que podríamos denominar "deposición seca" de los propágulos que se dispersan por al aire impulsados por el viento y depositados de una manera pasiva, y "deposición húmeda" de los propágulos que igualmente se dispersarían por el viento pero que su deposición ya no sería pasiva al estar facilitada por la acción de la lluvia. Para ello usamos colectores automáticos de deposición atmosférica capaces de discriminar entre deposición seca y deposición húmeda. Entre las muestras se encontraron huevos diapáusicos de rotíferos y ostrácodos, efípios de cladóceros, estatoblastos de briozoos y rotíferos bdelloideos en estado de anhidrobiosis. La cantidad de propágulos latentes dispersados recogidos en los colectores localizados en Doñana fue más alta que el que se instaló en Ruidera. La cantidad de huevos diapáusicos recogidos en los colectores de Doñana fue significativamente mayor en la deposición seca y se concentraron en los meses posteriores al verano. Estos resultados estarían indicándonos que la dispersión por el viento podría verse facilitada si la zona en la que se encuentra el banco de huevos diapáusicos queda desnuda y directamente disponible al efecto del viento. A pesar de que las cantidades de huevos diapáusicos recogidos en los colectores fueron escasos, su presencia refuerza la idea de la importancia que puede tener la dispersión por el viento y su potencial en la colonización a largo plazo.

Las aves acuáticas que habitan charcas y lagunas someras son un importante vector de dispersión de propágulos diapáusicos, siendo transportados en las plumas o alojados en el tracto digestivo, y en ambos casos sin pérdida de viabilidad. Por el momento todos los estudios de dispersión de propágulos diapáusicos de invertebrados acuáticos se han centrado en crustáceos, y tan sólo unos pocos han considerado los rotíferos, aunque de forma colateral, sin un suficiente

grado de resolución taxonómica y sin evaluar la capacidad de eclosión de huevos aislados de las heces. En el Capítulo 3 se aislaron e identificaron los propágulos diapáusicos de cladóceros y rotíferos encontrados en las heces de aves acuáticas localizadas en las lagunas de Dulce y Santa Olalla en el Parque Nacional de Doñana. También se evaluó su capacidad de supervivencia bajo condiciones ambientales locales. Los resultados mostraron unas tasas de dispersión muy superiores a las registradas por el viento, especialmente por el grupo de los rotíferos. Por tanto, la dispersión de los huevos diapáusicos por aves puede considerarse un mecanismo de dispersión relevante, especialmente para los rotíferos.

La resiliencia de las comunidades de zooplancton dependerá de la viabilidad de los huevos diapáusicos y de las condiciones ambientales que induzcan la eclosión. Distintos estudios han mostrado o sugerido que la concentración de oxígeno, los niveles de alimento, el fotoperiodo, la temperatura y/o la presencia de luz controlan la eclosión de los huevos diapáusicos de distintas especies de zooplancton. Otros factores como la salinidad se ha comprobado que algunas especies presentan un rango de tolerancia bastante amplio. Sin embargo, no se han hecho estudios sobre los efectos de la salinidad sobre la eclosión a distintas temperaturas, aislando los huevos de resistencia, y/o sobre la eclosión de huevos dispersados. En el Capítulo 4 abordamos las posibles capacidades de colonización de los huevos diapáusicos dispersados por aves. Además, se estimaron las tasas de eclosión de los huevos y efípios encontrados en los sedimentos de las distintas lagunas bajo diferentes condiciones de salinidad, temperatura y fotoperiodo. Los resultados mostraron que la capacidad de colonización de los huevos diapáusicos de rotíferos es dependiente de las concentraciones de salinidad y la dispersión por aves acuáticas, siendo la respuesta de los rotíferos a la eclosión dependiente de la temperatura. Además, el éxito de eclosión de algunas especies de rotíferos se ve favorecida por la ingestión de los huevos diapáusicos por aves acuáticas. Por tanto, ante las expectativas de incrementos en la salinidad y la temperatura de los sistemas acuáticos de agua dulce mediterráneos debido al cambio climático podría tener consecuencias sobre los patrones de colonización de las especies de rotíferos.

Los flujos de agua podrían considerarse un vector de dispersión que facilitaría el transporte tanto de huevos diapáusicos como de individuos que habitan la columna de agua, ya que los invertebrados de sistemas lénticos no suelen oponer resistencia a las corrientes de agua. Otro factor importante sería que al igual que la dispersión por aves acuáticas el transporte se produce de un cuerpo de agua a otro cuerpo de agua. En el Capítulo 5 estudiamos la diversidad de la comunidad de zooplancton de Santa Olalla y Dulce en Doñana y Tinaja y Morenilla en Ruidera. Los resultados mostraron que las especies más abundantes también fueron las que se encontraron en mayor número entre los huevos diapáusicos dispersados e identificados. Teniendo en cuenta en el caso de las lagunas de Ruidera un punto de conexión entre ellas comprobamos que no existían diferencias en cuanto a la composición de especies entre Tinaja y Morenilla, en cambio el punto de conexión la comunidad de zooplancton era ligeramente diferente. Posiblemente porque actúan como un río que conecta ambos puntos unificando las comunidades de zooplancton. En Doñana, entre las lagunas de Santa Olalla y Dulce suele producirse una conexión por inundación cuando las precipitaciones son muy altas. Este fenómeno también

permitiría el transporte de huevos diapáusicos. Por tanto, estas conexiones intermitentes podrían determinar la dinámica y estructura de las poblaciones de zooplancton que se encuentren próximas.

Entender las tasas y los mecanismos por los que el zooplancton se mueve entre hábitats tiene mucha importancia para comprender como las poblaciones de zooplancton se recuperan tras sufrir perturbaciones naturales o antropogénicas e interpretar los resultados que se obtengan de manipulaciones experimentales en sistemas acuáticos. Los resultados obtenidos muestran la importancia relativa de los mecanismos de dispersión de los propágulos de resistencia del zooplancton y su posible impacto en la biodiversidad de sistemas acuáticos. Las consecuencias de la dispersión del zooplancton sobre las comunidades lacustres y los bancos de huevos de resistencia pueden evaluarse en términos de la conservación de la biodiversidad y manipulación de sistemas acuáticos. Las predicciones sobre las tasas de recuperación de comunidades alteradas después de diferentes perturbaciones pueden ser más fiables estudiando los mecanismos por los que los organismos del zooplancton se mueven entre hábitats.

Summary

Inland freshwater bodies host a wide variety of aquatic invertebrates. The ponds and lagoons located in Mediterranean areas are subject to a high degree of temporality and environmental unpredictability that affects seasonal and interannual changes in zooplankton communities. Under these circumstances many zooplankton species during their life cycle may enter the diapause stage when environmental conditions are adverse, such as adults or through the production of diapausal eggs, remain viable for long periods of time and end the diapause period when conditions are again favorable. However, not all diapausing eggs hatch before the stimuli that indicate the return of favorable conditions, leaving a small fraction of diapausing eggs in sediment. This accumulation of diapausing eggs from different cycles is known as diapausing egg bank. Therefore, its characterization is fundamental, as well as knowing its capacity of survival and dispersion. To evaluate the efficiency of zooplankton dispersal, it is important to note that the dispersion may be limited by the viability of diapausing eggs. The identification of appropriate signals for hatching of diapausing eggs is of crucial fo importance in assessing such viability

The zooplankton dispersal capability may regulate the population dynamics and aquatic community structure and play a key role in the colonization of new water bodies. Zooplankton is potentially dispersed overland by abiotic vectors like wind, rain and water flow, or by organisms like waterfowls or insects. Although many studies have studied the dispersal vectors, quantitative measures of zooplankton dispersal have been rare and mainly focused on waterfowls. Unfortunately, little is known about dispersal rates by wind or rain due to the difficult to measure it. In chapter 2 we quantify the zooplankton propagules dispersal by wind and rain using two atmospheric deposition collectors, which permit to collect samples from dry atmosphere deposition and wet atmosphere deposition. Two of these collectors were placed beside the lakes Dulce and Santa Olalla, eutrophic lakes in Doñana Natural Park, and a third one was located close to the lakes Tinaja and Morenilla, oligotrophic lakes in the Ruidera

Natural Park. As the collectors located in Doñana gathered more quantity of zooplankton propagules, we built two nylon wind socks beside these collectors for measuring the particulates blowing in the wind. The results shown differences in the number and the species propagules collected between the lakes of different location. The higher abundance of dormant propagules was registered in the wind socks, but the presence of them in both collectors suggested the importance of the aerial dispersal. Our results suggest the relative importance of wind and rain as dispersal mechanisms of the zooplankton dormant propagules and their impact on the biodiversity of aquatic systems.

Waterfowl that inhabit ponds and shallow lagoons are an important vector of dispersion of diapseal propagules, being transported in the feathers or lodged in the digestive tract, and in both cases without loss of viability. At present, all studies of dispersal of aquatic invertebrate diabetic propagules have focused on crustaceans, and only a few have considered rotifers, although collaterally, without a sufficient degree of taxonomic resolution and without evaluating the hatchability of eggs isolated from feces. In Chapter 3, cladoceran and rotifers dispersed diapausing eggs found in feces of waterfowl located in *Dulce* and *Santa Olalla* lakes in the Doñana National Park were isolated and identified. Their ability to survive under local environmental conditions was also evaluated. The results showed dispersion rates much higher than those recorded by the wind, especially by the group of rotifers. Therefore, the dispersal of diapusa eggs by birds can be considered a relevant dispersion mechanism, especially for rotifers.

Salinity is becoming a serious threat to freshwater ecosystems due to global change, and it is known as a driving factor determining the presence and dominance of aquatic organisms. Zooplankton may produce resting eggs, which maintain its viability during long periods, being the egg banks research of interest to many biological phenomena, such as migration from the past, dispersal, temporal heterogeneity and biodiversity. In Chapter 4, the effects of salinity on zooplankton are mainly investigated by inducing hatching of zooplankton resting eggs. These eggs were isolated from surface sediment samples in lakes Santa Olalla and Dulce (Doñana National Park, Spain). To study factors affecting hatching rates, resting eggs were incubated at two temperatures (15 and 25 ^{o}C) under a gradient of salinity (0.3-9 psu), which includes salinities that were usually observed in these lakes. Additionally, the temporal dynamic of zooplankton in the lakes during several years was studied. Our results showed that salinity concentration affect the hatching rates of rotifers, but this effect was species-specific and could be modified by temperature. Moreover, we observed differences in the hatching response of zooplankton regarding of the origin of the eggs and, in some cases, the day of hatching was positively related with salinity. These results will be discussed with the dynamic of zooplankton in these lakes. The potential contribution of the resting eggs bank to freshwater zooplankton resilience after increased salinity will also be considered.

Water flows mighy be considered a dispersion vector that would facilitate the transport of both diapausing eggs and individuals which inhabit the water column, since the invertebrates of lentic systems do not usually resist water currents. Another important factor can be that like the dispersion by aquatic birds the transport takes place of a body of water to another body of water. In Chapter 5 we studied the diversity of the zooplankton community of *Santa Olalla* and

Dulce in Doñana and *Tinaja* and *Morenilla* in Ruidera. The results showed that the most abundant species were also found in the largest number among the dispersed and identified diapausing eggs. Taking into account the point of connection in Ruidera, *Hundimiento*, lakes we verified that there were no differences in the composition of species between *Tinaja* and *Morenilla*, in contrast in the point of connection the community of zooplankton was slightly different. Possibly because *Hundimiento* acts as a river that connects both points unifying the zooplankton communities. In Doñana, between *Santa Olalla* and *Dulce* lakes, a flood connection occurs when the precipitation is very high. This phenomenon might acts as a vector for diapausing eggs. Therefore, these intermittent connections may determine the dynamics and structure of nearby zooplankton communities.

Understanding the rates and mechanisms by which zooplankton moves between habitats is very important in understanding how zooplankton populations recover after natural or anthropogenic disturbances and interpret results obtained from experimental manipulations in aquatic systems. The results obtained show the relative importance of the dispersal mechanisms of zooplankton diapausing propagules and their possible impact on the biodiversity of aquatic systems. The consequences of dispersal of zooplankton on communities and diapausing egg banks can be assessed in terms of biodiversity conservation and aquatic systems manipulation. Predictions about recovery rates of disturbed communities after different disturbances may be more reliable by studying the mechanisms by which zooplankton organisms move between habitats.

Introducción general

El zooplancton de los sistemas acuáticos continentales engloba tres subgrupos principales (cladóceros, copépodos y rotíferos) cuyas especies, en su conjunto, forman parte del grupo más diverso de invertebrados acuáticos (Segers, 2008). Dentro del zooplancton, los cladóceros y rotíferos se caracterizan por presentar una reproducción partenogenética bajo condiciones favorables. Cuando pasan a ser desfavorables, tiene lugar la reproducción sexual en la que las hembras producen huevos haploides que tras ser fecundados dan lugar a huevos de diapáusicos o de resistencia que, en el caso de muchos cladóceros, quedan protegidos en la estructura llamada efipio. En algunos casos, la formación de huevos diapáusicos tiene lugar de forma asexual en poblaciones partenogenéticas obligadas tanto de cladóceros como de rotíferos (Conde-Porcuna et al., 2011; Stelzer, 2011). Estos huevos diapáusicos quedan en estado latente enterrados en el sedimento de los medios acuáticos hasta que las condiciones ambientales vuelven a ser favorables, momento en que eclosionan iniciándose nuevamente el ciclo partenogenético de reproducción. Una vez completado el periodo refractario de diapausa obligada sólo una fracción de los huevos diapáusicos completa su desarrollo del embrión hasta su eclosión de manera simultánea, y otra fracción de los huevos diapáusicos permanecen enterrados en el sedimento en estado de latencia, y aún viable, cuya acumulación tras numerosos ciclos forma un banco de huevos diapáusicos similar a los bancos de semillas de las plantas (Carvalho y Wolf, 1989; De Stasio, 1989; Hairston et al., 1996; Cáceres, 1998). Por tanto, podríamos distinguir una fase activa en la que la población ocupa la columna de agua, y una fase latente en la que la población se acumula en el sedimento en forma de banco de huevos diapáusicos (Figura 1).

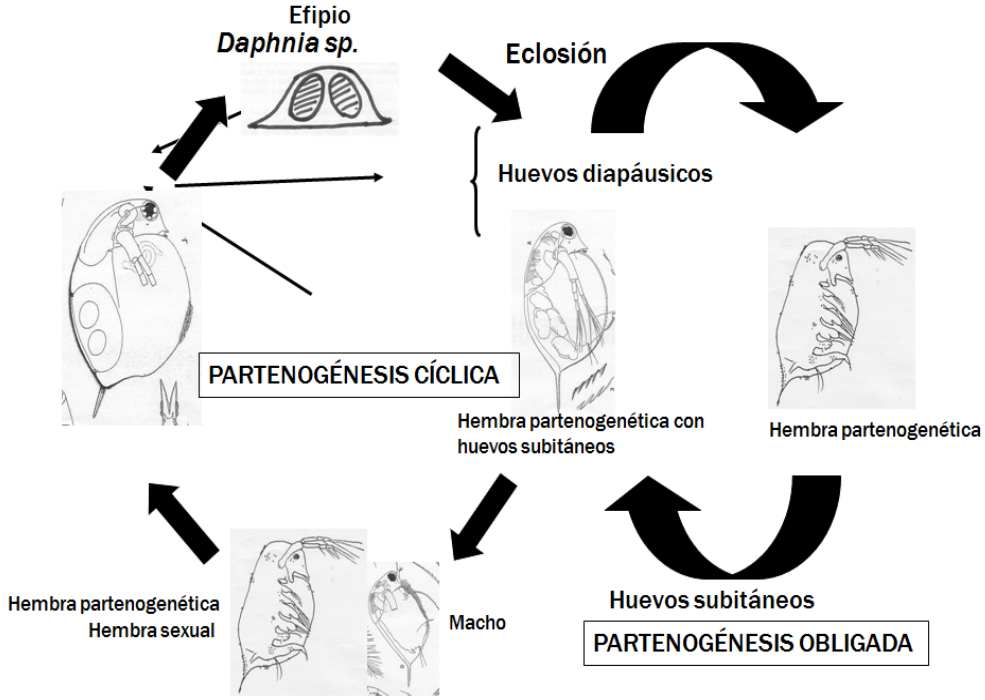

Figura 1. Ciclo reproductivo en el zooplancton partenogenético.

La producción de huevos diapáusicos asegura la persistencia de las poblaciónes, y les confiere una serie de ventajas adaptativas. En primer lugar, durante la reproducción sexual se produce recombinación meiótica que les confiere una mayor diversidad genética a la población. En segundo lugar, las estructuras de resistencia de los huevos diapáusicos los hace más resistentes a la desecación y transporte, facilitando su dispersión espacial.

Las distintas estrategias de producción e inversión en diapausa dependerán de las condiciones ambientales de cada hábitat (Hairston y Munns, 1984; Serra y King, 1999; Spencer et al., 2001; Schröder, 2005). Por tanto, la acumulación de huevos diapáusicos en el sedimento puede ser muy variable, dependiendo principalmente de las condiciones ambientales (Hairston, 1996). Además, los huevos diapáusicos se pueden ir acumulando enterrados en el sedimento y permanecer viables durante largos periodos de tiempo permitiendo la persistencia de la población. La eclosión de los huevos diapáusicos quedará restringida a los primeros centímetros del sedimento, el "banco activo" (Herzig, 1985; Cáceres y Hairston, 1998), que sería la porción de sedimento expuesta a los estímulos que inducen la eclosión (Hairston et al., 2000; Hairston y Kearns, 2002; Brendonck y De Meester, 2003).

La eclosión de los huevos diapáusicos cada nueva estación de crecimiento estará relacionada con el éxito reproductivo y de crecimiento que puedan tener las poblaciones volviendo a ser activas (García-Roger et al., 2014; 2016). En ambientes predecibles se espera una estrategia de producción de huevos diapaúsicos de forma sincrónica en la población del tipo 'todo o nada' o *bang-bang* previo al acontecimiento de cambio de las condiciones ambientales. En cambio, en un ambiente impredecible las poblaciones zooplanctónicas responden con una inversión temprana en la diapausa a tasas más bajas y de manera continuada (Carmona et al., 1995; Serra y King, 1999; Spencer et al., 2001). Este mantenimiento de huevos viables en el sedimento les permite enfrentarse a la impredecibilidad ambiental. Tanto la estrategia de producción intermedia de huevos diapáusicos como la estrategia de apuestas múltiples disminuye el riesgo de extinción local en ambientes impredecibles (Hairston y Munns, 1984; Stearns, 1992; Belmonte y Rossi, 1998).

La importancia de los huevos diapáusicos resulta, no solo al permitir la persistencia de las poblaciones bajo condiciones adversas, sino que también capacitan la dispersión espacial de poblaciones de cladóceros y rotíferos y, en definitiva, la colonización de nuevos sistemas acuáticos a través del aire, flujo de agua o vectores animales (Jenkins y Underwood, 1998; Brendonck y Riddoch, 1999; Michels et al., 2001; Green et al., 2008). Además, representan una herramienta más valiosa que los organismos activos para cuantificar la biodiversidad del zooplancton (Vandekerkhove et al., 2005a).

En cualquier caso, el primer paso para estudiar la dispersión espacial del zooplancton es **identificar de forma adecuada los huevos de resistencia**. Una vez hecho esto, se podrían **evaluar los distintos mecanismos dispersión espacial** de dichos huevos y **analizar las características ambientales que pueden afectar a la capacidad de eclosión** de estos huevos diapáusicos y, en definitiva, de colonizar o recolonizar los sistemas lacustres.

Identificación de los huevos de resistencia

Existe una gran diversidad morfológica de huevos diapáusicos entre las especies de cladóceros y rotíferos, lo que permitiría caracterizar el banco de huevos diapáusicos mediante la identificación de los mismos en base a sus diferencias morfológicas. Sin embargo, esta identificación es compleja ya que, generalmente, requiere el empleo del "método de eclosión" para relacionar la morfología de los huevos almacenados en los sedimentos con especies concretas de zooplancton que pueden ser identificadas tras eclosionar de dichos huevos. Este método ha sido aplicado con éxito a la hora de estimar la diversidad en comunidades de cladóceros (Declerck et al., 2005; Vandekerkhove et al., 2005a). Sin embargo, este método tiene limitaciones importantes, especialmente el hecho de que las condiciones ambientales para inducir la eclosión de los huevos pueden no ser las adecuadas, que la duración del periodo refractario de diapausa obligada no se haya cumplido o que exista una apuesta diversificada de las especies a la hora de eclosionar (De Meester y De Jagger, 1993; De Stasio, 2004). En consecuencia, aquellos huevos que no eclosionen no podrán ser asignados a especies concretas.

El uso de huevos diapáusicos para hacer estimas de diversidad, tienen otras limitaciones añadidas, ya que algunas especies no los producen, o lo hacen a muy pequeñas densidades (Jankowsky y Straile, 2003), o su producción puede estar limitada por los factores ambientales (Jeppesen et al., 2003). La variación espacial de la comunidad activa puede determinar una distribución parcheada de los huevos diapáusicos (Vandekerkhove et al., 2005a).

Un avance significativo para optimizar el análisis de las muestras del banco de huevos diapáusicos sería incluir el desarrollo de herramientas que permitan la identificación directa de especies a partir de los huevos diapáusicos, haciendo innecesario el procedimiento de incubación y de este modo evitar las limitaciones que puede presentar el método de la eclosión. Una alternativa puede ser la aplicación de técnicas moleculares, como la técnica del DNA barcoding (Hebert et al., 2003). Esta técnica se basa en la secuenciación y análisis de un fragmento de ADN que se usa como marcador molecular que permite diferenciar especies, el gen mitocondrial COI (citocromo oxidasa *c* subunidad I). Las regiones flanqueantes al gen están muy conservadas a nivel de especie, por lo que el uso de *primers* específicos como LCO1490 y HCO2198 (Folmer *et al.*, 1994) tienen una alta resolución. Su uso y efectividad ha sido ampliamente demostrada satisfactoriamente tanto con especies de cladóceros (Elías-Gutiérrez *et al.*, 2008), como con rotíferos (Gómez *et al.* 2002; Gilbert *et al.* 2005; Mills *et al.* 2016). Por tanto, la aplicación de estas herramientas al banco de huevos diapáusicos ayudarían a revelar toda la diversidad en un sistema acuático y que quedaría oculta usando únicamente el método de eclosión.

Dispersión de huevos diapáusicos

Las características de los huevos diapáusicos les confieren una protección mecánica y resistencia a la desecación que facilita su transporte a través del aire por el viento o al tránsito por la ingestión por animales (Jarnagin et al., 2000, Charalambidou y Santamaría, 2002; Figuerola y Green, 2002, Battauz et al., 2015). Sin embargo, se ha comprobado que sus

capacidades y tasas de dispersión varían entre las poblaciones de las especies de zooplancton (Cáceres y Soluk, 2002). A día de hoy no se sabe con certeza que vectores son más efectivos en la dispersión.

Dispersión por viento y lluvia

Aunque la dispersión por el viento ha sido ampliamente estudiada, aún sigue cargada de controversia la cuestión de si se trata de un vector que transporta numerosos propágulos diapaúsicos y por tanto es crucial para la dispersión de organismos zooplanctónicos entre masas de agua, o por el contrario es muy baja la dispersión de propágulos diapaúsicos por el viento limitando su capacidad para colonizar nuevos cuerpos de agua (Jenkins y Buikema, 1998; Jenkins y Underwood, 1998; Cohen y Shurin, 2003). Se ha visto que los factores más relevantes en la dispersión por el viento son la proximidad con el banco de huevos diapáusicos y la dirección del viento (Vanschoenwinkel et al., 2008a). Por tanto, en sistemas donde los niveles de agua fluctúen considerablemente dejando al descubierto y bajo el efecto del viento el bando de huevos diapaúsicos será más probable la dispersión de los mismos (Bilton et al., 2001; Vanschoenwinkel et al., 2008b; Tuytens et al., 2014). Por tanto, los eventos de dispersión se concentrarán durante las estaciones seca y húmeda (Hulsmans et al., 2007). Hasta el momento no se ha tenido en cuenta la dispersión por el viento desde el punto de vista de la deposición o sedimentación pasiva, discriminando entre lo que podríamos denominar "deposición seca" de los propágulos que se dispersan por al aire impulsados por el viento y depositados de una manera pasiva, y "deposición húmeda" de los propágulos que igualmente se dispersarían por el viento pero que su deposición ya no sería pasiva al estar facilitada por la acción de la lluvia.

Dispersión por aves

Las aves acuáticas que habitan charcas y lagunas someras son un importante vector de dispersión de propágulos diapáusicos, siendo transportados en las plumas (Proctor y Malone, 1965; Charalambidou y Santamaría, 2002; Charalambidou et al., 2003) o alojados en el tracto digestivo, y en ambos casos sin pérdida de viabilidad. Experimentos realizados con huevos diapáusicos del cladócero conocido como pulga de agua espinosa, *Bythotrephes longimanus*, demostraron experimentalmente que tras ser ingeridos por distintas especies de patos sobrevivían al paso del tracto digestivo, pero que las probabilidades de dispersión por cada individúo eran bajas, aunque a nivel de población podrían ser más altas (Charalambidou et al., 2003). Además, se ha comprobado que el anostráceo *Branchinecta lindahli* presenta unas tasas de eclosión de sus huevos diapáusicos más alta tras su ingestión por aves acuáticas, que los huevos presentes en el banco de huevos diapáusicos (Rogers, 2014).

Por el momento todos los estudios de dispersión de propágulos diapáusicos de invertebrados acuáticos se han centrado en crustáceos, y tan sólo unos pocos han considerado los rotíferos (Frisch et al., 2007; Green et al., 2008), aunque de forma colateral, sin un suficiente grado de resolución taxonómica y sin evaluar la capacidad de eclosión de huevos aislados de las heces.

Capacidad de eclosión de los huevos diapáusicos

La resiliencia de las comunidades de zooplankton dependerá de la viabilidad de los huevos diapáusicos y de las condiciones ambientales que induzcan la eclosión. Distintos estudios han mostrado o sugerido que la concentración de oxígeno, los niveles de alimento, el fotoperiodo, la temperatura y/o la presencia de luz controlan la eclosión de los huevos diapáusicos de distintas especies de zooplancton (Brendonck y De Meester, 2003; Gyllström y Hansson, 2004; Perez-Martinez et al., 2013). Las condiciones para la eclosión pueden ser diferentes entre sistemas, según sus características ambientales. En España, la luz induce la emergencia del zooplancton en lagos de alta montaña (Perez-Martinez et al., 2013), mientras el fotoperiodo sería un factor relevante para la eclosión de cladóceros en sistemas de baja altitud del sur de España (Vandekerkhove et al., 2005a). Sin embargo, el efecto de la salinidad sobre las tasas de eclosión del zooplancton apenas ha sido estudiado (Santangelo et al., 2014). Algunos estudios han mostrado la capacidad de los huevos diapáusicos de rotíferos de resistir elevadas salinidades durante periodos de exposición largos (Nielsen et al., 2012, Santangelo, 2014). Sin embargo, no se han hecho estudios sobre los efectos de la salinidad sobre la eclosión a distintas temperaturas, aislando los huevos diapáusicos, y/o sobre la eclosión de huevos dispersados.

El estudio de los efectos de la salinidad en la región mediterránea son especialmente relevantes, ya que se esperan cambios ambientales bastantes drásticos para las próximas décadas, cuyos efectos pueden verse reflejados en cambios en los patrones de precipitación, con una clara tendencia negativa (Giorgi, 2006; Giorgi y Lionello, 2008), y un incremento en la temperatura en lagos (Copetti et al., 2013). En su conjunto, estos cambios serán más acusados en las lagunas someras localizadas en zonas áridas, dando lugar a una reducción de los niveles del agua y un incremento en la salinidad (Jeppesen et al., 2011, Jeppesen et al. 2015). Bajo este escenario de cambio climático la riqueza de especies de invertebrados acuáticos de agua dulce podría verse afectada negativamente si se exceden los niveles de tolerancia a la salinidad (Nielsen et al., 2003). La biodiversidad de las comunidades zooplanctónicas podrían verse afectadas, En el caso de las especies de zooplancton los crustáceos serían los más afectados (Brucet et al. 2009, Brucet et al., 2010). Por todo ello, sería importante conocer los efectos que tiene la salinidad en la eclosión de los huevos diapáusicos, ya que podrían ser indicativos de la capacidad resiliente de los sistemas acuáticos, y de la capacidad de colonización de dichos sistemas por especies dispersadas, ante incrementos de la salinidad.

Objectives and thesis outline

The present thesis uses the zooplankton diapausing propagules as a tool to analyze the genetic biodiversity of rotifers in several lakes of the south of Spain, in order to morphologically identify diapausing eggs. This first step will allow to study dispersal of cladocerans and rotifers by wind, rainfall and waterbirds, analyzing the viability and hatching capabilities of both dispersed and sedimentary diapausing eggs in those lakes of the Mediterranean region under a climate change scenario. The main specific objectives of the present thesis are explained below:

1. To test the value of applying DNA barcoding to diapausing egg banks to characterize rotifer communities using a single sediment sample, and to morphologically identified rotifer resting eggs.

2. To evaluate wind and rainfall dispersal with the discrimination between dry deposition (sedimentation from the air) and wet deposition (from rainfall).

3. To quantify the diapausing egg dispersal of cladoceran and rotifers by waterbirds in relation with the zooplankton communities inhabiting the same geographic area, and to know the hatching response of those diapausing propagules to photoperiod and temperature.

4. To test the hatching response of local diapausing eggs from sediments and of diapausing eggs dispersed by waterbirds to salinity and temperature, and to know the effect of waterbird ingestion on the hatching success of rotifer resting eggs.

The structure of the thesis is explained below:

In **Chapter 1** we characterize the diapausing egg bank of rotifers applying DNA barcoding to a single sediment sample from Doñana and Ruidera lakes. We create a reference collection of DNA barcodes of water-column rotifers individuals. To detect the presence of detect potential cryptic species of rotifers we apply DNA taxonomy methods to delimit species, and describe species complexes in our dataset in the context of all published rotifer barcodes. The morphology of the resting eggs will be related with each species.

In **Chapter 2** we evaluate the dispersal of diapausing propagules of rotifers and cladocerans by wind and rainfall between Doñana National Park and Ruidera Natural Park using automatic atmospheric deposition collectors to examine differences between dry and wet passive deposition. We also investigate the relationship between meteorological variables and the passive deposition of propagules in Doñana.

In **Chapter 3**, we study the dispersal of rotifers and cladocerans by waterbirds in Doñana National park and we study the hatching response of dispersed propagules under different conditions of temperature and photoperiod.

In **Chapter 4**, we analyze the hatching response of rotifer resting eggs from the sediments and from facecal droppings of waterbirds to salinity and temperature to cope with future increases in salinity due to global change.

In **Chapter 5,** we relate the results of previous chapters with the biodiversity and dynamics of the zooplankton inhabiting the studied lakes.

General material and methods

Study site

The study was carried out in four lakes located in two regions with different limnological and geographic characteristics, and samples were taken from five locations (Figure 2).

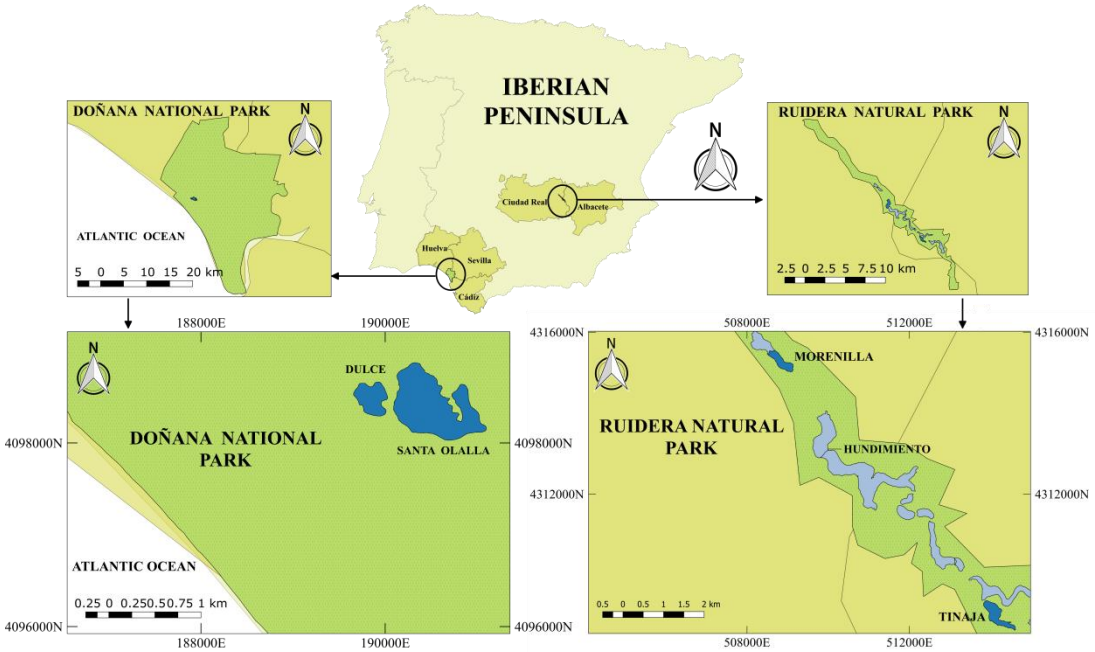

Figure 2. Map of the study area and water bodies location.

Doñana National Park in southwest Spain is a region with an extremely flat topography and a Mediterranean climate with an Atlantic influence that is characterized by mild winters and dry, hot summers and prevailing westerly winds (Bayán, 2005; Espinar y Serrano, 2009). Rainfall varies seasonally and annually, producing drastic fluctuations in water levels and in the physical-chemical and biological processes of freshwater bodies (Serrano *et al.,* 2006; Serrano y Zunzunegui, 2008; Espinar y Serrano, 2009; López-Archilla *et al.*, 2012). ***Santa Olalla*** lake and ***Dulce*** lake are contiguous coastal peridunal shallow ponds fed by groundwater and rainfall, permanent and semipermanent respectively (Figure 3). Although both ponds are usually isolated hydrologically and have different limnological characteristics, during high-precipitation seasons the ponds can be connected. The zooplankton composition changes seasonally and annually, with rotifers as predominant group, mostly from the genus *Brachionus*, followed by cladoceran and copepods (López et al., 1991; Serrano y Toja, 1998).

Figure 3. Sampling sites. *Santa Olalla* lake (left) and *Dulce* lake (right).

Ruidera Natural Park is in Central Spain, between Albacete and Ciudad Real provinces, and consists of 15 lakes connected in a chain, separated by travertine barriers and fed by groundwater and, when groundwater levels are high, by surface drainage from the upper to the lower lakes. The climate is continental Mediterranean with rainfall mostly in spring and autumn, and the prevailing wind is from the southwest. ***Tinaja*** lake is a warm monomictic mesotrophic and eutrophic lake and ***Cueva de Morenilla*** lake (***Morenilla*** lake hereafter) is a warm monomictic and eutrophic lake (Figure 4). Zooplankton species composition, abundance, and biomass changes seasonally and are variable throughout lakes, where rotifers are the most abundant taxon followed by cladoceran and copepods (Bort et al., 2005; Álvarez-Cobelas et al., 2007; Rojo et al., 2007).

Figure 4. Sampling sites. Sampling sites. *Tinja* lake (left) and *Morenilla* lake (right).

Figure 5. Sampling sites. *Hundimiento.*

Zooplankton sampling

Zooplankton samples were collected from ***Dulce*** and ***Santa Olalla*** lakes in Doñana, and ***Morenilla*** and ***Tinaja*** lakes in Ruidera. Additionally, zooplankton samples were collected from ***El Hundimiento*** (***Hundimiento*** hereafter), a water body which links ***Del Rey*** and ***Morenilla*** lakes (a discharge point from ***Del Rey*** lake waterfall) (Figure 4). From each lake and date, we collected a depth-integrated zooplankton sample in the open water at five randomly chosen points. For zooplankton abundance estimation we collected the quantitative zooplankton sample with a tube sampler (6.8 cm diameter) in Doñana. In Ruidera lakes we used a Van Dorn water sampler pulling it from near the bottom of the lake to the surface to different depths and integrating the vertical samples. We filtered a range of 0.5-12.5 and 6-27 litres of water in Doñana and Ruidera respectively, concentrated through a 45 µm mesh-size net and immediately fixed and preserved *in situ* in 70% ethanol. In *Hundimiento* we filtered a range of 2-5 litres of water and followed the same procedure described above.

Physical-chemical features

Contemporaneously with the zooplankton sampling, physical-chemical parameters were recorded from the water column by means of a multiparameter probe (Waterproof pH/CON 300 Meter, Oakton; USA). Chlorophyll *a* concentration (Chl *a*) was measured *in vivo* with a field fluorometer (Aquafluor, Turner Designs; Sunnyvale, California, USA), which measures total fluorescence as an indirect measurement of Chl *a* concentration. To do so, we first calibrated the fluorometer against a blank (distilled water). An integrated sample from the water column at the sampling location was emptied into a container. Then, a 1.5 ml of the integrated sample was placed in a plastic cuvette. The dual-channel fluorometer is able to test Chl *a* concentration by recording the intensity of fluorescence under blue light. An average of these measurements was

converted into chlorophyll *a* µg/L via a locally regression equation. We built a regression equation in order to convert total fluorescence measurements into Chl *a* concentrations (µg/L). For nutrients concentration analyses one litre of water were taken at the deepest point of each lake. Samples for nutrient concentration analyses were collected in acid-washed polyethylene bottles and immediately cooled until further analysis. Dissolved inorganic nitrogen (ammonium (NH4) and nitrate (NH3)) and soluble reactive phosphate (SRP) were analysed in the laboratory from previously filtered water samples with a Whatman® GF/F glass microfiber filters and total nitrogen (TN) and total phosphorus (TP) from unfiltered water samples following APHA (1992). Dissolved inorganic nitrogen (DIN) was computed as the addition of nitrate and ammonium concentrations for all calculations.

Sediment samples collection

The sediment cores were collected by means of a hand-operated sediment core sampler that is composed of a 2-m long shaft and a 50-cm long acrylic tube (diameter: 5.2 cm), which is screwed into a cylinder with a built-in silicone contra flap. Sediment cores of 10 cm depth were taken from the deepest part of each lake in Doñana and Ruidera. The first 4 cm of the core was sliced into 2-cm sections, and the slices were packed in ziplock plastic bags, labelled, wrapped in aluminium foil and stored at 4°C in the dark until processed. We only used the top four centimetres of the sediment, as this is likely to comprise the viable and responsive zooplankton egg bank (so-called active sediment). García-Roger et al. (2006) commented that 2 months is the time span needed to complete the mandatory refractory period for hatching induction in resting eggs of the rotifer *B. plicatilis*. For that reason, in our study, after 2-24 months of resting period.

Figure 6. Sediment samples collection.

Diapausing egg isolation

Sediment subsample of a known wet weight from the center of each 2-cm slice was taken for further processing. We used the modified sucrose flotation technique developed by Onbé (1978) and modified by Marcus (1990), to isolate diapausing eggs from the sediments. This technique have been used in previous studies on hatching success of zooplankton (Gómez et al., 2002, Vandekerkhove et al., 2005; García-Roger et al., 2006; Pérez-Martínez et al., 2013; Santangelo et al., 2014), and recently it has been demonstrated that this sugar flotation method has no effect on those hatching rates (Lukic et al., 2016). The supernatant was filtered through a 10 μm Nytal mesh and the filtrate washed with distilled water and transferred to a plankton counting chamber (Figure 7).

Figure 7. Sucrose flotation technique for diapausing eggs isolation.

Hatching experiments

Sediment samples and faecal samples were stored in cold and dark conditions just after their collection during several months to avoid hatching stimuli and to ensure the completion of diapause in recently produced diapausing eggs. All the experiments were performed in two Mettler incubation chambers to control their temperature (Figure 8). For the experiments in **Chapter 3** and **Chapter 4**, experimental temperature was kept at 15 °C in one chamber and at 25 ºC in the other one. Previously it has been observed that 15 ºC temperature is appropriate for analysing hatching rates of cladocerans in Spanish shallow lakes (Vandekherkove et al., 2005). Every day, wells were studied under microscope to record any hatching for 30 days, and the day of hatching and the species were noted. Diapausing eggs isolated from sediment samples and faecal samples were incubated at different conditions of photoperiod [light:dark (L:D) =14:10 h] and temperature (10, 14 and 20 ºC). Diapausing propagules were individually placed in a well of 96-well polyethylene microplate with mineral water. To perform salinity experiments mineral water adjusted at different salinities. More details of the salinity experiments are described in **Chapter 4**. These microplates were located in opaque chambers fitted with a zenith light, using white light with an intensity of 345.50 ± 20.54 lmol s^{-1} m^{-2} [mean ± standard error (SE)] as measured with a BQM photometer (Pérez-Martínez *et al.* 2013). The white light of the opaque chambers do not affect the temperature (Pérez-Martínez *et al.* 2013).

Figure 8. Mettler incubation chambers.

Dispersal sample collection

The dispersal of the dormant propagules reaching the lakes by wind and rainfall was measured using MTX® ARS 1010 automatic dry/wet passive collectors (MTX Italia SPA, Modane, Italy) equipped with two polyethylene buckets (surface area of 0.0667 m^2 each) and a hygroscopic sensor cell. This device activated an aluminium lid that covered the dry bucket and uncovered the wet bucket during rainfall and *vice versa* during periods without rain (Figure 9). The dispersal by wind was also evaluated using two windsocks, which were conical, 30-µm plankton nets fitted to a conical metal frame that rotated in the direction of the wind. Sampling collection procedures are described in detail in **Chapter 2.**

Figure 9. Automatic dry/wet atmospheric collectors and windsock (right) built in Doñana.

Fresh waterbird faeces were collected in the shore of those lakes or in islands where waterbirds roosted. Previously, those places were selected by using binoculars to localization and identification of the waterbird groups, and bird individuals were identified to species level. We ensured to collect each dropping removing the parts in contact with soil/sediment to avoid contamination (Figure 10). Waterbird faeces were stored in a tube kept in a cool box with ice until reach the laboratory, and they were mainly collected during autumn and winter. Sampling collection procedures and the isolation of diapausing eggs from waterbird faeces are described in detail in **Chapter 3.**

Figure 10. Waterbird faeces sample collection procedures.

DNA barcoding technique

DNA was extracted from single adult rotifers or diapausing eggs using the hot sodium hydroxide and Tris (HotSHOT) method (Montero-Pau et al., 2008). A region of the mitochondrial DNA from the cytochrome *c* oxidase subunit I gene (COI) was amplified using primers LCO1490 and HCO2198 (Folmer et al., 1994). PCR and DNA analyses procedures are described in detail in **Chapter 1**.

Chapter 1

Barcoding rotifer biodiversity in Mediterranean ponds using diapausing egg banks

Abstract

The biodiversity of Mediterranean freshwater bodies is among the most threatened worldwide; therefore, its accurate estimation is an urgent issue. However, traditional methods are likely to underestimate freshwater zooplankton biodiversity due to its high species seasonality and cryptic diversity. We test the value of applying DNA barcoding to diapausing egg banks, in combination with the creation of a reference collection of DNA barcodes using adult individual samples, to characterize rotifer communities. We use monogonont rotifers from two lakes in Doñana National Park and one from Ruidera Natural Park in Spain as models to create a reference collection of DNA barcodes for taxonomically diagnosed adult individuals sampled from the water column, to compare with the sequences obtained from individual eggs from the diapausing egg banks. We apply two different approaches to carry out DNA taxonomy analyses, the generalized mixed Yule coalescent method (GMYC) and the Automatic Barcode Gap Discovery (ABGD), to the obtained sequences and to publicly available rotifer sequences. We obtained a total of 210 new rotifer COI sequences from all three locations (151 diapausing eggs and 59 adults). Both GMYC and ABGD generated the same 35 operational taxonomic units (OTUs), revealing four potential cryptic species. Most sequences obtained from diapausing eggs (85%) clustered with sequences obtained from morphologically diagnosed adults. Our approach, based on a single sediment sample, retrieved estimates of rotifer biodiversity higher than or similar to those of previous studies based on a number of seasonal samples. This study shows that DNA barcoding of diapausing egg banks is an effective aid to characterize rotifer diversity in Mediterranean freshwater bodies.

Introduction

Mediterranean freshwater bodies are important reservoirs of biodiversity for aquatic invertebrates. They are among the most vulnerable and threatened habitats worldwide due to drastic changes in hydrological regime patterns and the introduction and natural invasions of exotic species (Myers et al. 2000; Sala et al. 2000; Oertli et al. 2005; Mergeay et al. 2005; Briski et al. 2011). Under this scenario, changes in the zooplankton community structure and composition can be noticeably dramatic, especially for passively dispersed species, highlighting the urgency of biodiversity assessments, which are challenging due to the time and cost of surveys. The application of conservation approaches usually fails as lakes and ponds are isolated islandlike habitats with high seasonal species turnover (Dudgeon et al., 2006). In addition, estimates of zooplankton diversity are often incomplete due to the large number of cryptic and undescribed species involved (Esteban and Finlay, 2010). Finally, freshwater biodiversity studies have often disregarded invertebrate species, which are essential for maintaining the natural dynamic of ecosystems (Muñoz, 2010).

Rotifera is one of the main groups of zooplankton and plays a key role as component of food webs in aquatic ecosystems, transferring energy from low to higher trophic levels (Wallace, 2002). The phylum is classified into three classes, Seisonidea, Bdelloidea, and Monogononta, and comprises over 2,000 described species, mostly microscopic. Monogononta, the most abundant and morphologically diverse class, includes 1,570 species (Segers, 2007) of globally distributed, short-lived organisms found in a wide range of continental water bodies from hypersaline to freshwater. However, their supposed ubiquity has been rejected because they have shown high levels of cryptic diversity. For example, previous studies focused on rotifer alpha diversity and biogeography showed seven putative species of *Brachionus plicatilis* in Spain and 15 described species worldwide (Gómez et al., 2002; Mills et al., 2016), six lineages of *Brachionus calyciflorus* from China and at least eight putative species from different countries (Schröder and Walsh, 2007; Xiang et al., 2011), six putative species of *Testudinella clypeata* from the UK (Leasi et al., 2013), 12 putative species of *Polyarthra dolichoptera* from different locations in Italy (Obertegger et al., 2014), and eight putative species of *Keratella cochlearis* from Italy (Cieplinski et al., 2016). In nearly 60% of the complexes described in the phylum Rotifera, at least two species of the same complex can occur (Gabaldón et al., 2016). A further challenge involved in assessing the diversity of rotifer communities is their short life cycle with a short generation time, temperature-dependent growth rate, and embryonic development which varies from population to population (Herzig, 1983). Monogonont rotifers complete their life cycle in 5–10 days and have a generation time threshold of 2–4 days in temperate lakes (20–22°C) (Gillooly, 2000; Ricci, 2001). Seasonal species replacement occurs faster in temporary and fluctuating environments; therefore, a higher sampling effort in terms of temporal frequency is needed to obtain unbiased assessments of species composition (Serrano and Fahd, 2005; Fahd et al., 2007).

The life cycle of monogonont rotifers includes diapausing eggs able to survive unfavorable periods of harsh conditions and prevent local extinctions (Ricci, 2001; Wallace, 2002). Diapausing eggs accumulate in the sediments, where they can persist for long periods of

time and establish egg banks, potentially integrating spatiotemporal patterns of community diversity, increasing generation time and decreasing population growth rate (DeStasio, 1989; Hairston, 1996; Brendonck and De Meester, 2003; Vandekerkhove et al., 2005a; Gabaldón et al., 2015;). Given these features, the study of diapausing egg banks could potentially become a cost-effective approach for assessment of zooplankton biodiversity, as a single egg bank sample may cover a period of several years, while plankton samples are highly variable temporally, implying that several samples are required for an accurate characterization of zooplankton communities (May 1986; Havel et al. 2000; Duggan et al. 2002; Vandekerkhove et al. 2005a; Mergeay et al. 2005). In other planktonic invertebrates, such as cladocerans, diapausing stages or ephippia can display very species-specific morphologies (e.g., size, shape, color, and external sculpturing or ornamentation) and they have been successfully used for species-level identification (Pourriot & Snell, 1983; Vandekerkhove et al., 2004). In contrast, although rotifer diapausing eggs can show genus-specific morphological features (Walsh, May, & Wallace, 2016), high intraspecific morphological variability and absence of useful diagnostic characters have precluded their use for species-level identification (Gilbert, 1974, 1995; Brendonck and De Meester, 2003), leading to biased diversity estimates between diapausing egg diversity and species diversity (Piscia et al., 2012). An early approach to the study of diapausing egg banks for assessing zooplankton species richness is the hatching method (May, 1986; Duggan et al. 2002; Brendonck & De Meester, 2003; Gleason et al. 2004; Vandekerkhove et al. 2005b; Palazzo et al. 2008). However, this method has limitations, and the identification of appropriate hatching cues (light, temperature, salinity, oxygen concentration, or others) and the latency period (which may vary from a few days to several months) are often species-specific and within species (Gilbert and Walsh, 2005), and potential biases introduced by bet-hedging, where only a fraction of the eggs hatches at a given time (Schwartz & Hebert, 1987; Schröder, 2005; Vandekerkhove et al. 2005c; García-Roger et al. 2014).

A significant improvement for the analysis of rotifer diapausing egg bank biodiversity would include the development of tools for the direct species identification of diapausing eggs. An alternative to morphological identification and hatching approaches is using molecular techniques, which can be applied on individual diapausing eggs or adults. DNA barcoding and DNA taxonomy offer a complementary tool to traditional taxonomy and to quantify global biodiversity, especially for groups whose morphology is uninformative, plastic, and/or difficult to describe (Hebert et al., 2003; Fontaneto et al., 2015). DNA barcoding consists of the sequencing and analysis of a DNA fragment of the mitochondrial gene COI (the cytochrome *c* oxidase subunit I) to identify species (Hebert et al., 2003). On the other hand, DNA taxonomy approaches, such as GMYC (generalized mixed Yule coalescent; Pons et al., 2006; Fujisawa and Barraclough, 2013) or Automatic Barcode Gap Discovery (ABGD; Puillandre et al., 2012), are based on the analysis of variation in genetic data for delimiting species. DNA barcoding and analytical tools of DNA taxonomy have offered a very productive approach to uncover cryptic species complexes (Hebert et al. 2004; Birky et al. 2005; Fontaneto et al. 2007; Puillandre et al. 2012; Fujisawa et al. 2013; Leasi et al. 2013). DNA taxonomy has indeed revealed that most rotifer taxonomic species analyzed are actually species complexes both in monogonont (Derry et al. 2003; Schröder and Walsh, 2007; Walsh et al. 2009; Xiang et al. 2011; Obertegger et al. 2012;

Leasi et al. 2013; Obertegger et al. 2014; Mills et al. 2016) and in bdelloids (Fontaneto et al. 2008, 2009, 2011), with 39 known species complexes identified so far in rotifers (21 monogononta and 18 bdelloidea), a number that is likely to increase (see Fontaneto, 2014 for a recent review). DNA barcoding has also been useful as a complementary tool for surveying rotifer biodiversity in larger geographic areas, revealing the presence of several potential cryptic species (García-Morales and Elías-Gutiérrez, 2013), and for studies on the dispersal and transport detection of invasive species (Mergeay et al., 2005; Briski et al., 2011).

Here, we test the value of applying DNA barcoding and DNA taxonomy to diapausing egg banks from a single sediment sample to characterize zooplankton communities. To do so, we collected sediment and water-column samples from two lakes in Doñana National Park and one from Ruidera Natural Park in Spain. We create a reference collection of DNA barcodes with the water-column monogonont rotifers taxonomically diagnosed as models to compare to barcodes obtained from their corresponding diapausing egg from sediment samples. We then use DNA taxonomy methods to delimit species and detect potential cryptic species, and describe species complexes in our dataset in the context of all published rotifer barcodes.

Materials and methods

Study site

Samples were taken from three lakes located in two regions with different limnological and geographic characteristics, *Tinaja* Lake located in Ruidera Natural Park, and *Santa Olalla* and *Dulce* located in Doñana National Park (Figure 2).

Sample collection

Sediment cores were taken from the deepest part of each lake (03/18/2009 in Doñana and 01/21/2009 in Ruidera). To construct a reference collection of rotifers found in the water column of the study sites water samples were collected. Sampling procedures are described in detail in the general methodology section.

Diapausing egg isolation and identification of rotifers from water samples

A sediment subsample of 6 g (wet weight) from the center of each 2-cm slice was taken for further processing. The technique is described in detail in the general methodology section.

Zooplankton water column samples and diapausing eggs isolated from sediment samples were examined under an inverted microscope. All individual rotifers were identified to species level using Koste's identification key (Koste, 1978), and each putative diapausing egg were classified according to their morphology. Each egg was photographed prior to DNA analyses.

DNA extraction, amplification, and sequencing

DNA was extracted from single adult rotifers or diapausing eggs using the hot sodium hydroxide and Tris (HotSHOT) method (Montero-Pau et al., 2008). A region of the mitochondrial DNA from the cytochrome *c* oxidase subunit I gene (COI) was amplified using

primers LCO1490 and HCO2198 (Folmer et al., 1994). Each 50 µl PCR contained 5 µl of template DNA, 2 mM of MgCl2, 0.2 mM of each nucleotide, 0.2 µM of each primer, 5 µl of 10× NH4 Bioline buffer, and 0.5 U of *BioTaq* DNA polymerase (Bioline). Amplification was performed in a Veriti thermocycler (Applied Biosystems) with the following cycling profile: 1 cycle of 3 min at 93°C; 40 cycles of 15 s at 92°C, 20 s at 45°C, and 30 s at 70°C; and 3 min at 72°C. Samples giving clear strong single bands were sequenced in both directions by Macrogen Inc. (South Korea) using 3730XL DNA analyzer (Applied Biosystems, USA). Chromatograms were checked and edited using CodonCode Aligner (CodonCode Corporation, Dedham, MA).

Phylogenetic analysis

We constructed a first dataset that included all newly obtained sequences in this study (Dataset S1 hereafter). The aim of this dataset is, firstly, to identify diapausing eggs matching with their adults, which served as a reference collection of the study sites, as all individuals were identified to morphospecies; and secondly, to estimate the hidden biodiversity by detecting potential cryptic species. Sequences were aligned using the ClustalW algorithm included in ARB (Ludwig et al., 2004) and trimmed to 499 base pairs, the length of the shortest sequence in our alignment. The alignment was individually checked and verified for protein coding frameshifts to avoid pseudogenes using MEGA5 (Tamura et al., 2011). All sequences obtained in this study were submitted to GenBank with accession numbers (KY749339–KY749548).

To construct the second dataset (Dataset S2 hereafter), we downloaded all the available monogonont rotifer COI sequences from GenBank retrieved using the following search criteria: Monogononta organism, sequences over 300 bp with COI, CO1, COX, or cytochrome *c* oxidase subunit I in title (downloaded on 8 June 2016). The purpose of this dataset is to potentially match the cryptic species in our dataset to previously described ones available in the GenBank reference collection. We generated an alignment following the same procedure as described for Dataset S1. Identical sequences in both datasets were collapsed into haplotypes using the online fasta sequence toolbox FaBox v. 1.4 (Villesen, 2007) before phylogenetic analyses.

For both datasets, we reconstructed phylogenetic trees with maximum likelihood (ML) and Bayesian inference (BI). ML reconstructions were carried out using MEGA5 (Tamura et al., 2011). Nucleotide substitution model was chosen using the find best DNA/Protein models option in MEGA5 (Tamura et al., 2011). We used a sequence from the bdelloid rotifer *Philodina roseola* as an outgroup (GenBank accession number: DQ078544). BI reconstructions were performed in BEAST v. 1.8.3 (Drummond et al., 2012) as implemented on the CIPRES Science Gateway (Miller et al., 2010). To do so, we used BEAUti v. 1.8.3 (Drummond et al., 2012) to create the xml input file needed for the BEAST runs with the following settings: a general time reversible with gamma distribution and invariable sites nucleotide substitution model (GTR + r + I) for Dataset S1 and a general time reversible with gamma distribution (GTR + r) for Dataset S2 with an uncorrelated lognormal relaxed clock. This model was implemented in BEAST, including an uncorrelated lognormal relaxed clock and coalescent prior, with the default settings of BEAUti for the remaining parameters. We created ultrametric phylogenies based on each COI dataset, and the phylogenetic analysis was run with two independent

searches for 80,000,000 generations with trees sampled every 10,000 generations, with a total number of trees of 8,000 for Dataset S1. For Dataset S2, the phylogenetic analysis was run with two independent searches for 100,000,000 generations with trees sampled every 5,000. For examining that the effective sample size (ESS) values for all parameters were above 200 and determining the burn-in, we used Tracer v. 1.6 in both datasets (Rambaut et al., 2013). We obtained a total of 60,000 trees and summarized with TreeAnnotator v. 1.8.3; the first 50,000 trees were discarded as burn-in.

DNA taxonomy

To identify the presence of independently evolving entities (putative cryptic species) in both COI datasets, we used the generalized mixed Yule coalescent method (GMYC), a robust tool for delimiting species using single-locus data (Pons et al., 2006; Fontaneto et al., 2007; Fujisawa and Barraclough, 2013). It is a likelihood method designed to delimit independently evolving species by fitting within-and between-species branching models to reconstructed gene trees. GMYC models were run on the previously constructed ultrametric maximum clade credibility consensus trees obtained with BEAST using R v. 3.3.1 (R Development Core Team, 2011) with the package *splits* v. 1.0-11 (https://r-forge.r-project.org/projects/splits/).

We used a second, alternative approach of DNA taxonomy for delimiting species, the ABGD (http://wwwabi.snv.jussieu.fr/public/abgd/ abgdweb.html). This method automatically identifies the threshold in genetic distances for species delimitation (a gap between intra-and interspecific diversity), the "barcoding gap"(Puillandre et al., 2012). We applied ABGD to Dataset S1 and Dataset S2 separately with all the sequences excluding the outgroup, because this method works better when there are more than 3–5 sequences per species. We used the uncorrected, JC69 and K2P distance matrices with default options (Pmin: 0.001, Pmax: 0.1, steps: 10, Nb bins: 20), except for the relative gap width (X) that was set to 1. Higher values than 1 recovered only one cluster. We considered only the results with a prior intraspecific divergence higher than 1.5%, as it has previously been described in rotifers for COI (Fontaneto, 2014).

DnaSP v4.0 (Rozas and Rozas, 1995) was used to calculate DNA divergence between the lineages delimited by both GMYC and ABGD analyses in Dataset S1 for groups of putative cryptic species.

Results

Sample processing and molecular analyses

The processing of 12 g (wet weight) of sediment in each lake resulted in a total of 193 putative rotifer diapausing eggs (Santa Olalla 6.08 eggs/g, Dulce 6.58 eggs/g, and Tinaja 3.41 eggs/g). These eggs were classified into 20 different groups according to their morphology (Table 1 and Figure 11). DNA was successfully extracted and COI amplified from 151 eggs (78.2% success rate), a high percentage given that it is difficult to identify healthy diapausing eggs in some species (García-Roger et al., 2006). Additionally, we identified 18 taxonomic

species from the water-column samples according to their morphology. Only 59 of 289 zooplankton samples yielded successful amplifications (20.4%), probably due to poor preservation (as opposed to poor primer match), as we managed to obtain high quality sequences from at least one specimen of all 18 taxonomic species identified. These samples were then used as a reference collection to assign diapausing eggs to particular taxonomic species by DNA barcoding.

Diapausing egg morphotypes	N seq	N DE	N AR	H	GMYC entities	Taxon	Location
DEM1	1	1	0	1	1	Fam. Flosculariidae	Tinaja
DEM2	15	12	3	2	1	*Filinia longiseta*	Santa Olalla, Dulce
DEM3	1	1	0	1	1	Fam. Collothecidae	Tinaja
DEM4	9	6	3	5	2	*Hexarthra fennica*	Santa Olalla, Tinaja
DEM5	7	5	2	1	1	*Hexarthra mira*	Dulce
DEM6	9	7	2	4	4	*Lecane* spp.	Dulce,Tinaja
DEM7	1	1	0	1	1	Fam. Notommatidae	Tinaja
DEM8	10	2	8	2	1	*Asplachna brightwelli*	Santa Olalla
DEM9	17	13	4	7	4	*Polyarthra vulgaris*	Santa Olalla, Dulce, Tinaja
DEM10	15	13	2	4	2	*Trichocerca* spp.	Tinaja
DEM11	17	12	5	3	2	*Keratella tropica*	Santa Olalla, Dulce
DEM12	2	1	1	1	1	*Proales* sp.	Tinaja
DEM13	2	1	1	2	1	*Brachionus budapestinensis*	Dulce
DEM14	46	28	18	3	2	*Brachionus plicatilis*	Santa Olalla, Dulce
DEM15	17	11	6	5	1	*Brachionus calyciflorus*	Santa Olalla, Dulce
DEM16	12	5	7	8	3	*Brachionus quadridentatus*	Santa Olalla, Dulce, Tinaja
DEM17	4	4	0	3	4	*Brachionus* spp.	Santa Olalla, Dulce
DEM18	10	7	3	1	1	*Brachionus angularis*	Santa Olalla, Dulce
DEM19	4	3	1	1	1	*Brachionus leydigi*	Santa Olalla
DEM20	7	6	1	3	1	*Brachionus variabilis*	Santa Olalla, Dulce

Table 1. Correspondence between diapausing egg morphotypes (DEM) and taxonomic species after DNA taxonomy.

Estimation of rotifer taxonomic diversity from the diapausing egg bank

We obtained a total of 210 new sequences from all three locations from both adults and diapausing eggs (listed in Appendix, Table A1). Sequences contained neither stop codons nor indels and were aligned unambiguously and collapsed into 65 haplotypes. A first approach for adult and diapausing egg morphotype assignation was based on ML and BI phylogenetic analyses (Figure 11). The consensus of 10,000 sampled trees from BI constructed with BEAST using the general time reversible + G + I model is shown in Figure 2. The GMYC model suggested the presence of 36 entities in Dataset S1 (clusters plus singletons and excluding outgroups with a confidence interval 34–38) and was the most likely model (likelihood of null model: 251.9922, ML of GMYC model: 293.8043, likelihood ratio: 83.62426, LR test: <0.0001). Sequences from diapausing eggs belonged to 35 GMYC entities, while zooplankton sample belonged to 24 entities (see Table 1 and Figure 11). These results showed how each diapausing egg morphotype belonged to a different GMYC entity, but also that diapausing egg morphotypes can include one or more GMYC entities. Diapausing eggs were assigned to a taxonomical species when haplotypes from both the egg and the identified adult belonged to the same GMYC entity. In this way, eleven of twelve Santa Olalla diapausing egg morphotypes belonged to the same operational taxonomic unit (OTU) as an adult in the dataset, and only a single diapausing egg belonging to the *Brachionus* sp. morphotype was unidentified. In Dulce, nine of thirteen diapausing egg morphotypes were assigned to the same OTU as rotifer adult morphospecies.

The remaining three, which had no assignment with adults, were classified into genera *Brachionus*, *Lecane*, and *Polyarthra*. In Tinaja, six of nine diapausing egg morphotypes were identified to species level with their adult rotifer isolated from the water samples. Sequences from diapausing egg morphotype *Brachionus quadridentatus* were grouped within a group of sequences from Dulce and Santa Olalla, and the *Proales* sp. diapausing egg morphotype were classified to genus level. In summary, 85% of sequences from diapausing eggs were assigned to a taxonomic species (17 of the 20 diapausing eggs morphotypes, Santa Olalla 91%, Dulce 69%, and Tinaja 66%; we excluded the adult rotifer *K. cochlearis*).

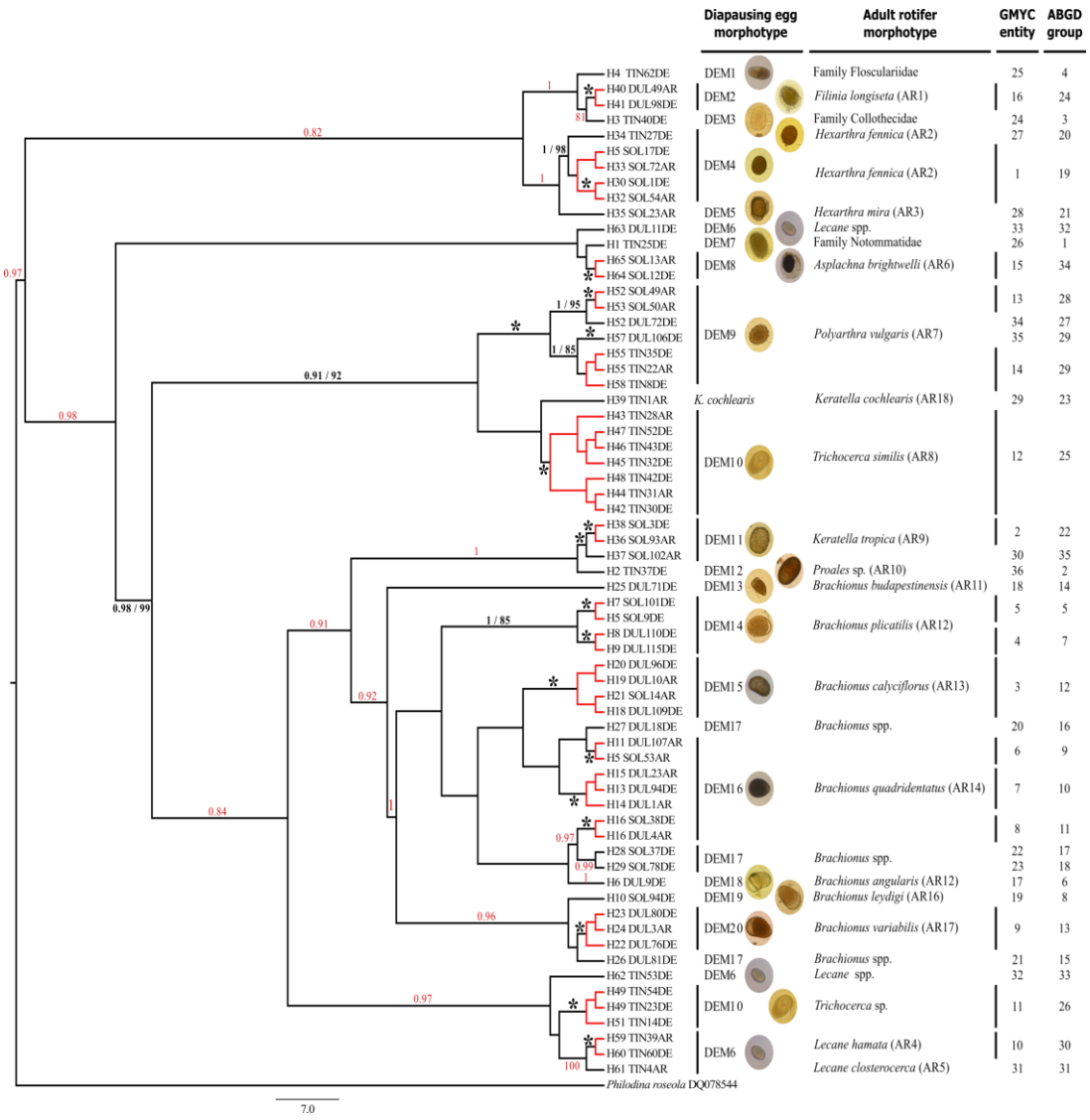

Figure 11. Phylogenetic relationships of the 65 COI rotifer haplotypes newly obtained, according to Bayesian Inference reconstructions. The consensus of 10,000 sampled trees from BI constructed with BEAST using the general time reversible + G + I model. Branch length indicates number or substitutions per site. Posterior probabilities values from the BI reconstruction above 0.8 and 80 for bootstrap support from the ML reconstruction are shown in each branch, respectively. (*) Asterisks indicate values of posterior probability and bootstrap support of 1 and 100 respectively. Values in red show posterior probability or bootstrap support above 0.8 and 80 respectively. Black branches indicate delineated OTUs, and red lines represent haplotypes belonging to the same GMYC entity (OTUs). Diapausing egg morphotype (DEM); adult rotifer morphotype (AR). The number of potential OTUs within each species according to the different methods in DNA taxonomy (ABGD and GMYC on different chronograms) is reported. Monophyletic groups in red indicate a single putative species recognized by the GMYC analysis. Note that the actual samples for each haplotype are detailed in Appendix Table A1.

The most widely represented genus in our dataset was *Brachionus* with 14 GMYC entities, 8 *Brachionus* species in Santa Olalla and Dulce lakes: Two are *B. plicatilis* which are followed in order of abundance by *B. calyciflorus*, *Brachionus angularis*, *Brachionus variabilis*, *Brachionus budapestinensis*, *Brachionus leydigi*, and *B. quadridentatus*, the latter containing three independent GMYC entities, one of them containing sequences from Doñana and Ruidera. The genus *Hexarthra* showed two main species, *Hexarthra mira* and *Hexarthra fennica* in Doñana, and a third *H. fennica* from Tinaja sediment samples, which corresponded to a different GMYC entity. From the genus *Polyarthra*, *Polyarthra vulgaris* was made of three GMYC entities from all study site samples and an unidentified *Polyarthra* single diapausing egg from Dulce. The common genus *Lecane* showed four GMYC entities, two of them from Tinaja Lake included *Lecane closterocerca* and *Lecane hamata* adult rotifers. The other two *Lecane* GMYC entities corresponded to the same diapausing egg morphotype and one included a single diapausing egg from Dulce Lake, and the other contained two diapausing eggs from Tinaja and Dulce lakes. *Keratella tropica* from both Doñana lakes split in two GMYC entities. A group of diapausing eggs identified as *Trichocerca* type formed an independent GMYC entity from *Trichocerca similis*. Seven diapausing egg sequences (three from Santa Olalla and two from Dulce with *Brachionus* diapausing egg morphotype, and three unidentified diapausing egg morphotypes from Tinaja) showed seven GMYC entities with no adult rotifer assignation. Additional species that did not split into different GMYC entities in our dataset were *Asplanchna brightwellii*, *Filinia longiseta*, and *Proales* sp. Similarly, sequences from adult *K. cochlearis* from Tinaja Lake lacked corresponding diapausing egg sequences. Uncorrected *p*-distances between each pair of GMYC entities considered as potential cryptic species varied from the lowest value 0.0378 of *K. tropica* to the highest 0.2431 of *H. fennica* (Supporting information Table S1).

The ABGD analysis indicated a clear barcode gap between 0.05 and 0.12 for Dataset S1 and grouped the sequences into 35 groups (see Supporting information Table S2 and Figure S1), 34 if we only considered the diapausing eggs. The choice between alignments or distance matrix (uncorrected, JC or K2P) had little influence on the results (see Supporting information Table S2). The groupings delimited by ABGD were highly congruent with the most conservative result of the GMYC analyses (Table 2).

Dataset	Clusters	GMYC entities	Likelihood-null	Likelihood-GMYC	Likelihood ratio	Threshold	GMYC entities	ABGD groups
1	16	36 (34-38)	251.9922	293.8043	83.62426	-0.020325	36	35
2	148	285 (271-306)	11089.35	11239.9	301.1003	-0.035099	271 /37*	252 /37*

Table 2. Number of GMYC entities includes the most likely solution and confidence interval of Dataset S1 and Dataset S2 Dataset. *Number of GMYC entities and ABGD groups obtained from our sequences in Dataset S2 analyses.

Cryptic species

For the second dataset, we downloaded the available 3,597 monogonont rotifer COI sequences from GenBank following the criteria described above. To this we added the 210 sequences obtained in this study both from diapausing eggs and adult rotifers. There were a total of 499 positions in the final alignment, after trimming and removing 120 shorter sequences. Sequence alignments and amino acid translations were unambiguous, with no gaps or stop

codons among the 3,686 sequences. A total of 427 polymorphic sites, including 352 parsimony informative sites, defined 1,275 unique haplotypes. The GMYC analysis for Dataset S2, resulted in 148 GMYC entities with strong support, and a total number of 285 GMYC entities (clusters plus singletons and excluding outgroups with a confidence interval 271–306) and it was the most likely model (likelihood of null model: 11089.35, ML of GMYC model: 11239.9, likelihood ratio: 301.1003, LR test: <0.0001) (see Figure S3). ABGD analyses generated 253 groups in the recursive partitions (see Table S2 and Figure S2). The two *B. plicatilis* GMYC entities found in both Doñana lakes were identified as "Almenara" and "Tiscar" belonging to the *B. plicatilis* complex. The GMYC entities with no adult rotifer assignation, TIN25DE, TIN40DE, and TIN62DE (DE, diapausing egg sequence), were identified to Notonmatidae, Collothecidae, and Flosculariidae family (*Floscularia melicerta*). The GMYC entity corresponding to the diapausing eggs DUL24DE and TIN53DE from Dulce and Tinaja respectively and the GMYC entity, which included the single DUL11DE sequence, belonged to the group of *Lecane* spp. species. The *Lecane* sp. diapausing egg morphotype identified as *L. closterocerca* and *L. hamata* was closely related with other *L. closterocerca* and *L. hamata*, supporting the idea that *Lecane* group is a huge species complex. *Polyarthra vulgaris* from Tinaja and Dulce were included in the same GMYC entity with an unpublished sequence of *Polyarthra* sp. The other *P. vulgaris* GMYC entity were closely related to the last GMYC entity and to the only *Polyarthra* diapausing egg morphotype from Dulce Lake. All three GMYC entities seem to be related to the *P. dolichoptera* species complex (Obertegger et al., 2014). *Keratella cochlearis* from Tinaja Lake belonged to the same GMYC entity that an unpublished *K. cochlearis* sequence. Our *B. calyciflorus* sequences were included in the same GMYC entity than *B. calyciflorus* sequences from the Netherlands and China. *Brachionus quadridentatus* showed three GMYC entities, one including a *B. quadridentatus* f. *cluniorbicularis* from Mexico. The four *Brachionus* diapausing egg morphotypes were confirmed.

We compared the number of taxa estimated from the diapausing egg banks and plankton sample with zooplankton species lists which included rotifer species compiled in previous studies from the three locations (Table 3). Our approach retrieved higher number of taxa for both Doñana lakes than in previous studies using conventional sampling techniques in the zooplankton (López et al., 1991; Galindo et al., 1994; Guisande et al., 2008). However, in Tinaja Lake, our approaches estimated fewer taxa both in the egg bank and in the water column than classical methods in a long term sampling campaign which included one sample every 2–3 months over 3 years in **Chapter 5** and other studies (Álvarez-Cobelas et al., 2007).

Location	GMYC entities			ABGD			Plankton samples (other works)
	Total entities	Sediment sample	Plankton sample	Total groups	Sediment sample	Plankton sample	
Tinaja	14	12	7	14	12	7	18 morphospecies and 15 samples (2008-2011)[a] 25 morphospecies and 4 samples (2001-2002)[b]
Santa Olalla	18	14	14	18	14	14	12 morphospecies and 17 samples (2008-2011)[a] 4 morphospecies and 23 samples (1985-1987)[b] 7 morphospecies and 21 samples (1989-1992)[c] 5 morphospecies and 6 samples (2004)[e]
Dulce	19	18	12	19	18	12	16 morphospecies and 17 samples (2008-2011)[a] 11 morphospecies and 23 samples (1985-1987)[b] 11 morphospecies and 21 samples (1989-1992)[c] 8 morphospecies and 6 samples (2004)[e]

Table 3. Total number of GMYC entities, GMYC entities retrieved from diapausing egg banks and from zooplankton samples. Number of taxonomic species and samples from previous work in our study sites.
[a]Moreno *et al.*, in prep.
[b]Álvarez-Cobelas *et al.*, 2007
[c]López *et al.*, 1991
[d]Galindo *et al.*, 1994
[e]Guisande *et al.*, 2008

Discussion

This is the first study using DNA barcoding on diapausing egg banks from a single sediment sample to characterize rotifer communities in continental aquatic systems. We generated a reference collection by barcoding individuals sampled from the water column, to which we assigned sequences obtained from diapausing eggs from sediments. Our DNA taxonomy analysis yielded 35 GMYC entities in the diapausing egg bank, a substantially higher number than the 20 types of diapausing eggs previously identified based on morphology. We identified 61% of GMYC entities to taxonomic species level and a 91% to likely genus level, with a combination of two reference data sets, one generated in this study and one downloaded from GenBank. Our approach, based on a single sediment sample, gave higher or similar estimates of rotifer biodiversity than previous studies based on a number of seasonal samples, reducing time and sampling effort.

Temporary versus permanent environments

The success of the application of barcoding to diapausing egg banks might differ between ponds with different hydrology, as this is known to influence the investment of rotifer species into diapausing eggs. Seasonal environments with fluctuating conditions may induce a higher diapausing egg production (Cáceres and Tessier, 2004; García-Roger et al., 2005, Pérez-Martínez et al., 2007; Altermatt and Ebert, 2008). Indeed, Santa Olalla and Dulce show drastic hydrological fluctuations between years as we described in **Chapter 2** and both Doñana Lakes present a higher abundance of diapausing eggs in comparison with Ruidera. In contrast, in permanent habitats dormancy might not be strictly necessary, and asexual reproduction would be favored (Serra and King, 1999; Schröder, 2005); therefore, some common rotifer species in more permanent environments may either lack a diapausing egg stage or produce few diapausing

eggs (for instance, as we observed for *K. cochlearis* from Tinaja Lake, and previously reported by Dumont, 1983). In consequence, DNA barcoding would be expected to be a more useful tool on diapausing egg banks in temporal–semipermanent systems than in permanent ones. Therefore, in the case of permanent and stable water bodies such as Ruidera Lakes, this molecular technique should be performed using both sediment and water samples simultaneously to include diapausing eggs and adults and obtain a good characterization of the rotifer community.

Reference collections

To aid in the identification of the diapausing eggs obtained, it is necessary to compare the sequences obtained to a curated reference collection of barcodes. For this, we (i) gathered a representative and taxonomically identified rotifer species list from the water column of the sampled lakes as a reference collection for rotifer diapausing egg bank characterization of the local area and (ii) we compared our dataset with rotifers sequences published in GenBank to identify cryptic species previously described. We avoided the limitations of using hatching from sediment samples, as optimal hatching cues can be species-specific and bet-hedging might limit the number of viable eggs that hatch from a given sample (Schröder, 2005; Vandekerkhove et al., 2005; García-Roger et al., 2014), as well as the undescribed diversity of diapausing egg morphologies (Gilbert and Wurdak, 1978; Snell et al., 1983; Munuswamy et al., 1996). Therefore, DNA barcoding might solve a misdiagnosis but we need a reference collection of barcodes.

Using diapausing eggs as proxies to estimate biodiversity (Pourriot and Snell, 1983; Ricci, 2001) might incur biases due to the lack of diapausing egg production in some species or habitat heterogeneity. Hence, some discrepancies between sediment samples and plankton samples are expected, they reflect the differences in sampling intensity (zooplankton survey vs sediment sample). A single sediment sample might reflect the biodiversity or composition of a diapausing egg bank, but cannot reflect their habitat heterogeneity (Chittapun et al.,2005). In addition, the usefulness of the combination of collecting plankton and sediment samples can be improved using DNA barcoding (Hebert et al., 2003).

Cryptic species

The species list made for our study sites reveals the presence of three rotifer taxonomic species that are part of well studied and described cryptic complexes (*B. plicatilis*, *B. calyciflorus* and *P. vulgaris*) and four potential cryptic complexes (*B. quadridentatus*, *K. tropica*, *H. fennica* and *Lecane* spp.). To verify the presence of cryptic species we used DNA taxonomy analyses, which provided higher taxonomic resolution than morphological identification. GMYC identified 36 entities from 17 adults and 20 different morphological diapausing eggs. ABGD also clustered the sequences into 35 groups congruent with the most conservative GMYC result. *Brachionus plicatilis* from Doñana samples and *B. quadridentatus* from Doñana and Ruidera samples belonged to different OTUs. The rest of *Brachionus* species with both diapausing egg and adult samples were *B. angularis*, *B. budapestinensis*, *B. leydigi*,

and *B. variabilis*. The remaining four *Brachionus* groups were singletons from diapausing eggs without adult confirmation. Those results reveal the widespread presence of this genus in these freshwater systems and its importance in zooplankton communities. In the *Keratella* group, *K. tropica* splits in two groups with strong support, suggesting the presence of potential cryptic species in both Doñana lakes. Other diverse group was the genus *Lecane*, which seems to comprise numerous undescribed cryptic species complexes (Walsh et al., 2009; García-Morales and Elías-Gutiérrez, 2013). The absence of Colothecaceae and Flosculariidae adults in the water-column samples is not surprising as these are may be related with the species of the family Collothecidae, which are mainly benthonic (De Manuel, 2000). The absence of corresponding sequences from adults from the water column for some of the GMYC entities found could also be due to the fact that a single plankton samples was used to build out reference collection.

The application of GMYC and ABGD analysis to Dataset S2 which included all the monogonont rotifer COI sequences available in GenBank in addition to our sequences, revealed that some cryptic species in our dataset belonged to previously described species complexes (Gómez et al., 2002; Xiang et al., 2011; García-Morales and Elías-Gutiérrez, 2013; Obertegger et al., 2014). The most studied rotifer cryptic species complexes, *B. plicatilis* and *B. calyciflorus*, split in 33 and 12 entities, respectively in Dataset S2. The *B. calyciflorus* entity found in Doñana samples belonged to the group of BcwII published by Xiang et al. (2011), probably a cosmopolitan species also collected in the Netherlands and China. In Doñana, Dulce, and Santa Olalla lakes, the most abundant monogonont rotifer was *B. plicatilis* in both sediment and freshwater samples, which split in "Almenara" and "Tiscar" species (Gómez et al., 2002; Ortells et al., 2006). *Brachionus* "Almenara" and *Brachionus* "Tiscar" were previously found by Ortells et al. (2006) in Mediterranean ponds, together with *B. plicatilis* "sensu stricto" also found in Doñana brackish water ponds (Badosa et al., 2017). Other *Brachionus* species which seems to form a species complex is *B. quadridentatus*, which includes three independent OTUs, two from Doñana lakes and a third one from Doñana and Ruidera. One of these OTUs includes a *B. quadridentatus* sequence from Mexico (García-Morales and Elías-Gutiérrez, 2013). In addition, sequences belonging to the morphospecies *P. vulgaris* from Doñana and Ruidera split into four OTUs. We also found for the first time a potential cryptic group of *Hexarthra* species, since that *H. fennica* from Doñana and Ruidera split in two OTUs and comprises a monophyletic group with strong support. The sequences we obtained for *Hexarthra* species are the first ones available (GenBank accession numbers KY749446– KY749454). All individuals of *H. fennica* from diapausing eggs and adult samples were morphologically homogeneous; nevertheless, we identified two potentially cryptic species based on GMYC and ABGD analyses, with genetic distances of 0.2431, one located in Tinaja Lake from Ruidera, and the other located in Santa Olalla Lake from Doñana.

Our study underscores the common coexistence of zooplanktonic cryptic species in rotifers (Gómez et al., 2002; Ortells, Gómez, & Serra, 2003; Gómez et al., 2007; Li et al., 2010; Obertegger et al., 2012; Gabaldón et al., 2016;). *Brachionus plicatilis* species complex is the best studied group of cryptic species with a well described representation of their coexistence in the Iberian Peninsula (Gómez et al., 2002). We found *Brachionus* "Tiscar" and *Brachionus*

"Almenara" coexisting in two nearby lakes (Santa Olalla and Dulce). These species have been previously described in Mediterranean lakes, but they have never been found coexisting in the same lake (Gómez et al., 2002; ; Ortells et al., 2006 Montero-Pau et al., 2011). "Almenara" was restricted to coastal lagoons of low salinity and "Tiscar" has been found in inland and coastal lakes. There is no evidence of both species co-occurring in the same water body. We show for the first time their coexistence of these cryptic species either in water column and sediments in Santa Olalla and Dulce lakes, although it is not clear if both cryptic species coexist at the same time in the water column.

An emerging and highly promising molecular approach and alternative technique to DNA barcoding zooplankton communities might be DNA metabarcoding (e.g., see Deiner et al., 2016). DNA metabarcoding is a high-throughput multispecies-identification tool from a single bulk sample containing entire organisms or degraded DNA (Taberlet et al., 2012). However, the application of DNA metabarcoding to rotifer diapausing eggs has some constraints. First, the multispecies-identification approach requires that the primers used are highly versatile to avoid biases in species amplification, but at the same time specific to the group of interest. Second, quantitative information is difficult to extract from the sequence information. Third, no morphological information can be linked to each individual. Despite these problems, the increasingly used and cost-effective of DNA metabarcoding might eventually become an effective tool applied to zooplankton diapausing egg banks characterization.

Conclusion

Our results highlight how an integrated taxonomic approach, combining DNA barcoding of diapausing eggs from a single sediment sample with the rapidly improving rotifer reference collection of DNA barcodes can be an efficient method to characterize rotifer communities from lentic aquatic systems. These results agree with previous studies that have shown how the zooplankton community characterization from diapausing egg banks is more effective than intensive samplings of active communities from different seasons (May, 1986; Duggan et al., 2002; Vandekerkhove et al., 2004), reducing time and sampling effort. Our molecular approach, based on DNA barcoding and DNA taxonomy, solve the problems related to diapausing eggs morphological identification and hatching cues and not only correctly classified a high percentage of the rotifer diapausing egg sequences, but also revealed the occurrence of cryptic species overlooked by traditional taxonomic methods. The incorporation of GMYC analysis for delimiting species boundaries as a complementary tool for DNA barcoding facilitated the identification of new cryptic species. Although GMYC has the tendency to oversplit in comparison with ABGD (Tang et al., 2012), both techniques gave similar results, resulting in accurate species delimitation and the presence of potentially undescribed cryptic species. DNA taxonomy for species delimitation should be considered as a first step for taxonomic identifications instead of a conclusive taxonomic tool (Kekkonen and Hebert, 2014). Nevertheless, we support the idea of integrative taxonomic approach as DNA barcoding might be a powerful complementary tool, in such cases where rotifer species do not produce diapausing eggs or they are produced in very low quantities and problems with the DNA preservation or amplification. Finally, our understanding of rotifer communities from diapausing egg banks

would benefit tremendously from the further development of the rotifer reference database of barcodes.

Supporting information

	P-distance	K2P	JC
Brachionus Almenara - *Brachionus* Tiscar	0.1600 ± 0.0167	0.1813 ± 0.0223	0.1800 ± 0.0212
B. quadridentatus I - *B. quadridentatus* II	0.1564 ± 0.0159	0.1771 ± 0.0205	0.1755 ± 0.0205
B. quadridentatus I - *B. quadridentatus* III	0.1621 ± 0.0166	0.1839 ± 0.0217	0.1826 ± 0.0212
B. quadridentatus II - *B. quadridentatus* III	0.1642 ± 0.0162	0.1866 ± 0.0214	0.1853 ± 0.0200
H. fennica I - *Hexarthra fennica* II	0.2431 ± 0,0195	0.3003 ± 0.0308	0.2939 ± 0.0286
K. tropica I - *K. tropica* II	0.0378 ± 0.0083	0.0392 ± 0.0094	0.0388 ± 0.0090
Polyarthra vulgarica I - *Polyarthra vulgarica* II	0.0449 ± 0.0089	0.0467 ± 0.0092	0.0463 ± 0.0092
Polyarthra vulgarica I - *Polyarthra vulgarica* III	0.1726 ± 0.0148	0.1805 ± 0.0208	0.1600 ± 0.0208
Polyarthra vulgarica I - *Polyarthra vulgarica* IV	0.1600 ± 0.0175	0.1973 ± 0.0224	0.1961 ± 0.0224
Polyarthra vulgarica II - *Polyarthra vulgarica* III	0.1726 ± 0.0171	0.1973 ± 0.0092	0.1961 ± 0.0216
Polyarthra vulgarica II - *Polyarthra vulgarica* IV	0.1782 ± 0.0175	0.2050 ± 0.0229	0.2035 ± 0.0228
Polyarthra vulgarica III - *Polyarthra vulgarica* IV	0.1284 ± 0.0148	0.1433 ± 0.0184	0.1408 ± 0.0176

Table S1. Uncorrected p-distances between each pair of GMYC entities considered as potential cryptic species, Jukes-Cantor model (JC) and Kimura's two-parameter model (K2P).

			Prior intraspecific divergence (*P*)										
Substitution model	**X**	**Partition**	**0.0028**		**0.0046**		**0.0077**		**0.0129**		**0.0215**		**0.0359**
			Dataset 1	Dataset 2	Dataset 1	Dataset 2	Dataset 1	Dataset 2	Dataset 1	Dataset 2	Dataset 1	Dataset 2	Dataset 1
		Initial	34	263	34	263	34	263	34	263	34	263	34
JC69	1.5												
		Recursive	43	418	42	359	42	325	41	287	38	253	35
		Initial	34	263	34	263	34	263	34	263	34	263	34
K80	1.5												
		Recursive	43	416	42	359	42	331	41	287	38	250	35
		Initial	34	263	34	263	34	263	34	263	34	241	34
Simple distance	1.5												
		Recursive	40	309	40	290	38	282	38	267	38	249	35

Table S2. Results of the Automatic Barcode Gap Discovery (ABGD) analyses for each substitution model, including the number of groups obtained in the initial and recursive partition in the different prior intraespecific divergences (*P*). **JC69**, Jukes-Cantor substitution model; **K80**, Kimura 2-parameter substitution model; **Simple distance**, p-distance. **X**, relative gap width.

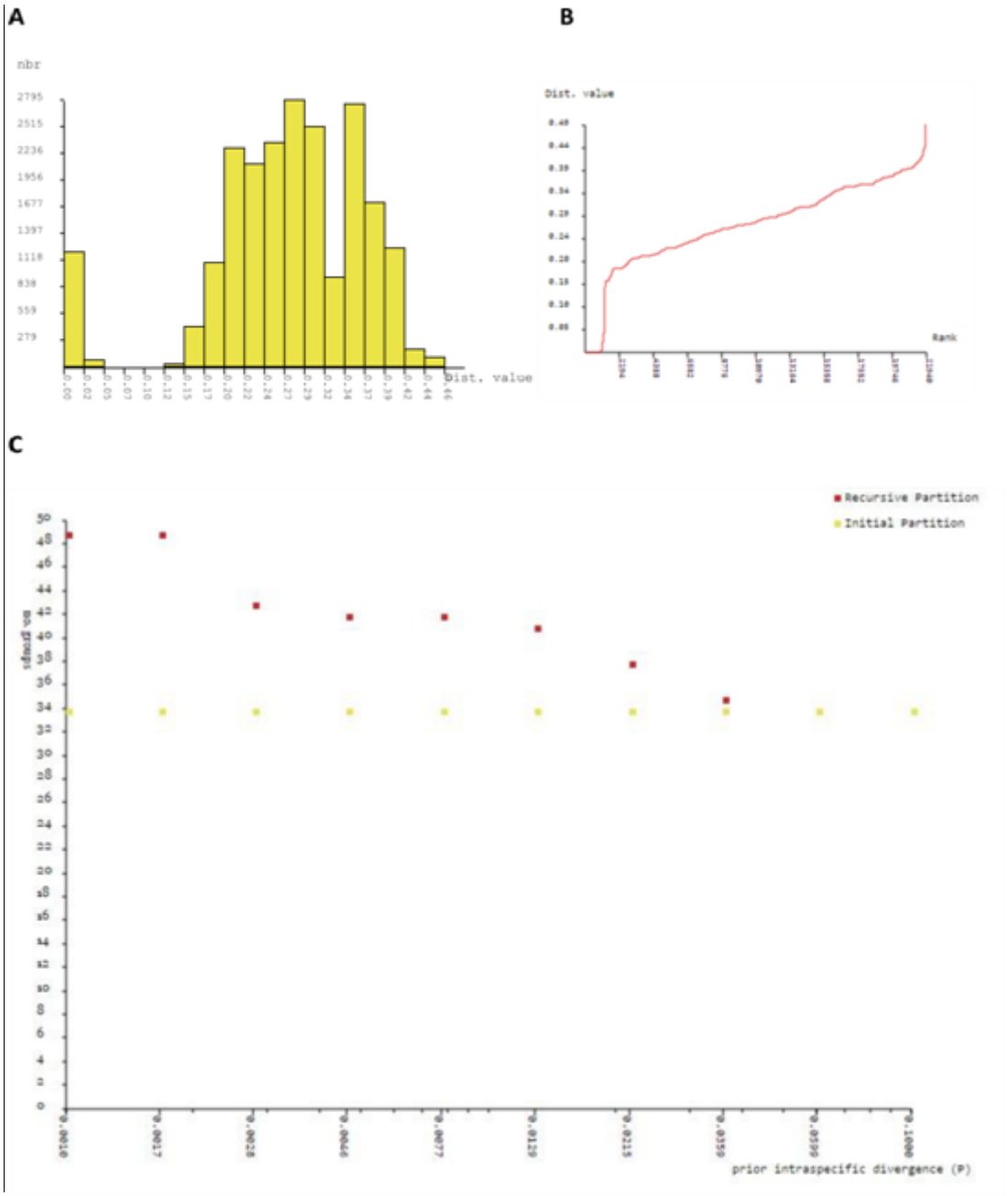

Figure S1. Barcode gap analysis generated by Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012). (A) histogram with distributions of Jukes-Cantor distances (Jukes & Cantor, 1969) between each pair of COI sequences, (B) ranked distances and (C) automatic partition based on Dataset1.

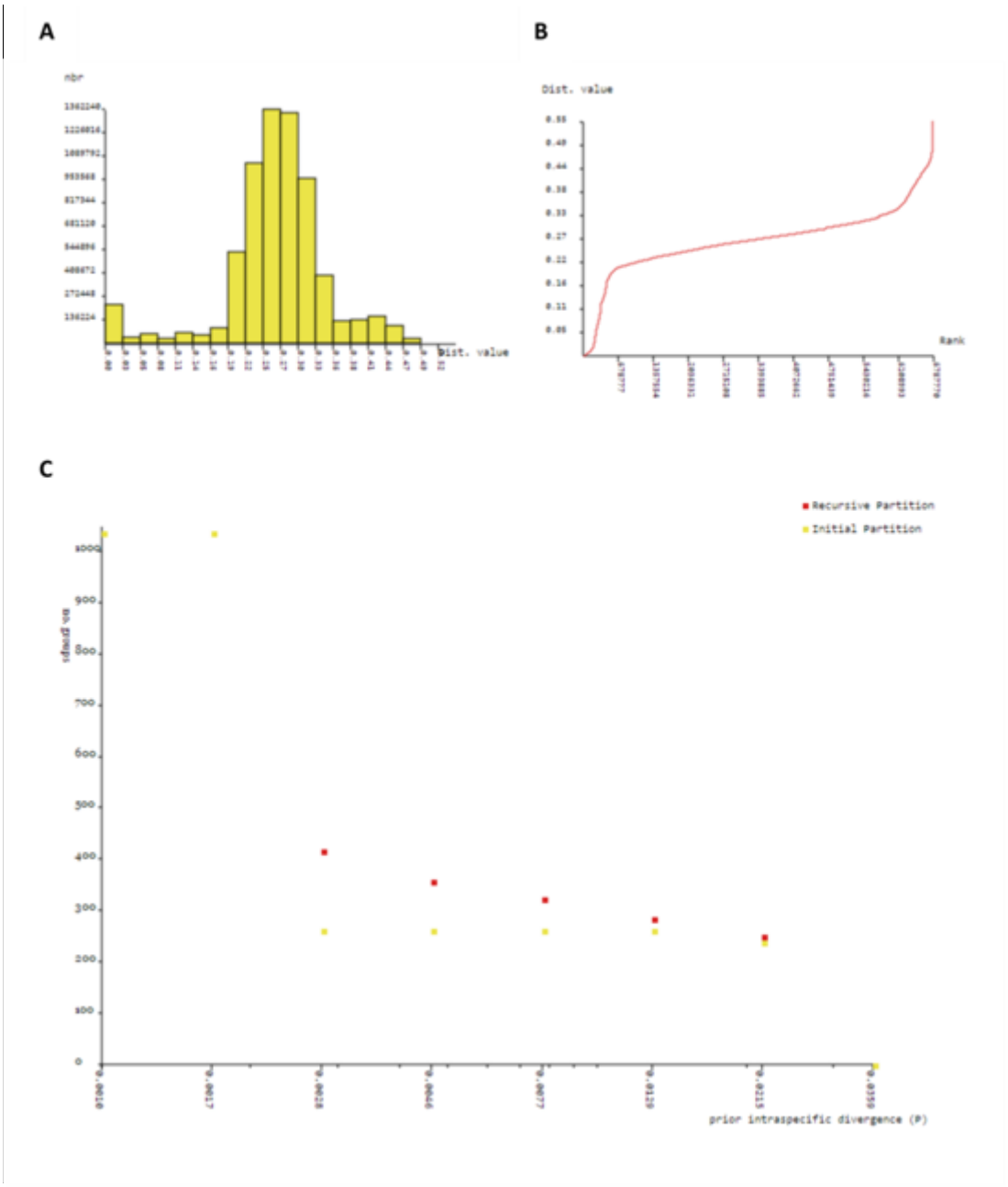

Figure S2. Barcode gap analysis generated by Automatic Barcode Gap Discovery (ABGD) (Puillandre et al. 2012). (A) histogram with distributions of Jukes-Cantor distances (Jukes & Cantor, 1969) between each pair of COI sequences, (B) ranked distances and (C) automatic partition based on Dataset2.

Chapter 2

Dispersal of Zooplankton Dormant Propagules by Wind and Rain in Two Aquatic Systems

Abstract

Zooplankton dispersal may regulate population dynamics and the structure of aquatic communities. Zooplankton and other aquatic invertebrates, such as freshwater bryozoans, are potentially dispersed overland by abiotic vectors (e.g., wind, rain and water flow) or by organisms (e.g., waterfowl and insects), and although these dispersal vectors have been widely studied, the importance of dispersal by wind remains controversial. In addition, little information is available on passive deposition rates and the differentiation between dry deposition (sedimentation from the air) and wet deposition (from rainfall). In the present study, we quantified zooplankton propagule dispersal by passive deposition from the air and rainfall using deposition collectors designed to gather samples from dry or wet atmospheric deposition. The collectors were located in two regions with distinct limnologic and topographic characteristics: Doñana National Park and Ruidera Natural Park. In Doñana, we also used windsocks to intercept the dormant propagules dispersed by wind, and a larger number were collected in the dry atmospheric deposition collector than in the wet one. Moreover, the deposition of dormant propagules was only related to the meteorological variables, wind direction and temperature, and most of the propagules appeared to arrive in Doñana from the northwest.

Our results indicate that overland dispersal by wind and rain is relatively infrequent and probably limited to a few zooplankton species. Despite the few dormant propagules that were collected, they were present in passive deposition collectors and windsocks. Aerial overland dispersal at low rates implies long-term relevance to the genetic structure of zooplankton and their colonization of water bodies.

Introduction

The dispersal of freshwater invertebrates is crucial to the development of aquatic communities, and its role in shaping their structure is an important research question for aquatic ecologists. Freshwater organisms have traditionally been considered cosmopolitan due to their wide distribution and their capacity for dispersal between water bodies (Darwin, 1859), although differences in dispersal capacity have been observed among taxa. Zooplankton and freshwater bryozoans produce numerous dormant propagules that resist desiccation and passively disperse overland between water bodies in diapause as resting eggs, cysts, or statoblasts, among other cryptobiotic stages (Hairston, 1996; Brendonck and Riddoch, 1999; Bilton et al., 2001; Panov et al., 2004). These propagules can be dispersed by wind (Jenkins and Underwood, 1998; Brendonck and Riddoch, 1999; Caceres and Soluk, 2002; Cohen and Shurin, 2003; Vanschoenwinkel et al., 2008b; Vanschoenwinkel et al., 2008a), water flow (Michels et al., 2001; Vanschoenwinkel et al., 2008b), and biotic agents (Maguire, 1963; Green et al., 2008; Vanschoenwinkel et al., 2008c). Their dispersal has been quantified and the effectiveness of the dispersal vectors has been evaluated by various means, including the interception of dormant propagules borne overland by wind and rain (Jenkins and Underwood, 1998; Brendonck and Riddoch, 1999; Vanschoenwinkel et al., 2008a); the performance of colonization experiments taking wind, rainfall, and/or animals into account (Caceres and Soluk, 2002; Cohen and Shurin, 2003; Frisch et al., 2012; Sciullo and Kolasa, 2012); and the study of water flows (Allen, 2007; Sciullo and Kolasa, 2012). However, the results of these investigations have been inconsistent.

The low wind dispersal rates found for zooplankton dormant propagules led some researchers to question the importance of this vector (Jenkins, 1995, Jenkins and Underwood, 1998, Brendonck and Riddoch, 1999), and it has been proposed that zooplankton passively dispersed by wind are relatively slow to colonize new habitats (Jenkins and Buikema, 1998; Jenkins and Underwood, 1998; Cohen and Shurin, 2003). However, observations of passive dispersal are influenced by several methodological factors, including the proximity of dispersal collectors (artificial mesocosms, windsocks, or sticky traps) to water bodies with egg bank sources, their elevation, and their orientation relative to the prevailing winds (Cáceres and Soluk, 2002; Vanschoenwinkel et al., 2008a; Vanschoenwinkel et al., 2008b). Higher wind dispersal rates of dormant propagules were observed (using windsocks and sticky traps placed very near sources) in studies of rock pool metacommunities (Vanschoenwinkel et al., 2008b; Vanschoenwinkel et al., 2008b), but the collected samples did not accurately represent the zooplankton community as there was an absence of rotifers (Vanschoenwinkel et al., 2008b; Vanschoenwinkel et al., 2008b; Sciullo and Kolasa, 2012). Overall, the most abundant passive dispersers appear to be monogonont and bdelloid rotifers followed by cladocerans (Cáceres and Soluk, 2002; Cohen and Shurin, 2003). The proximity and exposure of the resting egg bank and the direction rather than speed of the wind may be the most relevant factors in wind dispersal (Vanschoenwinkel et al., 2008b). Egg banks may be more exposed to the wind during the dry season in aquatic systems with highly fluctuating water levels (Bilton et al., 2001; Vanschoenwinkel et al., 2008b; Tuytens et al., 2014), so the key dispersal vectors may be wind

during dry seasons and water flow during wet seasons (Hulsmans et al., 2007). However, only a few studies have taken the passive deposition of resting eggs from the air into account (Jenkins and Underwood 1998; Sciullo and Kolasa, 2012), and these studies did not discriminate between passive wet deposition (due to rainfall) and passive dry deposition (due to sedimentation from the air).

The main objectives of the present study were i) to compare the dispersal of zooplankton, including rotifers and bryozoans, by wind and rainfall between two regions with different limnologic and topographic characteristics (Doñana National Park and Ruidera Natural Park) using automatic atmospheric deposition collectors to examine differences between dry and wet passive deposition; ii) to investigate the relationship between meteorological variables and the passive deposition of propagules in Doñana; and iii) to measure the dormant propagules dispersed by wind in Doñana using two windsocks.

Materials and methods

The dispersal of zooplankton dormant propagules by wind was studied in Ruidera Natural Park and Doñana National Park. More details of the study site are described in the general methodology section of this thesis. The dispersal of the dormant propagules reaching the lakes by wind and rainfall was measured using MTX® ARS 1010 automatic dry/wet passive collectors (MTX Italia SPA, Modane, Italy) equipped with two polyethylene buckets (surface area of 0.0667 m^2 each) and a hygroscopic sensor cell. This device activated an aluminium lid that covered the dry bucket and uncovered the wet bucket during rainfall and *vice versa* during periods without rain. One collector was located in Ruidera between the *del Rey* (Ciudad Real, Spain) and *Colgada* (Ciudad Real/Albacete, Spain) lakes (38°57'48.70"N, 2°53'15.35"W; elevation 790 m; Ciudad Real, Spain), and two were located in Doñana (Huelva, Spain), one on the east side of *Santa Olalla* lake (Doñana A; 36°58'47.38"N, 6°28'22.40"W; elevation 8 m) and the other between the *Dulce* and *Santa Olalla* lakes (Doñana B; 36°58'43.60"N, 6°28'59.82"W; elevation 6 m), which are two coastal shallow peridune ponds and natural eutrophic to hypereutrophic systems surrounded by a number of temporary ponds (see Figure 12). The distances of the Doñana A and Doñana B collectors from the nearest water body were 30 m and 100 m, respectively, and the Ruidera collector was located 30 m from the nearest lake. Dry and wet deposition buckets were collected every one to three months during 2008, 2009 and 2010 in Doñana and during 2008 and 2009 in Ruidera. On each sampling date, the dry and wet deposition buckets from the three collectors were replaced and taken to the laboratory. First, the dry deposition was carefully and thoroughly examined to detect and gather any bird faeces or large insects, which were then inspected for the presence of dormant propagules. We also found an important amount of plant seeds, but we did not take them into account. Next, the deposition bucket was rinsed with distilled water, and this solution and the contents of the wet deposition bucket were pre-filtered with a 500-µm nylon mesh. The particles retained in the nylon mesh were examined under a stereoscopic microscope. The pre-filtered water was then filtered through a 10-µm nylon mesh, and the contents were rinsed with distilled water and examined under a stereoscopic microscope to detect dormant propagules.

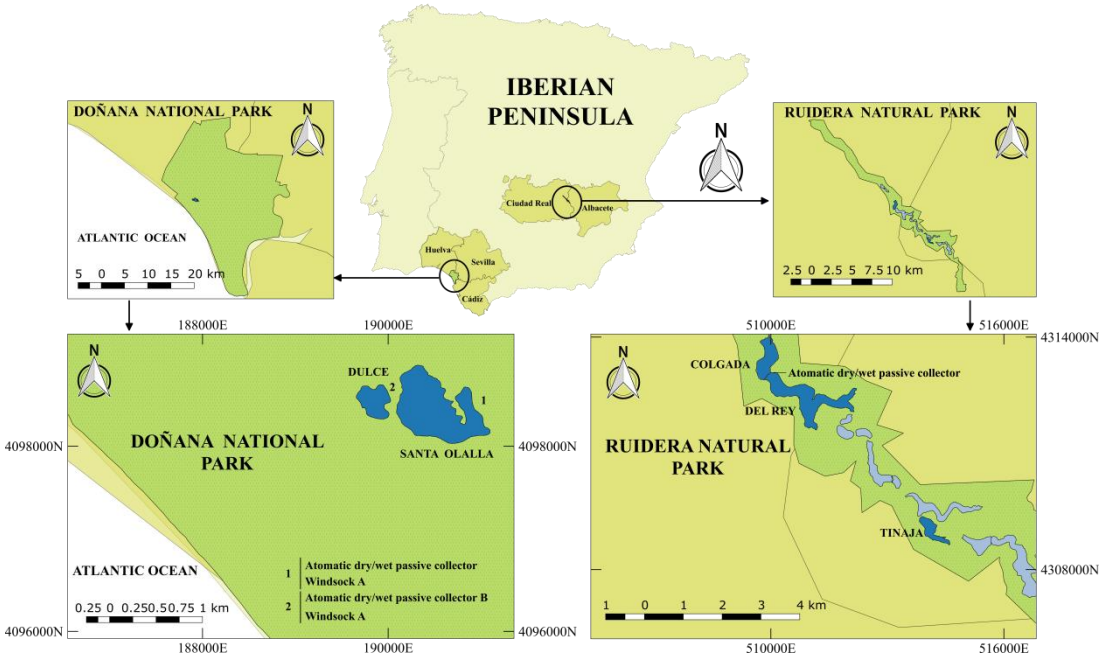

Figure 12. Map of the study areas and the locations of the automatic dry/wet passive collectors and windsocks in Doñana (left) and the automatic dry/wet passive collector in Ruidera (right).

Because of the consistently higher density of dormant propagules collected in Doñana Park (see Results), the dispersal by wind was also evaluated during the last four months of the study using two windsocks, which were conical, 30-µm plankton nets fitted to a conical metal frame that rotated in the direction of the wind (Vanschoenwinkel et al., 2008b). One was placed close to the Doñana A collector (Windsock A) and the other close to the Doñana B collector (Windsock B). They were in place from June to October 2010 and were collected and replaced monthly, except for Windsock B during the last month (due to tearing). Each collected windsock was carefully rinsed with distilled water to obtain all of the intercepted propagules, and the water was then collected in bottles and kept in dark, cold conditions during transport to avoid breaking diapause. Each of the dormant propagules obtained from the collectors and windsocks and identified under the stereoscopic microscope was photographed and then placed in a well of a 96-well polyethylene microplate under a 14 h light/10 h dark-photoperiod and a temperature of 20 ºC, which simulated summer conditions for hatching (Jenkins & Underwood, 1998; Vandekerkhove et al., 2004).

Infraestructura Científica y Tecnológica Singular (ICTS; http://icts.ebd.csic.es) provided daily meteorological data including wind speed and direction, precipitation (mm), and air temperature (°C) from the *Control* RM1 Station (37°1'21"N, 6°33'19"W; elevation 6 m), which was located 8 km from the Doñana B collector and 8.70 km from Doñana A. ICTS also provided accumulated evaporation (mm) and temperature (ºC) data from the *Cancela Millan* RMN2 Station (37º 1' 9" N, 6º 21' 55"W; elevation 2 m), which was located 10.50 km from Doñana A and 11.40 km from Doñana B. Meteorological data were not gathered in Ruidera Park because of the small number of dormant propagules collected (see Results).

Statistical analysis

Statistical analyses were performed using program R 2.14.0 (R Foundation for Statistical Computing). For these analyses, we used the number of dormant propagules collected per day, accounting for the total number of days that the automatic dry/wet passive collectors were working, and the average values of the meteorological variables (wind direction, wind speed, precipitation and temperature) between sampling days. The meteorological variables related to the number of dormant propagules were selected by model selection, which was based on the second-order Akaike information criterion (AIC_c) because of the small sample sizes (Burnham and Anderson, 2002). The AIC_c values were compared following the convention that if the ΔAIC_c (differences in AIC_c between each model and the model with the minimum AIC_c) is less than 2, the two models have relatively equal support. Otherwise, the model with the lowest AIC_c value was considered to be the most plausible model (Burnham and Anderson, 2002). As we have no previous information about the possible models to explain propagule deposition, we include all of the possible models with the independent variables as well as the null model with only an intercept term. The normality of the residuals of the selected models was tested with the Shapiro test. Additionally, the Akaike weights were summed (cumulative AIC_c weights) over all possible models containing a given variable to measure the relative importance of each independent variable (Burnham and Anderson, 2002; Burnham and Anderson, 2004). The larger the cumulative AIC_c weight, the more important the variable is relative to the other variables. Barbieri and Berger (2004) suggest that variables with a cumulative weight ≥ 0.5 show strong evidence of inducing a response in the dependent variable. All of the meteorological variables were previously log-transformed because, in all cases, the models showed a higher explanatory power (higher R^2). Model selection was conducted using the AICcmodavg package (Mazerolle, 2015).

Additional correlation analyses were used to explore the relationships between some meteorological variables and for studying the propagules collected in windsocks because of the few data available. The non-parametric Spearman's test was used when the normality assumption was violated, and the Oriana program (Kovach Computing Services) was applied to obtain the vector-averaged wind direction for each period and a circular histogram. The circular wind direction covariate was accommodated by including the sine and cosine of the wind direction rather than the wind direction itself during model selection (Johnson and Wehrly, 1978; Mardia and Jupp, 2000; Jammalamadaka and Lund, 2006). STATISTICA (Statsoft) was used for the remaining histograms and graphs.

Results

A total of 47 zooplankton and bryozoan dormant propagules were collected in the passive automatic deposition collectors and windsocks (Table 1). Considering the number of days that the collectors were working, an average of 0.076 dormant propagules/day/m^2 (per collector bucket) were passively deposited in Doñana, and 0.040 dormant propagules/day/m^2 (per collector bucket) were passively deposited in Ruidera. In the windsocks, an average of 0.163

dormant propagules/day per windsock were intercepted in Doñana. The propagules collected in the Doñana passive collectors were gathered between June and November in 2008 and between June and September in 2010, while those in the Ruidera collectors were gathered between July and September in 2008 and in January and February in 2009. The most abundant zooplankton dormant propagules were rotifers followed by ostracods and cladocerans. Dormant propagules of rotifers of the genus *Brachionus* were found in the Doñana A wet, Doñana B dry, and Ruidera dry collectors and those of the genus *Hexarthra* in the Doñana B wet and dry collectors. Cladocera ephippia of *Daphnia magna* were collected in Doñana, and bryozoans were represented by *Plumatella* statoblasts collected in both collectors and windsocks. Finally, two live bdelloids were found in the Ruidera wet collector. A larger amount of propagules was collected in the collectors in Doñana than in Ruidera (Figure 13), and the maximum detection rate was 0.548 dormant propagules/day/m^2 in the Doñana collectors *versus* 0.713 dormant propagules/day/m^2 in the Ruidera passive collector, corresponding to the sampling in October 2008 and January 2009, respectively. The two windsocks deployed in Doñana collected a higher density of propagules with greater taxonomic richness over a shorter time period in comparison with the passive deposition collectors (Table 4). Rotifers from the genus *Brachionus* and resting eggs from two different *Hexarthra* species were intercepted in both windsocks, and one resting *Keratella tropica* egg was found in Doñana windsock A, which had a healthy appearance and was the only dormant propagule hatched under the present study conditions. Ostracod dormant propagules were collected in both windsocks, and cladocerans were represented by a *Daphnia longispina* ephippium. The most abundant species in the windsocks were the same as in the atmospheric deposition samplers.

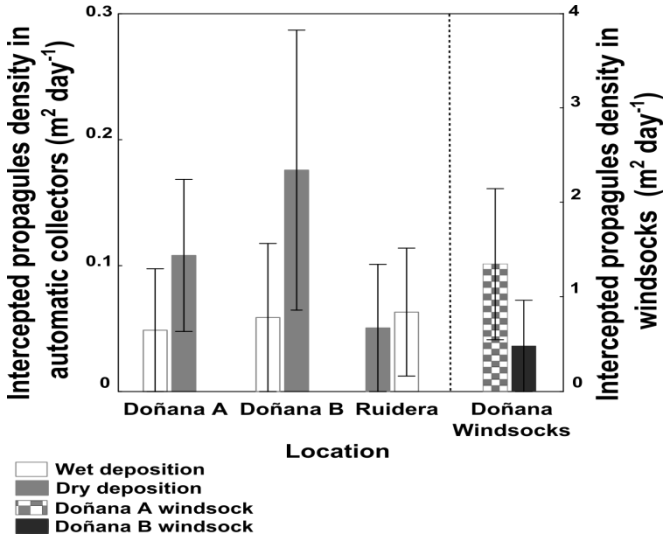

Figure 13. Dispersal rates of zooplankton dormant propagules intercepted by the automatic dry/wet passive collectors located in Doñana and Ruidera (left side of the figure) and the wind-socks located in Doñana (right side of the figure). Error bars correspond to standard errors. Distances of collectors and wind- socks from the main sources of dormant propagules: 30 m to Doñana A and Windsock A, 100m to Doñana B and Windsock B and 30 m to Ruidera.

	Brachionus spp.	*Hexarthra* spp.	*Keratella* spp.	Bdelloids	*Plumatella* spp. statoblast	Cladoceran ephippia	Ostracods	Total
Doñana A Wet	1				1			2
Doñana A Dry					3	1		4
Doñana B Wet		2						2
Doñana B Dry	3	2			4	1		10
DoñanaWindsock	2	6	3		3	1	3	18
DoñanaWindsock	1	2			2	1	2	8
RuideraWet				2				2
RuideraDry	1							1
Total								47

Table 4. Zooplankton dormant propagules collected in the automatic dry/wet passive collectors located in Doñana and Ruidera and in the windsocks located in Doñana.

According to the AIC_c, the best model to explain the dry deposition of propagules is the model using the cosine of wind direction and temperature as independent variables (see Supporting information Table S3). However, other models are also plausible candidates, and even the null model was selected, especially for the total propagule deposition (see Supporting information Table S3). However, when we analysed the relative importance of each independent variable, we found that the temperature and the cosine of wind direction are more important than the other variables according to their Akaike weights (Table 5). As a consequence, in our study, propagule deposition in the dry deposition containers seems to be mainly related to temperature and wind direction, although the results were unclear when considering total deposition (including wet containers) (Table 5). Similar results were observed for zooplankton dormant propagules alone (ostracods, cladocerans, and rotifers) and for rotifer resting eggs alone (Table 5). As shown in Figure 3, wind speeds ranged from 2.3 m/s to 3.1 m/s in Doñana, and the prevailing wind was from the west. However, wind speed seems to not be relevant in our study. The accumulated evaporation was significantly correlated with temperature (Spearman's nonparametric correlation, rho = 0.5408, n = 220, $p < 0.0001$) and wind speed (Spearman's nonparametric correlation, rho = 0.2204, n = 220, $p = 0.0008$). No analyses were performed on the data for dormant propagules collected in Ruidera because of their scarcity (Table 4).

Collectors	Response variable	Variable AIC_c weights				
		tem	cosw	sinw	wspeed	rain
	Zooplankton propagules + bryozoan statoblasts	**0.71**	**0.66**	0.15	0.18	0.25
Doñana B dry	Zooplankton propagules	**0.72**	**0.59**	0.19	0.18	0.33
	Rotifer resting eggs	**0.68**	**0.53**	0.25	0.21	0.32
	Zooplankton propagules + bryozoan statoblasts	0.48	0.33	0.16	0.18	0.31
Total dry (collectors A and B)	Zooplankton propagules	**0.86**	**0.81**	0.12	0.14	0.27
	Rotifer resting eggs	**0.68**	**0.54**	0.24	0.21	0.31
	Zooplankton propagules + bryozoan statoblasts	0.46	0.28	0.27	0.26	0.15
Total Donaña B (dry and wet)	Zooplankton propagules	0.46	0.17	0.31	0.21	0.15
	Rotifer resting eggs	0.44	0.17	0.34	0.22	0.15
	Zooplankton propagules + bryozoan statoblasts	0.41	0.26	0.23	0.23	0.17
Total Doñana A-B (dry and wet)	Zooplankton propagules	0.50	0.23	0.32	0.26	0.14
	Rotifer resting eggs	0.42	0.17	0.35	0.25	0.14

Table 5. Relative importance of meteorological variables in predicting the abundance of collected propagules. For each variable, we report the sum of AIC_c weights for all models in which the variable occurred. cosw: cosine of wind direction, sinw: sine of wind direction, tem: temperature (log-transformed), wspeed: wind speed (log-transformed), rain: rainfall (log-transformed). In bold: Variable was in top model based on AIC_c (see table S3, page 75).

The relationship between the time of year and the amount of dormant propagules collected was analysed by converting the day of the year into a circular variable (Jammalamadaka and SenGupta, 2001), which was excluded from the previous model selection to avoid redundancy with other variables. The sine and cosine of the day were used in a circular–linear correlation analysis of the total number of dormant propagules gathered in each collector. The day of the year was only significantly related to the total number of dormant propagules collected in the Doñana B dry deposition container ($r = 0.522$, $p < 0.05$) and to the mean number in the Doñana A and B dry deposition containers ($r = 0.516$, $p < 0.05$).

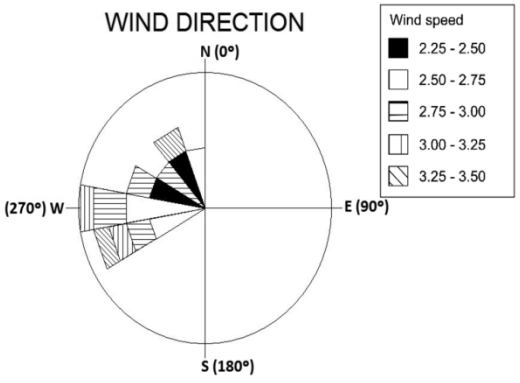

Figure 14. Raw data on wind direction and speed throughout the study period (15 intervals) (from 26 May, 2008 to 23 October, 2010).

Discussion

This is the first study to use automated wet/dry collectors in combination with windsocks to collect dormant zooplankton propagules. The total number of resting propagules gathered per collector was higher in Doñana than in Ruidera, although the difference was not statistically significant due to the small sample size in Ruidera. The advantage of using the automated wet/dry collectors is that they allowed for discrimination between dry and wet atmospheric deposition. According to this we collected more dormant propagules in the dry deposition buckets than in the wet deposition buckets (0.119 dormant propagules per day/m^2 and 0.034 dormant propagules per day/m^2, respectively) in Doñana. In Ruidera, the wet deposition buckets collected more propagules (0.054 dormant propagules per day/m^2 in the wet deposition buckets and 0.027 dormant propagules per day/m^2 in the dry deposition buckets).These differences in the number of dormant propagules collected in each deposition bucket, the proximity of the collectors to water bodies (30 or 100 m), and the scarceness of dormant propagules collected during our study period support the idea that overland dispersal by wind and rain is infrequent and operates as a regional-scale process (Jenkins and Underwood, 1998; Brendonck and Riddoch, 1999). Although the total number of dormant propagules collected was very low in both systems, wind and rain could be relevant dispersal vectors that play a key role in community assembly in new aquatic systems if we consider wind dispersal to be a stochastic process and that a single or a few individuals may colonize new water bodies (Ortells et al., 2014).

Given that almost all of the dormant propagules were collected between June and November in the passive deposition collectors in Doñana, our results may also be related to the exposure of egg banks to wind during the dry season. During the study period, the depth of the lakes in Doñana was reduced by 45-75 % in the summer months (high temperatures and virtually no rainfall) with a major reduction in their surface area, although they did not become completely dry. Therefore, the egg banks in the dried areas were potentially exposed to the action of the wind (Galindo et al., 1994), but in contrast, the water levels and the surface areas of the *del Rey* and *Colgada* lakes in Ruidera, which are permanent water bodies with an average depth of 20 and 16 m, respectively, fluctuated less. Our observation of a significant relationship between higher temperatures and a larger number of collected propagules supports the influence of the dry season in these dispersal events. However, the long dry phase and the desiccation of egg banks exposed to the sun may also increase the mortality of diapausing eggs in temporary compared to permanent water bodies, which may reduce viable dispersal (Bohonak and Jenkins, 2003).

Jenkins and Underwood (1998) observed low wind dispersal rates for zooplankton propagules and collected only rotifer species (bdelloids) in their windsocks, which were deployed in sites distant from as well as near to potential sources of zooplankton propagules (400 m and 150 m, respectively). Higher dispersal rates and a greater variety of taxa were collected in our windsocks in Doñana, although the results were considerably lower than those reported by Vanschoenwinkel and co-workers in collectors adjacent to rock pool metacommunities

(Vanschoenwinkel et al., 2008b; Vanschoenwinkel et al., 2009). Previous authors collected several hundred thousand wind-dispersed dormant propagules on sticky traps in only one month, a larger amount than we collected in the wet/dry atmospheric deposition collectors in Doñana over approximately three years and in the windsocks over four months. This difference may be because the rock pools in the previous studies become totally dry during the summer season (Vanschoenwinkel et al., 2008b; Altermatt and Ebert, 2008; Pellowe-Wagstaff and Simonis, 2014), and their substrate may favour the lifting and subsequent movement of the dormant propagules relative to the muddy lake sediments. Additionally, sticky traps were located very close to sources of dormant propagules and at ground level in a flat open area, where they could receive heavier propagules that arrive by rolling and saltational movements (Vanschoenwinkel et al., 2009).

Several studies have demonstrated similarities between established zooplankton communities and dispersing organisms, suggesting that the relative abundance of the species in the local community is reflected in the dispersing community (Jenkins and Buikema, 1998; Ives et al., 2004; Sciullo and Kolasa, 2012). Although the dispersed propagules did not represent all of the taxa in the Doñana lakes, the most abundant genera were also those observed with the greatest frequency in the water column (Conde-Porcuna et al., 2009). The composition of the species collected in the atmospheric deposition collectors and the windsocks was similar, suggesting that the propagules passively deposited in the collectors adequately reflect the species dispersed by the wind with the exception of ostracods, which were only observed in the windsocks. Rotifers were the most abundant taxa in all the collector systems, while cladocerans were only collected in the dry deposition collector. Rotifers are often prominent among the early colonists of experimental water bodies (Jenkins and Buikema, 1998; Cáceres and Soluk, 2002; Badosa et al., 2010; Frisch et al., 2012), so the size of the propagules and their dispersal ability may play a key role in the colonization processes.

Wind speed seems to be irrelevant in the deposition of propagules in Doñana, although other studies found a significant relationship between wind speed and propagule dispersal (Vanschoenwinkel et al., 2008b, Parekh et al., 2014). During our study period, the minimum wind speed in Doñana was approximately 5 km/h (1.3 m/s), and the average wind speed was two-fold higher, suggesting than eggs could readily be lifted and dispersed by the wind (Parekh et al., 2014). Thus, the majority of dormant propagules collected in the present study were small, zooplankton resting eggs (rotifers and ostracods) and bryozoan statoblasts, with a lesser proportion of cladoceran ephippia, which are generally heavier. Wind speed influenced the aerial transportation of propagules but appeared to have no effect on their passive deposition in the present study, although this might be due to the relatively small sample size.

Wind direction seems to play a greater role than wind speed. We observed that the cosine of wind direction and temperature were the most relevant variables in relation to the propagules collected in the dry containers, but the relative importance of both variables, especially the cosine of wind direction, was clearly lower when including both dry and wet

collectors (Table 5). This suggests that wind direction could be more relevant to dry deposition than to wet deposition.

Little is known about the average number of propagules deposited by rainfall during a given time period in a region (Vanschoenwinkel et al., 2008b). The passive wet deposition rate was lower (0.029 dormant propagules per day/m^2) than the passive dry deposition rate (0.112 dormant propagules per day/m^2) in Doñana, but the wet deposition rate was higher (0.053 dormant propagules per day/m^2) than the passive dry deposition rate (0.026 dormant propagules per day/m^2) in Ruidera. Our results support the conclusion that overland zooplankton dispersal by wind and rainfall is infrequent and occurs in a few species (Jenkins and Underwood, 1998), at least in the aquatic systems of Doñana and Ruidera. Extrapolating from the passive deposition rate of dormant propagules in the Doñana A collector (0.051 dormant propagules per day/m^2), the nearest lake to the west, *Santa Olalla* with an area of 32.7 ha, could potentially receive 16415 dormant propagules per day. Based on the rate in the Doñana B collector (0.102 dormant propagules per day/m^2), the neighbouring *Dulce* Lake, with an area of 6.3 ha, might receive 6325 dormant propagules per day. The automated wet/dry collector from Ruidera was located between the *del Rey* and *Colgada* lakes with surface areas of 38 and 100 ha, respectively, so the extrapolated passive deposition rate of dormant propagules in both lakes might be 15086 and 39700 dormant propagules per day, respectively. Although this type of extrapolation is contentious and might ignore distance decay and the viability of dormant propagules, it suggests that a large number of propagules can be dispersed by the wind and reach the lake surface, highlighting the potential of resting eggs and desiccated bdelloids dispersed by wind to be colonizers (Frisch et al., 2012; Ortells et al., 2014). The study of the dispersal of dormant propagules by wind with windsocks is useful, but the impact of propagule dispersal on aquatic systems depends on their passive deposition. In contrast, wind direction played a significant role in their deposition, and based on the positive relationships obtained (with the coefficient of the cosine term and the cosine of angles in quadrants between 0°-90° and 270°-360°; see Table 6) and the absence of winds from the northeast in Doñana during the study period, a larger number of dormant propagules appear to be carried by winds from the northwest (between 270° and 360°) than from the southwest (wind directions between 180° and 270°). This is consistent with the predominant wind direction observed in Doñana and the greater number of temporary ponds in the northwest direction.

Quadrant/Angle	Sine of wind direction	Cosine of wind direction
1º Quadrant: 0°- 90° (0 to π/2)	+	+
2º Quadrant: 90°- 180° (π/2 to π)	+	-
3º Quadrant: 180°- 270° (π to 3π/2)	-	-
4º Quadrant: 270°- 360° (3 π /2 to 2 π)	-	+

Table 6. Signs of the function in each quadrant, and function values in the first quadrant of the parameters of wind direction.

Evidence of the viability of dormant propagules transported by wind, rain, birds or other animals has been published (Jenkins and Underwood, 1998; Bohonak and Whiteman, 1999; Figuerola and Green, 2002; Vanschoenwinkel et al., 2008a). Jenkins and Underwood (1998) incubated particles collected with windsocks and rain samplers, hatching only bdelloid rotifers from the wind samples and monogonont rotifers from the rain samples over one year. Although our study design was not appropriate to determine propagule viability because of the long periods between sampling days, the collected dormant propagules were individually isolated and tested under hatching conditions. The scarceness of viable dormant propagules in the automatic wet/dry collectors could be related to the long and harsh environmental conditions suffered by the dormant propagules passively deposited between each sampling date (Moghraby, 1977; Raikow et al., 2007; Branstrator et al., 2013). Despite this, we collected one viable resting rotifer egg in the windsocks and two viable bdelloids in the Ruidera wet collector. If dormant propagules had reached a water body, they might survive for subsequent hatching, but further research on the hatching rates of dispersed dormant propagules is required with shorter intervals between samplings to establish the colonization potential of zooplankton dormant propagules transported through the air.

In summary, this is the first study of the aerial dispersal of zooplankton and bryozoan dormant propagules to differentiate between dry and wet deposition using automated wet/dry collectors. In Doñana, more dormant propagules were collected in dry than in wet atmospheric deposition collectors, which might be related to the difference in the number of days that each wet and dry sampler was opened, and more dormant propagules were intercepted by windsocks than by passive deposition in the collectors. The composition of the propagules collected in the windsocks and those in the atmospheric deposition collectors was similar, suggesting that zooplankton dispersal may be effectively studied with windsocks alone, although some taxonomical groups may not be detected. Most of the collected propagules were resting rotifer eggs, and we also provided limited evidence of their viability following overland dispersal, including the hatched egg in Doñana and the live bdelloids found in Ruidera. Based on these findings, overland movement of zooplankton dormant propagules by wind appears to be infrequent but may influence colonization over long time intervals.

Supporting information

Doñana B dry						**Total dry (collectors A and B)**					
	AICc	AICcW	Adj R^2	*p*	norm		AICc	AICcW	Adj R^2	*p*	norm
Response variable: Zooplankton propagules + bryozoan statoblasts	-62.72	0.38	0.40	0.02		*Response variable: Zooplankton propagules + bryozoan statoblasts*					
y= -0.36 + 0.08** cosw + 0.28** tem					yes	null model	-79.41	0.21	-	-	
						y= -0.16 + 0.03* cosw + 0.13* tem	-78.62	0.14	0.23	0.08	yes
						y= -0.09 +0.01ns rain + 0.10ns tem	-77.73	0.09	0.18	0.12	yes
						y=0.05 – 0.01ns wspeed	-77.62	0.09	0.02	0.28	no
Response variable: Zooplankton propagules						*Response variable: Zooplankton propagules*					
y= -0.19 + 0.04** cosw + 0.16** tem	-80.78	0.31	0.40	0.02	yes	y=-0.06 + 0.01** cosw + 0.04** tem	-125.20	0.48	0.47	0.01	yes
y= -0.21 + 0.03* cosw + 0.01ns rain + 0.18** tem	-79.23	0.14	0.40	0.02	yes	y=-0.06 + 0.01* cosw + 0.002ns rain + 0.05** tem	-123.30	0.18	0.52	0.01	yes
Response variable: Rotifer resting eggs						*Response variable: Rotifer resting eggs*					
y=-0.17 + 0.04** cosw + 0.14* tem	-83.71	0.28	0.37	0.02	yes	y=-0.04 + 0.01** cosw + 0.03* tem	-126.13	0.28	0.37	0.02	yes
y=-0.18 + 0.03* cosw + 0.01ns rain + 0.16** tem	-81.78	0.11	0.43	0.03	yes						
Total Donaña B (dry and wet)						**Total Doñana A-B (dry and wet)**					
	AICc	AICcW	Adj R^2	*p*	norm		AICc	AICcW	Adj R^2	*p*	norm
Response variable: Zooplankton propagules + bryozoan statoblasts						*Response variable: Zooplankton propagules + bryozoan statoblasts*					
null model	-78.32	0.20	-			null model	-90.73	0.23	-	-	
y=-0.16 + 0.03* cosw + 0.14* tem	-77.60	0.14	0.23	0.08	yes	y=-0.10 + 0.02ns cosw + 0.09* tem	-89.40	0.12	0.20	0.10	no
y = 0.06 - 0.13ns wspeed	-77.46	0.13	0.08	0.16	no	y=-0.10 + 0.02ns cosw + 0.09* tem	-89.37	0.12	0.05	0.22	no
y=-0.07 + 0.05ns sinw + 0.10* tem	-76.95	0.10	0.20	0.11	yes						
Response variable: Zooplankton propagules						*Response variable: Zooplankton propagules*					
null model	-90.96	0.21	-			null model	-107.14	0.18	-	-	
y=-0.06 + 0.03ns sinw + 0.07* tem	-90.39	0.16	0.24	0.08	yes	y=-0.03 + 0.02* sinw + 0.04* tem	-106.90	0.16	0.26	0.07	yes
y=-0.03 + 0.03ns tem	-89.83	0.12	0.06	0.19	no	y= 0.03 – 0.05ns wspeed	-106.21	0.11	0.07	0.17	no
y= 0.04 – 0.07ns wspeed	-89.23	0.09	0.02	0.27	no	y=-0.06 + 0.01ns cosw + 0.05* tem	-105.93	0.10	0.21	0.10	no
						y=-0.02 + 0.02ns tem	-105.62	0.08	0.04	0.24	no
Response variable: Rotifer resting eggs						*Response variable: Rotifer resting eggs*					
null model	-92.35	0.21	-			null model	-107.75	0.21	-	-	
y=-0.06 + 0.03ns sinw + 0.07* tem	-91.99	0.18	0.25	0.07	yes	y= -0.03 + 0.02* sinw + 0.04* tem	-107.30	0.17	0.25	0.07	yes
y=-0.03 + 0.03ns tem	-90.98	0.11	0.05	0.22	no	y= 0.02 - 0.04ns wspeed	-106.60	0.12	0.06	0.19	no
y= 0.03 - 0.06ns wspeed	-90.63	0.09	0.02	0.02	no						

Table S3. Results of models predicting the abundance of collected propagules; only models with ΔAICc < 2 are presented. Adj R2: adjusted R2 (proportion of the variance explained); AICcW: model weight; norm: normality of residuals; cosw: cosine of wind direction; sinw: sine of wind direction; tem: temperature (log-transformed); wspeed: wind speed (log-transformed); rain: rainfall (log-transformed). No model selection was performed for the Doñana A collectors and the Doñana B wet collector due to the small number of propagules collected (see Table 4).*$*p < 0.05$, $**p < 0.01$*

Chapter 3

Dispersal of rotifers and cladocerans by waterbirds: Seasonal changes and hatching capability

Abstract

Geographical dispersal of aquatic invertebrates may occur through transport by abiotic vectors (wind, rain and waterflow) or by organisms (mainly by insects, fishes and waterbirds). However, there is a lack of information on dispersal by waterbirds of some zooplankton groups as cladocerans and, mainly, rotifers. Moreover, the hatching success of those dispersed propagules has been poorly studied, and no information is available on the hatching success of directly isolated propagules. In the present study, we quantify the dispersal of cladocerans and rotifers through waterbirds by collecting faecal droppings of waterbirds around two lakes located in Doñana National Park (south of Spain). We also analyse the hatching success of dispersal propagules under different conditions of photoperiod and temperature. We found diapausing propagules in waterbird faecal samples belonging to 22 species of rotifers or cladocerans, and a mean number of 0.9 diapausing propagules per faecal dropping was found. No effect of photoperiod nor temperature was observed on the hatching success of these propagules. Although the hatching success was low, rotifer dispersal by waterbirds has been undervalued. Moreover, in the studied area, dispersal of rotifers by waterbirds may be, at least, as relevant as dispersal by air currents. Further studies are needed to evaluate the relative importance of dispersal mechanisms in different areas.

Introduction

Zooplankton populations vary in their dispersal and colonization ability (Cáceres & Soluk, 2002). Increased attention should be focussed on the potential role of dispersal limitation and its importance for understanding the structure and function of aquatic communities. Experiments in artificial systems (experimental ponds, wading pools) suggest that dispersal limitation is likely important in structuring zooplankton assemblages (Jenkins, 1995; Jenkins & Buikema, 1998; Holland & Jenkins, 1998), while experiments in natural fishless ponds and larger lakes conclude that dispersal limitation plays a minor role (Lukaszewisky et al., 1999; Shurin, 2000).

Diapausing propagules of zooplankton can be dispersed by wind (Jenkins & Underwood, 1998, Vanschoenwinkel et al.., 2008, Moreno et al., 2016), water flow Michels et al., 2001), fishes (Battauz et al., 2015), and waterbirds (Green et al., 2013). Several studies have shown that waterbirds may carry resting propagules of plants and invertebrates both in their digestive tract and in their feathers, a substantial proportion of which are viable (Proctor & Malone, 1965; Charalambidou & Santamaría, 2002; Charalambidou et al., 2003, Figuerola et al., 2003; Frisch et al., 2007; Green et al., 2008; Brochet at al., 2009; Green et al., 2013; Muñoz et al., 2014; Rogers, 2014; Soons et al., 2016). However, more studies that quantify dispersal by waterbirds in the field are needed (Green et al., 2013), and most of those previous studies were focused on aquatic plants, anostracans or other invertebrates, while scarce or absent information was provided for cladocerans or, especially, rotifers. Previous studies have only described some few species and many unknown species of cladocerans and rotifers (Figuerola et al., 2003; Frisch et al., 2007; Green et al., 2008; Green et al., 2013), and their hatching capability was not directly estimated.

Cáceres & Soluk (2002) concluded, in an experimental study, that dispersal is primarily the result of wind and rain rather than by animal vectors. However, the viability of dispersal by waterbirds seems to be related with the propagule size (De Meester et al., 2002) and with the gizzard size of waterbird species (Sánchez et al., 2002). In this sense, we hypothesize that the small size of resting eggs of rotifers would facilitate their internal transport by waterbirds and they should suffer less physical damage by waterbird's gizzard. For that reason, more studies on rotifer dispersal are needed.

Hatching rates of diapausing propagules of zooplankton have been related with photoperiod and temperature (Gyllström & Hansson, 2004; Vandekerkhove et al., 2005; Dupuis & Hann, 2009), although the relevance of photoperiod may change between regions

(Vandekerkhove et al., 2005) or just the presence of light may be more relevant in some systems (Pérez-Martínez et al., 2013). However, previous studies on hatching capability of propagules dispersed by waterbirds did not consider different conditions of light and/or temperature.

In the present study, we will evaluate (1) waterbird capability to transport zooplankton (rotifers and cladocerans) diapausing propagules in two lakes of a semi-arid region (Doñana National Park), (2) seasonal differences in the transport of zooplankton propagules by waterbirds, and (3) hatching response of zooplankton dispersal propagules under different conditions of temperature and photoperiod. We hypothesized that dispersal of cladocerans and rotifers by waterbirds will be relevant for aquatic systems, especially in the case of rotifers.

Material and methods

Study area

Samples of waterfowl faeces were obtained from two lakes, *Santa Olalla* and *Dulce*, located in Doñana National Park (southwest Spain) (Figure 2).

Collection and analysis of waterfowl faecal samples

Fresh waterbird faeces (one dropping) were collected in the shore of those lakes or in islands where waterbirds roosted. Previously, those places were selected by using binoculars to localization and identification of the waterbird groups, and bird individuals were identified to species level. We ensured to collect each dropping removing the parts in contact with soil/sediment to avoid contamination. Waterbird faeces were stored in a tube kept in a cool box with ice until reach the laboratory, and they were mainly collected during autumn and winter. These periods overlapped with the southwards and northwards waterfowl migrations. Additionally, we also obtained some samples during spring. These samplings were spread across four years (2008, 2009, 2010 and 2012). In the laboratory, the tubes were store at 4°C until processed. As it was not possible to distinguish between faeces from different individuals, all droppings were pooled in the laboratory for each waterbird species each sampling day.

Diapausing propagules of the pooled samples were isolated by means of the sugar flotation method developed by Onbé (1978) and modified by Marcus (1990). This method is used for isolation of diapausing propagules from sediments (Vandekherkhove et al., 2004),

and we found that it is useful for isolation of propagules from faecal samples, because, previously, we checked that no eggs were remaining in the faeces after employing this method of isolation. Isolated diapausing propagules were counted and identified under a microscope. Identification was done to genus level according to Koste (1978), Moreno-Linares et al. (2016) and to Moreno et al., (2017), and only apparently healthy propagules were considered.

Hatching experiment

Some of the diapausing propagules found in the faecal samples were incubated at different conditions of photoperiod [light:dark (L:D) =14:10 h] and temperature (10, 14 and 20 ºC). This incubation was done after those propagules were kept in cold conditions during several months. Diapausing propagules were individually placed in wells of 96-well polyethylene microplates with mineral water. These microplates were located in opaque chambers fitted with a zenith light, using white light with an intensity of 345.50 ± 20.54 lmol s-1 m-2 [mean ± standard error (SE)] as measured with a BQM photometer (Pérez-Martínez et al., 2013). These opaque chambers were introduced in Mettler incubation chambers to control their temperature. The white light of the opaque chambers do not affect the temperature (Pérez-Martínez et al., 2013). This experiment was conducted for 20 days. The rest of the collected diapausing propagules were used in for the experimental design in **Chapter 5**.

Statistical analyses

Statistical analyses were performed using R 3.3.3 (R Foundation for Statistical Computing) program. To detect differences in the presence of zooplankton diapausing propagules in the waterbird faecal pellets regarding the time of the year, we analyzed the relationship of those diapausing propagules and the day of the year by using generalized mixed linear models. The number of diapausing propagules was corrected by the number of faecal droppings of each pooled sample by including them as a covariate in the models, while the waterfowl species and sampling year were entered in the models as random factors. To analyze changes in the zooplankton richness in the faecal samples, a similar model was performed but using the number of diapausing propagules in the faecal samples as a covariate instead of using the number of waterbird faecal droppings. In all cases, we modelled assuming a Poisson distribution and overdispersion assumption was tested using *overdisp_fun* (Bolker *et al.*, 2009). As more than 40% of our data were zeros, and overdispersion was clear in all cases, zero-inflated negative binomial models were used

instead of Poisson models (Martin et al., 2005). Our analyses were performed using the *glmmADMB* R package (Fournier et al., 2012; Skaug et al., 2016), as this handles zero-inflated data.

The circular day of the year variable was accommodated by including the sine and cosine of the day of the year in all models rather than the day of the year variable itself, as done for other circular variables (Johnson and Wehrly, 1978; Mardia and Jupp, 2000; Jammalamadaka and Lund, 2006, Conde-Porcuna et al., 2014).

For the hatching experiment, a linear mixed model was performed using hatching rates as a binary dependent variable, while photoperiod and temperature were considered as independent factors. The zooplankton species, waterfowl species and the opaque chambers used in the experiment were included in the model as random factors. In this case, the analysis was modelled with a binomial distribution, and the results were expressed with the function *Anova* of the R package "car" (Fox & Weisberg, 2011). The relevance of the random factors was evaluated using the likelihood ratio tests (LRT) (Pinheiro & Bates, 2000).

Results

We collected 327 waterfowl faecal samples during the sampling study. From these samples, we obtained 287 diapausing propagules of zooplankton (261 of rotifers and 26 of cladocerans). Table 1 shows the total number of diapausing propagules of each zooplankton species found from each waterfowl species. A total of 22 zooplankton species were found in faecal samples, and *Brachionus* was the main zooplankton genus (Table 7). Additionally, we found 37 bryozoan statoblasts and 23 ostracod eggs in total faecal droppings, although we do not focus on them in this study.

Most of the faecal samples were obtained during winter, although the higher numbers of zooplankton propagules per faecal sample were observed during autumn (Figure 15). The number of faecal samples collected was similar between lakes (mixed model, Wald $z = 0.42$, $p=0.67$; waterfowl species and year as random factors). It is striking that we did not find any propagule in 29 faecal pellets of *Chariadius alexandrina* (kentish plover), while the higher number of diapausing propagules per faecal sample was found in *Sterna hirundo* (common tern).

Table 7. Dipausing propagules of zooplankton isolated from waterbird faecal samples. The propagules used in the hatching experiment are indicating in brackets.

	Waterbird species																		
	A. cly	*A. pe*	*A. pla*	*C. ale*	*C. alp*	*E. gar*	*F. atr*	*M.* sp	*N. ruf*	*P. car*	*P. his*	*P .leu*	*P. por*	*P. ros*	*Ster sp*	*V. va*	U1	U2	Total
Number of samples	**18**	**2**	**89**	**29**	**6**	**5**	**79**	**13**	**2**	**1**	**16**	**4**	**17**	**10**	**13**	**7**	**12**	**4**	**327**
Rotifer species																			
Brachionus sp.	-	-	1	-	-	-	-	-	-	-	1 (1)	-	-	-	-	-	-	-	2
B. angularis	2	-	42 (1)	-	-	1	16 (2)	4 (3)	-	-	1 (1)	2	-	3	54	1	-	-	126
B. budapes	-	-	4	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	5
B. calyciflorus	-	-	22	-	1	-	2	-	-	-	-	-	-	1	2	2	-	2	32
B. plicatilis	1	-	6	-	-	-	1	-	-	-	-	-	-	-	1	-	-	-	9
B. urceolaris	-	-	-	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	2
B. variabilis	2	-	7 (5)	-	-	-	-	2 (2)	-	-	1 (1)	-	-	-	-	-	1 (1)	-	13
Cephalodella sp.	-	-	-	-	-	-	-	-	1 (1)	-	-	-	-	-	-	-	-	-	1
Dicranophorus sp.	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
Filinia sp.	-	-	2	-	-	-	6	2 (2)	-	-	1 (1)	-	-	-	2	1	-	1	15
Hexarthra fennica	1	-	4	-	-	-	3	-	2 (2)	-	-	-	1	-	4	-	-	-	15
Keratella tropica	-	-	2	-	-	1	1	1 (1)	2 (2)	-	-	-	-	-	-	1	-	-	8
Lecane sp.	2	-	1	-	1	-	-	-	-	-	-	-	-	-	-	1	-	-	5
Polyarthra sp.	-	-	3	-	-	-	9	-	-	-	-	-	-	-	1	2	-	-	15
Proales sp.	-	-	-	-	-	-	1 (1)	-	1 (1)	-	-	-	-	-	1	-	-	-	3
Unknown Rotifera	-	-	-	-	-	-	1	-	1 (1)	-	-	-	1	-	2	4	-	-	9
Cladoceran species																			
Alona sp.	-	-	1 (1)	-	-	-	11 (10)	-	1 (1)	-	-	-	-	-	-	-	-	2	15
Daphnia sp.	-	-	1 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
D. longispina	-	-	1 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1
D. magna	-	-	2 (1)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	2
Diaphanosoma sp.	-	-	-	-	-	-	-	-	-	1 (1)	-	-	-	-	-	-	-	-	1
Unknown Cladocera	1	-	3 (2)	-	-	-	2 (1)	-	-	-	-	-	-	-	-	-	-	-	6

Waterfowl species: *A. cly* = *Anas clypeata* (Northern Shoveler); *A. pe* = *Anas penelope* (Eurasian Wigeon); *A. pla* = *Anas platyrhynchos* (Mallard Duck); C. ale = *Charadius alexandrinus* (Kentish Plover); *C. alp* = *Calidris alpina* (Dunlin); *E. garzetta* = *Egretta garzetta* (Little Egret); *F. atr* = *Fulica atra* (Common Coot); *M.* sp = *Motacilla* sp. (Wagtail); *N. ruf* = *Netta rufina* (Red-crested Pochard); *P. car* = *Phalacrocorax carbo* (Great Cormorant); *P. his* = *Passer hispaniolensis* (Spanish Sparrow); *P. leu* = *Platalea leucorodia* (Common Spoonbill); *P. por* = *Porphyrio porphyrio* (Purple Swamphen); *P. ros* = *Phoenicopterus roseus* (Flamingo); *Ster sp* = *Sterna sp.* (Tern); *V. va* = *Vanellus vanellus* (Northern Lapwing); U1 = Unknown species 1; U2 = Unknown species 2.

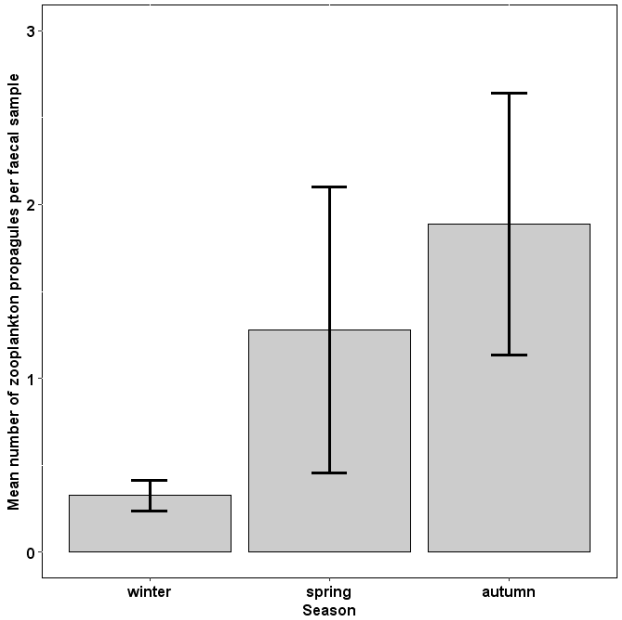

Figure 15. Abundance of total zooplankton propagules from faecal samples of waterfowl during the study period.

The results from a mixed model suggest that most of the zooplankton propagules were obtained at the beginning of autumn, because the amount of zooplankton propagules was negatively related with the sine and the cosine of the day of the year (Table 8) and no samplings were done in summer. Results were similar when we removed the factor "lake" (Table 8). The random factor waterfowl species did not significantly affected to the number of propagules, because the AIC in the model without that random factor was lower than in the full model (Δ AIC= 2.0).

As most of the zooplankton diapausing propagules found belonged to *Brachionus angularis*, this species can condition the overall pattern of zooplankton propagules. For that reason, we performed separate analysis for *Brachionus angularis* and cladocerans. According to them, *Brachionus* propagules were also more abundant in autumn (Table 8, Figure 16). Results were also similar when we removed the factor "lake" (Table 8). However, the majority of the cladoceran diapausing propagules were found in spring (Table 8), being the highest number of cladoceran propagules found during that season (Fig 16).

Table 8. Mixed-models for explaining the abundance of zooplankton diapausing propagules. The table includes the meaning of signs of the sine and cosine functions for the four quadrants of a circle (four periods of a year).

Quadrant/Angle	Time of the year (aprox.)	Sine of Julian day	Cosine of Julian day
1º Quadrant: 0°- 90°	1 January /1 April	+	+
2º Quadrant: 90°- 180°	2 April / 1 July	+	-
3º Quadrant: 180°- 270º	2 July / 30 September	-	-
4º Quadrant: 270°- 360º	1 October / 31 December	-	+

	Total zooplankton propagules					***Brachionus angularis* resting eggs**					**Cladoceran ephippia**				
	Value	SE	Wald z	*p*	AIC	Value	SE	Wald z	*p*	AIC	Value	SE	Wald z	*p*	AIC
Model					212.6					119.3					77.1
Intercept	1.67	0.53	3.13	**0.002**		-2.46	1.33	-1.86	0.063		-1.53	0.87	-1.76	0.078	
Number of samples	0.12	0.04	2.82	**0.005**		0.22	0.08	2.74	**0.006**		0.10	0.07	1.45	0.146	
cosine (julian day)	-1.63	0.70	-2.32	**0.021**		0.24	1.24	0.20	0.844		-1.68	0.98	-1.71	0.088	
sine (julian day)	-1.01	0.30	-3.42	**0.001**		-2.56	0.64	-3.98	**<0.001**		1.63	0.81	2.00	**0.046**	
Lake (Santa Olalla)	<0.01	0.51	<0.01	0.998		0.51	0.83	0.62	0.538		-0.87	0.94	-0.93	0.352	
Without Lake factor					210.6					117.7					76.0
Intercept	1.67	0.51	3.27	**0.001**		-2.18	1.23	-1.78	0.075		-0.95	0.70	-1.35	0.176	
Number of samples	0.12	0.04	2.84	**0.005**		0.22	0.08	2.72	**0.007**		0.12	0.05	2.33	**0.020**	
cosine (julian day)	-1.63	0.67	-2.43	**0.015**		0.31	1.25	0.25	0.803		-2.96	0.89	-3.35	**0.001**	
sine (julian day)	-1.01	0.27	-3.73	**<0.001**		-2.41	0.57	-4.27	**<0.001**		1.04	0.67	1.56	0.118	

Values that are statistically significant are indicated in bold

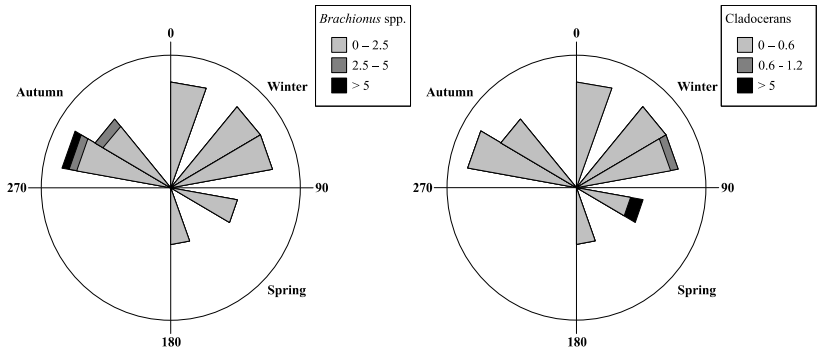

Figure 16. Temporal variability of diapausing propagules in faecal samples during the study period for zooplankton groups. The day of the year is represented as a circular variable.

We also evaluate the temporal pattern of the zooplankton richness in the faecal samples through a mixed model, in which we observed that the zooplankton richness of faecal propagules was independent of the time of the year (cosine of Julian day: Wald z = -0.05, p = 0.96; sine of julian day: Wald z = 0.87, p = 0.39). As expected, that zooplankton richness increased with increasing the number of zooplankton propagules (Wald z = 2.18, p = 0.03).

Respect to the hatching experiment, we only observed 6 hatched propagules of different species (*Alona* sp., *Daphnia* sp., *Diaphanosoma* sp., *Brachionus variabilis*, *Proales* sp., and an unknown rotifer species), so, globally, the hatching success was only of 12.5%, regardless of the temperature and photoperiod used in the experiment. Despite of the low hatchlings, we performed the statistical analysis showing that, in fact, no significant effect was neither observed for temperature (Chi-square = 0.73, df = 3, p = 0.86) nor for photoperiod (Chi-square = 0.55, df = 1, p = 0.46). The random factor "waterfowl species" was not significant (LRT, deviance = 1.21, p = 0.27), while the influence of zooplankton species and the opaque chambers was negligible (p ≈ 1). The hatched propagules were obtained from faecal samples of *Anas platyrhynchos* (*Alona* sp., *Daphnia* sp., *Brachionus variabilis*), *Phalacrocorax carbo* (*Diaphanosoma* sp.) and *Netta rufina* (*Proales* sp., and an unknown rotifer species).

Discussion

Abundance of dispersed zooplankton propagules

This is the first study to analyze interespecific and seasonal variations in the abundance of healthy diapausing propagules of rotifers and cladocerans in waterbirds faecal droppings. This study shows evidence for the dispersal of, at least, 16 rotifer species and 6 cladoceran species. For the most rotifer species that we found, this is the first record in waterbird faecal droppings. Other studies have shown the presence in waterbird faecal droppings of propagules of *B. plicatilis* and unidentified bdelloid and monogonont rotifers (Frisch et al., 2007), *Brachionus* sp. and *Cephalodella* sp. (Green et al., 2008). Respect to cladocerans, previous studies have found *D. magna* and *Moina brachiata* (Figuerola et al., 2003; Frisch et al., 2007), *Daphnia* sp. (Green et al., 2013) and unidentified cladoceran ephippia (Figuerola et al., 2003; Green et al., 2008). In our study, we also found *D. longispina*, *Alona* sp. and *Diaphanosoma* sp. According to our

study, dispersal of zooplankton species by waterbirds is very relevant, especially for rotifers. Resting eggs of rotifers are not well known because keys are still lacking, but, in addition to the hatching techniques, the development of genetic tools facilitates the morphological identification of resting eggs (Moreno et al. 2017).

Other studies also refer many unidentified invertebrate eggs in faecal droppings of waterbirds (Figuerola et al., 2003; Green et al., 2008; Green et al., 2013). In our case, 261 rotifer resting eggs apparently healthy were identified in faecal droppings. Probably most of those unidentified invertebrate eggs could be rotifer eggs. In fact, rotifer resting eggs were much more abundant than cladoceran propagules in our study, and we expect the same result in other studies.

Nevertheless, Figuerola et al. (2003) found more than 500 ephippia of cladocerans in 401 facecal droppings examined, although most of them were not identified to genus. Those ephippia were mainly found in *Anas clypeata* and *Fulica atra*. However, we only found 26 viable propagules of cladocerans in 327 faecal droppings, and most of them were found in *Anas platyrhynchos*. Nevertheless, Green et al. (2013) also found few cladoceran ephippia, just one *Daphnia* ephippia in 9 samples of killdeer. These differences may be related with seasonality, differences in waterbird species and differences in the availability of diapausing propagules in a region.

For other aquatic groups, it has been described that distribution patterns of dispersal propagules are highly specific and vary over time (Green et al., 2002). Frisch et al. (2007) found more hatchlings of aquatic invertebrates in winter than in autumn, and they consider that this could be related with a seasonal change in the availability of propagules or with differences in the waterbird species. We also observed seasonal differences in zooplankton propagules from waterbirds faecal samples. Cladocerans propagules were mainly found during early spring, while resting eggs of *Brachionus angularis*. (the most abundant rotifer) were mainly found during early autumn. No seasonality was observed for zooplankton richness. Although zooplankton diapausing propagules use to be produced during summer, dispersal of propagules may occur even propagule production and waterbirds movements are not coincident in time (Figuerola et al., 2003). For that reason, Figuerola et al. (2003) related seasonal differences of dispersed propagules of plants and invertebrates with feeding changes of waterbirds. They include different feeding methods for waterbird species and changes in feeding depth (Figuerola et al., 2003). Nevertheless, in their study, it seems that there was not a clear seasonal difference for cladoceran ephippia and, at least for corixid eggs, no seasonal differences were observed in *A. platyrhynchos* and *F. atra* droppings (Figuerola et al., 2003). These authors also found differences in propagule abundance due to waterbird species, observing also an interaction between seasonality and waterbird species. However, we did not find a relevant effect of waterbird species on the abundance of zooplankton dipausing propagules.

Hatching experiment

The low hatching success of zooplankton propagules we observed could be related with several factors. First, hatching experiments were performed using mineral water with low

salinity (<0.2 g l^{-1}), while in Doñana water bodies or in other aquatic systems of that region, salinity use to be higher. In consequence, salinity levels could have limited the hatching capability. Second, the behaviour of these zooplankton species from semi-arid aquatic systems may follow a bet-hedging strategy reported for many organisms (Evans & Dennehy, 2005), so species may distribute the hatching over several periods in order to increase the likelihood of survival. This strategy has been suggested for alpine *Daphnia* (Pérez-Martínez et al., 2013) and for temporary rotifer populations inhabiting unpredictable habitats (García-Roger et al., 2014).

It is difficult to compare our hatching success with those from other studies with waterbird faecal samples, because of the scarcity of such studies on zooplankton populations (rotifers and cladocerans) and because those few studies did not isolated resting eggs, because they estimate the proportion of faecal droppings with hatchlings as an indication of hatching rates (Frisch et al., 2007; Green et al., 2008). Nevertheless, according to those studies, global hatching rates of rotifers ranged between 10 and 31 % (Frisch et al., 2007; Green et al., 2008) and those for cladocerans were about 21% (Frish et al., 2007). These values are relatively similar to those we obtained, and differences may be related with the fact they used temperatures of 15 ^{o}C or upper.

Anyway, this is the first study showing the hatching success of zooplankton diapausing propagules from waterbirds faeces isolating directly those propagules. Moreover, no previous studies have checked the possible influence of photoperiod and temperature on those propagules, although we did not find a significant effect of these factors on the hatching success. Previous studies on sedimentary propagules have shown that cladocerans may be affected by photoperiod and temperature (Stross 1966, Vandekerkhove et al., 2005; Dupuis & Hann, 2009), although in alpine lakes, which freeze for short periods, *Daphnia* was not affected by these factors (Pérez-Martínez et al., 2013). In rotifers, Dupuis & Hann (2009) did not observe effect of photoperiod on hatchling abundance, and suggest that temperature may be the relevant cue to terminate dormancy. Anyway, it is difficult to conclude with our results because of the scarcity of hatchlings that we had.

Consequences of zooplankton dispersal by waterbirds

Waterbirds may ingest not only resting eggs recently produced, but also older propagules from the upper sediment layers. Many plant seeds will be destroy during disgestion (Figuerola et al., 2003), but the much more smaller rotifer eggs should suffer much less damage than plant seeds or even cladoceran ephippia. In consequence, viability of rotifer resting eggs could be potentially higher than for other aquatic groups. In fact, most of the rotifer resting eggs that we found looked healthy (all of them included in Table 7). Further studies are needed to have more estimates of the hatching capability of dispersal rotifer resting eggs. The relationship between zooplankton dispersal ability and dormancy, or the ecological traits related to dispersal ability in zooplankton (Havel & Shurin, 2004), is unresolved yet. Anyway, our study shows that dispersal by waterbirds should be condidered a relevant mechanism of rotifer dispersal. In **Chapter Synthesis** (see page)we discuss our results with those from **Chapter 1**.

Chapter 4

Variations in the hatching response of rotifers to salinity and waterbird ingestion

Abstract

Climate change is increasing aridity in Mediterranean region, and as a consequence, salinity concentrations are increasing in aquatic systems of this region. This effect may negatively affect the resilient capacity of aquatic communities and modify regional dynamics favouring the colonization of salt-tolerant species The objective of our study is to test the effects of salinity concentration on the resilient capacity and dispersal response of rotifers at different temperatures through the study of their hatching success. This hatching response will be compared between resting eggs isolated from the sediments of two peridunal lakes of Doñana National Park (Santa Olalla and Dulce) and from waterbird faecal samples collected in that area. Those resting eggs were used in hatching experiments performed at two temperatures (15 and 25 ºC) and four salinity treatments (0.2, 1, 4 and 8 g l^{-1}). Results showed species-specific hatching response of rotifers to salinity, although a global negative and linear trend was observed between the hatching success and the salinity concentration at 15 ºC of temperature, while a global quadratic trend was observed at 25 ºC. The only resting eggs that hatched from waterbird faecal samples were isolated from facecal droppings of *Anas platyrhynchos* and *Anas clypeata*, although a high number of resting eggs were isolated from other waterbird species as *Fulica atra*. Anyway, at least for the species *Brachionus angularis*, we observed that gut passage through waterbirds may increase the hatching success regardless of salinity concentration.

Introduction

The study of the effects of the climate change and the environmental disturbances on the biodiversity represents one of the fields of major interest at present. Changes in precipitation patterns worldwide are expected by many climate models (Knutti & Sedlacek, 2013, Jeppesen et al., 2014). Net rainfall tends to decrease, especially in the Mediterranean region where it is expected a 25-30% drop in freshwater runoff for the period 2040-2061 (Giorgi, 2006; Giorgi & Lionello, 2008). Respect to temperature, it is expected that temperature in Mediterranean lakes would increase 1.5 ºC for the period 2001-2050 (Copetti et al., 2013).

The ecological state of shallow lakes is expected to change in the future because of climate change, which will cause changes in water level and salinity in arid areas (Jeppesen et al., 2011). Jeppesen et al. (2015) show that an increase in salinity/conductivity is associated with a decline in lake water levels in several semi-arid Mediterranean shallow lakes. Simulations of climate change indicates significant reductions in groundwater resources in Doñana wetland at the South of Spain (Guardiola-Albert & Jackson (2011). According to these effects, shallow lakes may shift to oligosaline or mesosaline conditions.

As freshwater biota, in general, do not extend into saline or slightly saline water (Nielsen et al., 2003), the species richness of freshwater biota will decrease if salinity increases. Nielsen et al. (2003) suggest that aquatic biota (bacteria, algae, aquatic plants and invertebrates) will be negatively affected if salinity exceeds 1 g/l.

Brucet et al. (2009) showed that crustacean zooplankton was strongly affected by salinity, while temperature had no clear effect. A decline in phytoplankton and macrophyte richness has also been observed with an increase in conductivity (Muylaert et al., 2010, Lauridsen et al., 2015). However, Declerck et al. (2005) did not find a negative relationship between conductivity and the richness of zooplankton groups in similar lakes, although salinity has been positively related to rotifer abundance and negatively related to the abundances of cyclopoids (Brucet et al., 2010).

Zooplankton may be a good proxy for the effects of climate change (Jepessen et al., 2011). Touran (2012) suggests that can be used to estimate the ecosystem's sensitivity to salinity. The absence of fish in most of temporary wetlands reinforces the value of zooplankton as a tool for the evaluation of ecological quality in Mediterranean wetlands (Gilbert et al., 2015).

In last years several studies have assessed the richness of zooplankton taxa through the analysis of egg bank samples. Although changes in environmental conditions could reduce biodiversity of the zooplankton active community, the diapausing propagules in the sediment could reflect a higher biodiversity, including viable resting eggs waiting for better conditions to hatch. This may favour the resilience of water systems once they recover their initial conditions.

Zooplankton resilience will depend on the viability of resting eggs and on the environmental conditions that will trigger resting egg hatching. Studies have shown or suggested that the oxygen concentration, food levels, photoperiod, temperature and/or light presence determine the hatching of resting eggs in different species of zooplankton (Brendonck & De Meester, 2003; Gyllström & Hansson, 2004; Vandekerkhove et al., 2005; Perez-Martinez et al., 2013). The conditions for hatching may be different between systems according to their environmental characteristics. In Spain, light is determinant to trigger the emergence of zooplankton in high mountain lakes (Perez-Martinez et al., 2013), while the photoperiod would be a relevant factor for zooplankton hatching in systems located at low altitude in the south of Spain (Vandekerkhove et al., 2005). However, the effect of salinity on hatching rates of zooplankton has hardly been studied. Santangelo et al. (2014) showed an inhibition of rotifer hatching rates at high salinities at 24 ºC of temperature, although they did not study those hatching rates at other temperatures nor specific patterns between hatching rates and salinity.

The effect of climate change is not restricted to altering local systems, but also may modify regional dynamics (Tuytens et al., 2014). Changes in salinity may favour the colonization of salt-tolerant species (Jeppesen et al., 2015), but also zooplankton resilience may also be favoured by dispersal of resting eggs from unaffected areas (Santangelo et al., 2014). Thompson & Shurin (2012) showed experimentally that dispersal may increase the resistance of local ecosystems to environmental stress by providing regional species adapted to stress conditions.

Anecdotal evidence documenting overland transport of zooplankton by vectors such us wind, rain, insects and vertebrates has been accumulating for over a century (Darwin, 1859; Proctor & Malone, 1965; Bohonak & Whiteman, 1999). Experiments in artificial systems (experimental ponds, wading pools) suggest that dispersal limitation is likely important in structuring zooplankton assemblages (Jenkins & Buikema, 1998; Holland & Jenkins, 1998), while experiments in natural fishless ponds and larger lakes conclude that dispersal limitation plays a minor role (Lukaszewisky et al., 1999; Shurin et al., 2000).

Several studies have also shown that waterfowl may carry resting propagules of invertebrates both in their digestive tract and in their feathers, and many of them are viable (Proctor & Malone, 1965; Charalambidou & Santamaría, 2002; Charalambidou et al., 2003). Charalambidou et al. (2003) experimentally investigated the survival of diapausing eggs after their passage through the digestive tract of four species of ducks. They conclude that the probability of endozoochorous dispersal by individual ducks is low, although in population terms should be high. Several studies have shown, studying helophytes, gut passage significantly changed seed response to salinity (Espinar et al., 2004; 2006). Espinar et al. (2004) point out the relevance of considering salinity or other environmental factors when dealing with the effects of gut passage on aquatic plants. In anostracans, it has also been shown that, at least for *Branchinecta* genus, the waterbird ingestion may be benefiting (Rogers, 2014).

However, it is not clear if diapausing propagules dispersed by waterbirds could get successfully established in water bodies of different salinity conditions. Moreover, there is scarce information about rotifer dispersal by waterbirds, and, as far as we know, there is no published information about the effect of ingestion of rotifer resting eggs by waterbirds nor about possible interaction effects between salinity and waterbird ingestion on rotifer hatching capability.

In this study, we will analyse the hatching response of resting eggs of different rotifer species to salinity concentrations, and also to waterbird ingestion by comparing the hatching response from sediment egg banks and from waterbird faecal droppings. We hypothesized (1) that rotifer communities would show a negative trend with salinity concentrations, (2) that rotifer species would show different responses to salinity concentrations, (3) that the rotifer hatching response from waterbirds faecal samples could be dependent of the waterbird species, and (4) that, at least for some rotifer species, consumption of resting eggs could favour/disfavour egg hatching. All these responses will be evaluated at two temperatures (15 and 25 ^{o}C) to check for possible temperature influence. We will discuss the relevance of our results to know the relative importance of rotifer dispersal by waterbirds and the rotifer capability to cope with future increases in salinity due to global change.

Material and methods

Sediment cores were obtained in *Dulce* and *Santa Olalla* Lakes located in Doñana National Park (Figure 1). Sediment core sampling method and hatching experimental conditions described in the general methodology section of this thesis.

Diapausing propagules samples

Only the top four centimetres of the sediment cores were used, as this is likely to comprise the viable and responsive zooplankton egg bank (active sediment). Moreover, it has been described bioturbation in those lakes (Conde-Porcuna et al., 2009), which mix the upper layer of sediments. The collection of the sediment cores is described in the general methodology section of this thesis.

Fresh waterfowl faeces were collected in the shore of both shallow lakes or in islands where waterfowl roosted. Samples were collected during autumn and/or winter in several years (2008, 2009, 2010 and 2012). The sampling periods overlapped with the southwards and northwards waterfowl migrations. Once, we also obtained samples in April (2009). Faecal samples were stored and processed as described in the general methodology sectionof this thesis. Faecal samples were pooled by waterfowl species each sampling day because they were clearly identifiable by bird species but not by bird individuals. More details about faecal sampling and the found-diapausing propagules of rotifers and also cladocerans are describeb in more details in general material secciton and in **Chapter 3**.

Most part of the healthy resting eggs isolated from sediments and faecal samples were used in hatching experiments. Other resting eggs isolated from these waterfowl faecal samples were used in the experimental design in **Chapter 3**. Rotifer resting eggs (from the sediments and from the waterfowl faeces) were microscopically taxonomic identified according to Koste (1978), and the previously identified in **Chapter 1** and **Chapter 3** and to hatching experiments of the current study.

Hatching experiments

To test the effect of salinity and the origin of diapausing propagules (sediments or faecal pellets) on the hatching capability of those propagules, six hatching experiments were performed at different times under the same conditions. Four of them were performed using diapausing propagules from the sediments and the other two using diapausing propagules from waterfowl faecal material. In each of these experiments, the diapausing propagules

(regardless of the species) were individually placed in a well of a 96-well polyethylene microplate with mineral water adjusted at different salinities (0.2, 1, 4 and 8 g l^{-1}) on a long-day photoperiod [light:dark (L:D) =14:10 h]. Salinity solutions were prepared adding Instant Ocean Sea Salt (see Atkinson & Bingman, 1998) to mineral water. The pH values in the artificial solutions were stable and alkaline (mean ± SD; 8.14 ± 0.30). Water pH is also usually alkaline in Santa Olalla and Dulce lakes (Serrano et al., 2006), especially in Santa Olalla lake with pH values higher than 9 (López-Archilla et al., 2004).

All the experiments were performed in two Mettler incubation chambers. For each experiment, experimental temperature was kept at 15 °C in one chamber and at 25 °C in the other one. Previously it has been observed that 15 °C temperature is appropriate for analysing hatching rates of cladocerans in Spanish shallow lakes (Vandekherkove et al., 2005). Every day, wells were studied under microscope to record any hatching for 20 days, and the day of hatching and the species were noted. In total, we used 764 diapausing propagules from sediments (sedimentary eggs) and 223 from waterbird faecal samples (faecal eggs).

Statistical analyses

Statistical analyses were performed using R 3.3.3 (R Foundation for Statistical Computing) program. We analyzed the overall hatching success of zooplankton resting eggs using generalized linear mixed models with Markov chain Monte Carlo (MCMC) methods. MCMC methods can easily handle complex models with multiple random effects when large data sets are used (Bolker et al., 2009, Hamra et al., 2013), and they may be more robust than other strategies used in complex generalized linear mixed models (Hadfield 2010). These analyses were performed in the Bayesian R package MCMCglmm (Hadfield, 2010). The hatching success was a binary response variable (success=1 or failure=0), while predictors were the origin of the diapausing propagules (sediments or faecal samples), the salinity concentration, considered as a continuous variable, and the interaction between the origin of the eggs and salinity to know if the egg response to salinity could be depending on the origin of the eggs. We tested both linear and quadratic relationships with salinity because we assumed that either hatching rates might change linearly with salinity concentration or optimal hatching rates might be achieved around an intermediate value of salinity. We used sampling date, experiment number, zooplankton species, and waterfowl species as random factors. We also used "lake" as a fixed factor to correct for potential site effects, as we could not consider it as a random factor because it only has two levels (Santa Olalla or Dulce). This is a useful strategy for nested effects if the number of groups is low

(Shielzeth & Nakagawa, 2013). We also performed these analyses by considering only the mallard duck (*Anas Platyrhyncos*), because most of faecal eggs used in the experiments belonged to mallard samples, and/or by separately considering the origin of the diapausing eggs. Estimates of the predictors were considered statistically significant if the 95% credible intervals did not cross zero and pMCMC values calculated in MCMCglmm were less than 0.05 (Fisher et al., 2013). We used DIC (deviance information criterion) for comparing linear and quadratic models in the analysis of relations between hatching rates and salinity concentration. DIC is similar to the AIC, and it is used in Bayesian approaches (Bolker et al., 2009). We also tested the statistical significance of random effects using this deviance information criterion (Bolker et al., 2009; Wilson et al., 2010). More information about these Bayesian models is provided in the Appendices section (Appendix2, page 164).

We also performed simpler generalized linear mixed models on hatching rates by considering just main zooplankton species as a fixed factor. For these simpler models with less than 3 random effects, we used a frequentist approach with the function *glme*r of the R package "lme4" (Bates et al., 2015). We modeled assuming a binomial distribution. We assumed that all the effects were present regardless statistical significance (Bolker et al., 2009), although we removed the interaction term of the model (salinity:origin) if it was not significant. Linear and quadratic models using salinity concentration were compared, and we choose the model with the lower AIC value. The relevance of the random factors was evaluated using the likelihood ratio tests (LRT) (Pinheiro & Bates, 2000). Pairwise comparisons within main effects with more than two categories were made by the function *glht* of the R package "multcomp" (Hothorn, 2008). We also tested simple main effects for interactions to evaluate contrasts across the levels of one factor, when the values for other factors are fixed at a certain level. These tests were done with the function *testInteractions* of the R package "phia" (De Rosario-Martínez, 2015a, 2015b).

The day of hatching was also analysed through generalised linear mixed models, but now assuming a Poisson distribution. Overdispersion assumption was tested in *glmer* models using *overdisp_fun* (Bolker *et al.*, 2009). *MCMCglmm* models include individual-levels random effects, which solve overdispersion problems.

As there was no true replication of the two temperature treatments (Hurlbert, 1984), all analyses on the zooplankton hatching rates were preformed for each temperature separately. Results were further compared among the two temperatures.

Results

Overall description of propagules and hatching variability

Table 9 shows the species or genus of the zooplankton diapausing propagules experimentally used according to their origin. The most diapausing propagules were belonging to the genus *Brachionus*. Table 9 also shows the waterbird species from which we obtained faecal eggs, observing that most of the faecal eggs used in the experiments were found in faecal pellets of the mallard duck (*Anas platyrynchos*), the common tern (*Sterna hirundo*) and the common coot (*Fulica atra*).

In relation to the hatching experiments, most of hatchlings corresponded to the genus *Brachionus* in both the sediment and the waterbird faecal samples (Table 9). Other species that hatched from sediments were *Polyarthra* sp., *Hexarthra fennica*, *H. mira*, *Filinia longiseta* and *Cephalodella* sp., while those that also hatched from faecal samples were *Polyarthra* sp. and *Dicranophorus* sp. No hatchlings were observed at 8 g l^{-1} of salinity from propagules isolated from faecal samples at 15 ºC, while some hatchlings were observed at 8 g l^{-1} in the other treatments (Figure 17). Almost all hatchlings from faecal eggs corresponds to propagules isolated from mallard duck faecal samples, while no zooplankton hatchlings were neither observed from propagules isolated from common coot faecal samples nor from common tern faecal samples at any salinity treatment (Table 9). Although no zooplankton hatchling was also observed for many bird species, diapausing propagules were not as abundant in their samples as they were in samples from common coot or common tern (Table 9).

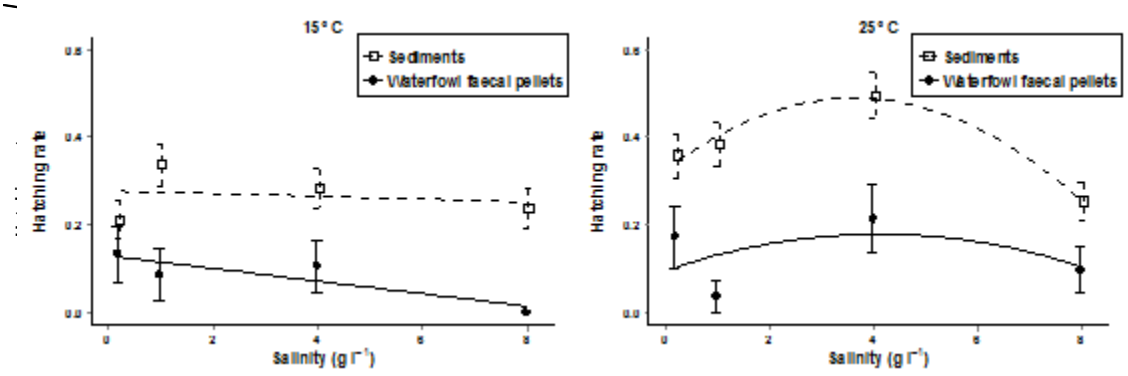

Figure 17. Relationships between hatching rates of resting eggs and salinity at each temperature, according to the origin of the eggs. Mean ± SE.

Rotifers	Lake sediments	Faecal samples of waterfowl species									Total
		A. clypeata	***A. platyrhyn.***	***C. alpina***	***E. garzetta***	***F. atra***	***P .leucorodia***	***P. porphirio***	***S. hirundo***	***V. vanellus***	
Brachionus angularis	233 (23)	2 (1)	41 (13)	-	1	14	2	-	54	1	348
B. budapes	-	-	4	-	-	1	-	-	-	-	5
B. calyciflorus	31 (9)	-	22 (8)	1	-	2	-	-	2	2	60
B. leydigi	3 (2)	-	-	-	-	-	-	-	-	-	3
B. plicatilis	170 (85)	1	6	-	-	1	-	-	1	-	179
B. urceolaris	-	-	-	-	-	2	-	-	-	-	2
B. variabilis	11 (6)	2	2	-	-	-	-	-	-	-	15
Cephalodella sp.	11 (2)	-	-	-	-	-	-	-	-	-	11
Dicranophorus sp.	-	1 (1)	-	-	-	-	-	-	-	-	1
Filinia sp.	57 (6)	-	2	-	-	6	-	-	2	1	68
Hexarthra fennica	111 (66)	1	4	-	-	3	-	1	4	-	124
H. mira	34 (8)	-	-	-	-	-	-	-	-	-	34
Keratella tropica	44 (8)	-	2	-	1	1	-	-	-	1	49
Lecane sp.	-	2	1	1	-	-	-	-	-	1	5
Polyarthra sp.	59 (29)	-	3 (1)	-	-	9	-	-	1	2	74
Proales sp.	-	-	-	-	-	-	-	-	1	-	1
Unknown rotifer	-	-	-	-	-	1	-	1	2	4	8

Table 9. Rotifer resting eggs used in the hatching experiments indicating their origin. Total hatchlings in experiments are indicated in brackets.

Waterfowl species: *A. clypeata* = *Anas clypeata* (Northern Shoveler); *A. platyrhyn*= *Anas platyrhynchos* (Mallard Duck); *C. alpina* = *Calidris alpina* (Dunlin); *E. garzetta* = *Egretta garzetta* (Little Egret); *F. atra* = *Fulica atra* (Common Coot); *P. leucorodia* = Platalea leucorodia (Common Spoonbill); *P. porphirio* = *Porphyrio porphyrio* (Purple Swamphen); *S. hirundo* = *Sterna hirundo* (Common Tern); *V. vanellus* = *Vanellus vanellus* (Northern Lapwing).

Hatching success from propagules of sedimentary vs. faecal samples

Globally, regardless of the rotifer species, salinity treatment or temperature, hatching success of rotifer isolated from sediments tended to be higher (mean ± SD; 0.32 ± 0.47, n=764) than those isolated from waterbird faecal samples (mean ± SD; 0.11± 0.31 , n=223). Anyway, different types of results were obtained using statistical analyses. According to the overall model, we observed, at 15 ºC, a clear linear negative effect of salinity on hatching success, while the interaction term (salinity x origin) was "marginally" significant because of the credible intervals (Table 10). Figure 18 shows the negative effect of salinity on egg hatching at 15 º C and that the slope for resting eggs from waterbird faecal samples is slightly higher than for those coming from sedimentary samples. As *A. platyrynchos* was the only bird species from which zooplankton hatchlings were obtained at 15 ºC, there was a significant effect of waterbird species on hatching success (Table 10). For that reason, we also performed the analysis by considering sedimentary propagules and propagules from faecal samples of just that bird species. Anyway, the results of this analysis (Salinity: posterior mean = -0.45, CI = -0.91 to -0.04, pMCMC = 0.022; Origin: posterior mean = -0.28, CI = -3.21 to 3.20, pMCMC = 0.806; Salinity x Origin: posterior mean = 0.42, CI = -0.03 to 0.86, pMCMC = 0.044) were similar to that including all bird species (Table 2). Salinity concentration was also significant at 25 ºC, but with a quadratic relationship (Table 10, Figure 18), because the DIC value of the model was higher when using a linear relationship with salinity (Δ DIC= 9.1).

As the random factor "zooplankton species" was significant (Table 10), we checked the different hatching responses of the most representative species in both sediment and mallard faecal samples (*B. angularis* and *B. calyciflorus*) through a binomial mixed model including sedimentary resting eggs and resting eggs from mallard faecal samples. The analysis showed a negative effect of salinity, a significant effect of the origin of the eggs (higher rates from waterbird samples) and a marginally significant interaction effect between the origin of the propagules and the rotifer species on the hatching success at 15 ºC (Table 11). In a previous model, as the interaction between salinity and rotifer species was clearly not significant (p = 0.65), it was removed from the final model in Table 11. At this temperature, a post-hoc analysis of the interaction between the origin of the propagules and the rotifer species revealed that *B. angularis* resting eggs coming from faecal samples showed significantly higher hatching success than those coming from the sediments, while no significant effects were observed for *B. calyciflorus* eggs (Table 11). However, there was neither effect of salinity nor different hatching responses of rotifer species at 25 ºC of

temperature (Table 11). Although the interaction between the origin of the eggs and the species was clearly not significant in this case, we decided to maintain it for species comparison purposes. Anyway, the model without the interaction was similar (AIC = 176.2). An individual linear mixed model just for *B. angularis* also showed, at 15 ºC, higher hatching success for faecal eggs than for sedimentary eggs (Wald z = 2.13, p = 0.033, Figure 18), while no effect of salinity was observed (Wald z = -1.60, p =0.11). No significant effect was observed at 25 ºC (salinity effect: Wald z = -0.54, p = 0.59; origin of the eggs: Wald z = 1.25, p = 0.21). Other species were too scarce or absent in faecal samples to be specifically considered (Table 9).

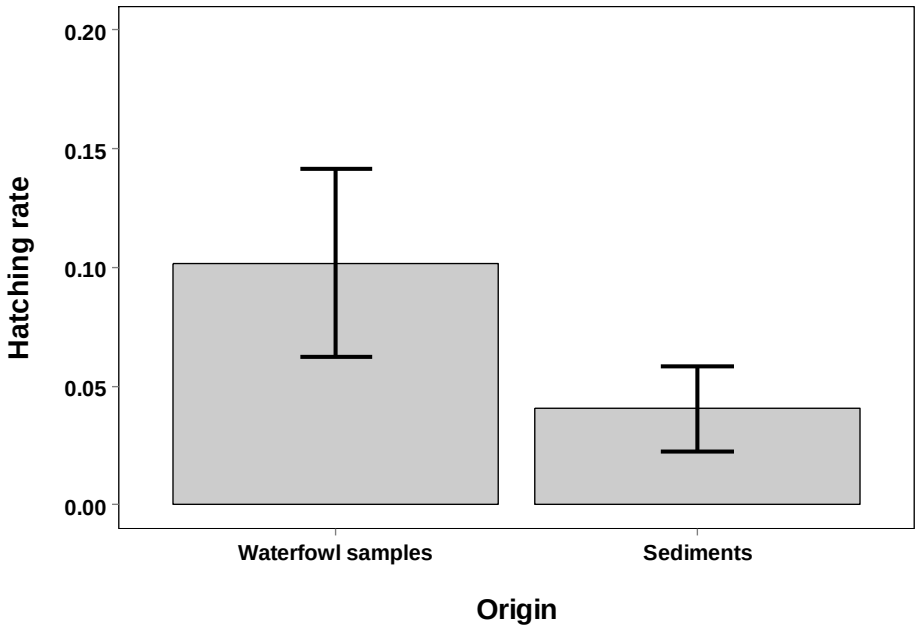

Figure 18. Mean hatching rates of resting eggs of *Brachionus angularis* at 15 ºC from sediment and waterfowl samples. Mean ± SE.

Table 10. Mixed-models for the effect of salinity and the origin of the eggs on the hatching rates of resting eggs.

	Temperature 15 ºC -DIC = 399.8				Temperature 25 ºC -DIC = 531.7			
	Post.mean	low-CI 95%	up-CI 95%	pMCMC	Post.mean	low-CI 95%	up-CI 95%	pMCMC
Main effects								
Intercept	-4.54	**-8.71**	**-0.79**	**0.011**	-4.01	**-6.52**	**-1.66**	**<0.001**
Salinity	-0.43	**-0.84**	**-0.01**	**0.022**	0.49	**0.16**	**0.84**	**0.005**
Salinity^2					-0.07	**-0.11**	**-0.03**	**0.001**
Origin (sediments)	3.71	-1.82	9.81	0.163	2.91	-1.18	7.33	0.127
Lake (Santa Olalla)	-0.23	-1.03	0.58	0.580	0.11	-0.48	0.73	0.737
Salinity x Origin	0.40	-0.03	0.83	0.047				
				ΔDIC*				ΔDIC*
Random factors								
Waterfowl species	4.04	0.28	9.16	-17.4	3.17	0.09	7.37	-15.1
Rotifer species	1.96	0.48	3.94	-70.4	0.87	0.11	2.01	-31.8
Date of collection	0.62	<0.01	2.43	0.3	0.31	<0.01	1.41	0.7
Experiment number	1.44	<0.01	4.17	-5.0	0.17	<0.01	0.76	0.9

*The DIC of the full model minus DIC of the reduced model without that term. Large negative numbers indicate strong support for keeping the term in the model. Values that are statistically significant are indicated in bold.

Table 11. Mixed-models for the effect of salinity, the origin of the eggs, and the egg species (*Brachionus angularis* and *B. calyciflorus*) on the hatching rates of resting eggs.

	Temperature 15 ºC -AIC = 99.9				Temperature 25 ºC -AIC = 178.1			
	Estimate	SE	Wald z	*p*	Estimate	SE	Wald z	*p*
Main effects								
Intercept	-0.31	0.56	-0.56	0.578	-0.07	1.06	-0.07	0.949
Salinity	-0.25	0.12	-2.08	**0.037**	-0.03	0.07	-0.52	0.600
Origin (sediments)	-3.67	1.37	-2.67	**0.008**	-1.51	1.39	-1.09	0.274
Species (*B. calyciflorus*)	0.31	0.89	0.35	0.729	0.09	0.76	0.13	0.900
Lake (Santa Olalla)	1.63	1.28	1.28	0.201	-0.75	0.59	-1.26	0.206
Origin x species	2.82	1.53	1.85	0.065	0.43	1.21	0.36	0.720
Post-hoc analysis								
	B. angularis		*B. calyciflorus*		*B. angularis*		*B. calyciflorus*	
	Chi-square	*p*	Chi-square	*p*	Chi-square	*p*	Chi-square	*p*
Faeces vs. sediments	7.14	**0.015**	0.59	0.443	1.20	0.548	0.36	0.550

Values that are statistically significant are indicated in bold.

Table 12. Mixed-models for the effect of salinity on the hatching rates of resting eggs from sediments.

	Temperature 15 ºC -DIC = 352.0				Temperature 25 ºC -DIC = 445.9			
	Post.mean	low-CI 95%	up-CI 95%	pMCMC	Post.mean	low-CI 95%	up-CI 95%	pMCMC
Main effects								
Intercept	-1.11	-2.83	0.67	0.195	-1.22	-2.53	0.06	0.053
Salinity	-0.03	-0.14	0.08	0.573	0.54	0.17	0.91	**0.002**
Salinity^2					-0.07	-0.12	-0.03	**0.001**
Lake (Santa Olalla)	0.08	-0.79	0.94	0.856	0.19	-0.50	0.82	0.577
				ΔDIC*				ΔDIC*
Random factors								
Rotifer species	2.25	0.55	4.53	-75.2	1.16	0.19	2.53	-44.3
Date of collection	0.49	<0.01	1.93	0.5	0.41	<0.01	1.90	0.5
Experiment number	0.95	<0.01	2.99	-3.4	0.20	<0.01	0.88	0.9

*The DIC of the full model minus DIC of the reduced model without that term. Large negative numbers indicate strong support for keeping the term in the model. Values that are statistically significant (main effects) or are clearly relevant (random factors) are indicated in bold.

Hatching success from propagules of sedimentary samples

Beside all these models, analyses were also performed using just the sedimentary eggs to look for different patterns respect to the overall analyses and to study the relationship of the salinity with the hatching success of those rotifer species which are scarce in faecal samples. These analyses showed no significant effect of salinity on hatching success of zooplankton at 15 ºC, but there was a significant quadratic relationship of salinity with hatching success at 25 ºC (Table 12). At 25 ºC, the DIC value was higher if a linear relationship with salinity was considered (Δ DIC= 10). The random factor "zooplankton species" was again significant at both experimental temperatures (15 and 25 ºC) (Table 12). Because of that, we repeated that analysis using "zooplankton species" as a fixed factor, but including only the main zooplankton species in both lakes (*B. plicatilis*, *B. angularis* and *B. calyciflorus*). Results indicated that salinity only affected to hatching success of *B. plicatilis* at both 15 ºC (positive linear trend) and 25 ºC (quadratic trend) of temperature (Table 13, Figure 19). Moreover, the hatching success of *B. calyciflorus* were significantly higher than those of *B. angularis* at 15 ºC of temperature (Table 13, Figure 19). As *Hexarthra fennica* was relevant in Santa Olalla lake, but absent in Dulce lake, we performed an additional linear mixed model for this species showing a quadratic effect of salinity on their hatching success at 15º C (LRT: Chi-squared = 8.99, df = 2, p = 0.011), while no effect was observed at 25 ºC (p = 0.276).

As hatching success of *B. plicatilis* were positively related with salinity at 15 ºC, while salinity had a negative effect on the hatching success of the overall data at that temperature (Table 10), we repeated that MCMC analysis excluding *B. plicatilis*. The result showed that salinity was again affecting negatively to the rotifer hatching success (posterior mean = -0.40, CI = -0.83 to -0.02, pMCMC = 0.032), but now the interaction term (salinity x origin of the eggs) was clearly not significant (posterior mean = 0.22, CI = -0.21 to 0.65, pMCMC = 0.306). This indicates that the higher slope observed for faecal eggs in Table 2 may be due to the scarcity of *B. plicatilis* eggs in the faecal samples. If we exclude *B. plicatilis* eggs from the overall analysis of Table 10 at 25 ºC, results remain the same, because we also observed a quadratic relationship of salinity with the rotifer hatching success.

Table 13. Mixed-models for the effect of salinity and the egg species (*Brachionus angularis*, *B. calyciflorus and B. plicatilis*) on the hatching rates of resting eggs from sediments. The relationships of salinity with hatching rates were linear (15 ºC) and quadratic (25ºC). Main results are expressed with the function *Anova* of the R package "*car*" (Fox & Weisberg, 2011).

	Temperature 15 ºC -AIC = 163.8				**Temperature 25 ºC -AIC = 244.4**			
	Chisq	df		*p*	Chisq	df		*p*
Main effects								
Intercept	14.34	1		**<0.001**	7.86	1		**0.005**
Salinity	0.53	1		0.466	1.46	2		0.481
Species	8.25	2		**0.016**	0.98	2		0.612
Lake (Santa Olalla)	0.30	1		0.582	0.07	1		0.781
Salinity x Species	4.91	2		0.086	11.19	4		**0.025**
Post-hoc analysis								
Pairwise contrasts	Estimate	SE	Wald z	*p*	Estimate	SE	Wald z	*p*
B. calyciflorus - *B. angularis*	4.48	1.80	2.49	**0.031**	1.05	1.07	0.98	0.580
B. plicatilis – *B. angularis*	1.87	0.82	2.30	0.051	0.30	0.65	0.46	0.887
B. plicatilis– *B. calyciflorus*	-2.61	1.66	-1.58	0.240	-0.75	1.04	-0.73	0.743
Interaction with salinity	Value	df	Chisq	*p*	Value (Salinity)	df	Chisq	*p*
B. angularis	-0.13	1	0.53	0.561	0.09	1	0.10	0.987
B. calyciflorus	-2.29	1	1.16	0.561	0.33	1	0.47	0.987
B. plicatilis	0.24	1	7.57	**0.018**	1.57	1	20.86	**<0.001**
					Value (Salinity^2)	df	Chisq	*p*
B. angularis					-0.03	1	0.48	0.732
B. calyciflorus					-0.04	1	0.34	0.740
B. plicatilis					-0.17	1	15.77	**<0.001**

Values that are statistically significant are indicated in bold.

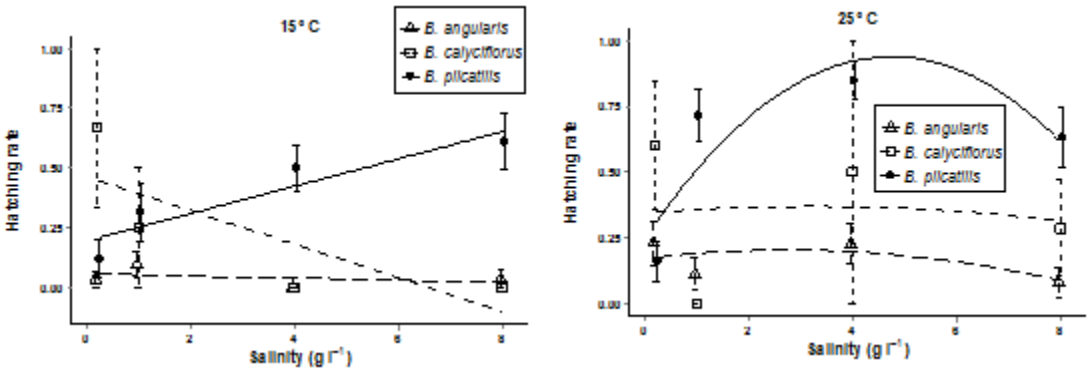

Figure 19. Relationships between hatching rates of resting eggs of *Brachionus angularis*, *B. calyciflorus* and *B. plicatilis* with salinity concentration at 15 and at 25 ºC of temperature. Mean ± SE.

Paired t-test showed no difference for the hatching success from sedimentary eggs (regardless rotifer species) across the salinity range between 15 ºC and 25 ºC of temperature ($t = 2.31$, $df = 3$), $p = 0.10$). Nevertheless, to avoid sacrificial pseudoreplication due to the significant effect of rotifer species on hatching success, additional paired t-test were also performed for the main hatched species from sedimentary eggs. These analyses also did not show significant results for *B. plicatilis* ($p = 0.137$), *B. angularis* ($p = 0.11$) and *H. fennica* ($p = 0.532$).

Hatching success from propagules of faecal samples

Finally, we analysed faecal eggs separately. In this case, as the random factor "experiment number" has only two levels, we performed the analyses separately for the two experiments. Additionally, there were some convergence problems with the function *glmer*. For that reason, we also perform the analyses separately for both lakes and/or we used the *MCMCglmm* function, if resulted in a good convergence of parameters and absence of autocorrelation despite there were fewer cases that in previous analyses. Results showed a marginal effect of salinity on hatching success at 15 ºC (marginally significant), while salinity was not significantly related with the hacthing rates at 25 ºC (Table 14), and the relationship was linear. Waterbird species were slightly relevant at 15 ºC in experiment 5 because of the hatchlings from the mallard samples. When we repeated the analysis using just mallard faecal samples, the negative effect of salinity was significant because 95% credible intervals did not cross zero (posterior mean = -0.40, CI = -0.83 to -0.01, pMCMC =

0.035). No hatching was recorded from faecal eggs of Santa Olalla lake at 15 ºC. In experiment 6, salinity negatively affected the hatching success in Santa Olalla lake, and there were differences in the response of rotifer species (Table 14).

Paired t-test showed no difference for the hatching success of faecal eggs (regardless rotifer species or waterbird species) across the salinity range between 15 ºC and 25 ºC of temperature ($t = 1.35$, $p = 0.270$). An additional analysis for the main hatched species from faecal eggs (*B. angularis*) showed similar results ($p = 0.249$).

Day of egg hatching

The day of hatching ranged between 1 and 19 days. Neither effect of salinity concentration nor the origin of the eggs was observed on the day of hatching of resting eggs at any experimental temperature ($p > 0.05$ in all cases). There were also no differences between rotifer species. Similar results were observed analysing just sedimentary eggs or just faecal eggs, except if we compare the hatching time of *B. angularis* and *B. calyciflorus* from faecal eggs at 25ºC. In that case, we observed that the hatching time of *B. angularis* was shorter than that of *B. calyciflorus* (Chi-squared = 4.38, $p = 0.038$).

Table 14. Mixed-models for the effect of salinity on the hatching rates of resting eggs from all faecal samples. Results (*MCMCglmm*) for experiment 5 are focused on Dulce lake because of the lack of hatchlings in Santa Olalla lake. Results (*glmer*) for experiment 6 were obtained from Santa Olalla lake at 25 ºC, because there were no hatchlings, neither at 15 ºC nor in Dulce lake. Random factors were analysed according to the likelihood ratio test for the experiment 6.

	Experiment 5								Experiment 6			
	Temperature 15 ºC -DIC = 42.1				Temperature 25 ºC -DIC = 56.7				Temperature 25 ºC -AIC = 13.2			
	Post.mean	low-CI 95%	up-CI 95%	pMCMC	Post.mean	low-CI 95%	up-CI 95%	pMCMC	Estimate	SE	Wald z	*p*
Main effect												
Intercept	-2.43	-5.46	0.27	0.047	-3.44	**-6.43**	**-0.92**	**0.003**	24.41	9.68	2.52	**0.012**
Salinity	-0.37	-0.80	0.01	0.047	0.07	-0.20	0.32	0.604	-41.48	12.95	-3.20	**0.001**
				ΔDIC*				ΔDIC*			Chisq	
Random factors												
Waterfowl species	2.38	<0.01	6.39	-6.1	2.12	<0.01	6.12	-4.8			1.30	0.254
Rotifer species	0.60	<0.01	2.46	0.6	0.64	<0.01	2.61	0.5			5.79	**0.016**

*The DIC of the full model minus DIC of the reduced model without that term. Large negative numbers indicate strong support for keeping the term in the model. Values that are statistically significant are indicated in bold.

Nevertheless, an increase of temperature could reduce the hatching time, according to a paired t-test performed across the four salinity treatments of sedimentary eggs regardless of the zooplankton species ($t = 7.86$, $p = 0.004$; Figure 20). Similar analysis on the main hatched species from the sediments revealed significant results for *B. plicatilis* ($p = 0.019$) and *H. fennica* ($p = 0.001$) and marginally significant for *B. angularis* ($p = 0.074$). No significant result was obtained for faecal eggs ($p = 0.483$), although fewer data were available in this case.

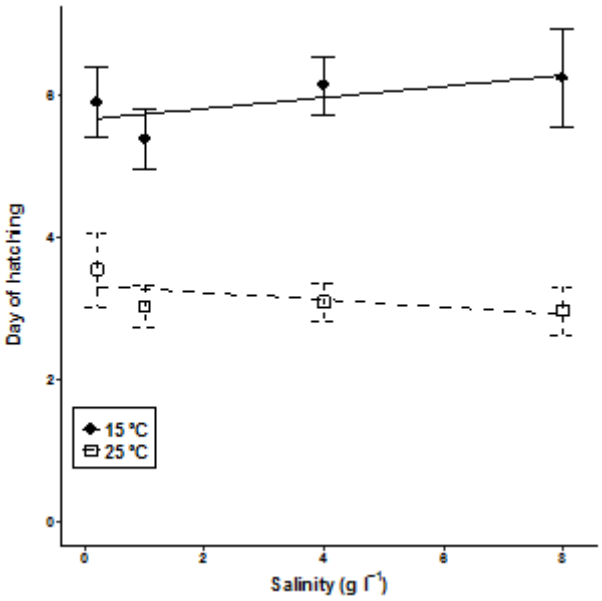

Figure 20. Mean day of hatching of resting eggs from sediments across the salinity range at each experimental temperature. Mean ± SE.

Discussion

This is the first study comparing the hatching response of rotifer species between resting eggs from the sediments and resting eggs from waterbird faecal samples. Moreover, we also evaluate that response at different salinity concentrations, but we did not find an interaction between salinity and the origin of the eggs. Nevertheless, the hatching response changed between rotifer species and between waterbird species. We also showed that, at least for one rotifer species, the hatching success may change as a result of waterfowl ingestion.

The range of germination rates of plant species collected from waterbirds faecal samples ranges between 0 and 28.5 % (Wongsriphuek et al., 2008), while in aquatic leaf beatles the hatching is about 20% (Laux & Kölsch, 2014). In our study, the overall mean of the hatching success of rotifers from faecal eggs was 0.11, but, as far as we are aware, there are no previous studies on rotifers resting eggs from waterbird faecal samples. In previous studies, the hatching response of invertebrates was estimated incubating inundated waterbird faecal samples, so the propagules were not previously isolated (Frisch et al., 2007; Green et al., 2008; Laux & Kölsch, 2014). In those studies, the number of waterbird droppings with rotifer hatchlings was about 10-13%, which could be similar with our overall mean. Nevertheless, in our study, resting eggs were

individually isolated from faecal samples, and we studied their hatching capability in isolated units.

Other studies have only found hatchlings of *B. plicatilis*, *Brachionus* sp., *Cephalodella* sp., some few bdelloid rotifers in coot, ducks or black swan (Frisch et al., 2007; Green et al., 2008). However, it is not possible to know the real hatchlings in these studies because reproduction after hatching may have occurred. In our case, we found hatchlings of *B. angularis*, *B. calyciflorus*, *Dicranophorus* sp. and *Polyarthra* sp., and we were able to estimate the hatching success. Moreover, we also found other 13 rotifer species in the resting eggs of waterbird droppings (Table 9), although they did not hatch.

We showed that rotifer populations were negatively affected by our moderate increases of salinity at 15 ºC, and that response is similar regardless of the origin of the resting eggs (sediments or waterfowl faecal samples). Previous studies have shown negative effects of salinity on rotifer hatching rates from sediment egg banks, but using higher salinity ranges (García-Roger et al., 2008; Nielsen et al., 2012, Santangelo et al., 2014). However, in those studies, resting eggs were not individually isolated to study their hatching success, and no information about the number of resting eggs per species was provided. Santangelo et al. (2014) studied hatchlings abundance and species richness at 24 ºC of temperature, and they found significant reductions in abundance and species richness at high salinities (>16 g l-1). In our study, we found different hatching responses to salinity concentration depending on the experimental temperature. Overall, we observed a negative effect of our moderate concentration range of salinity on hatching success at 15 ºC, while a quadratic relationship between hatching success and salinity was observed at 25 ºC. Kim et al. (2011) showed that the salinity experienced during resting egg formation is the optimal one for egg hatching in *Brachionus*. Our resting eggs may have been formed not only at medium salinity concentrations but also to the high temperatures typical of summer time in this area. These conditions could stimulate hatching. Nevertheless, further studies should clarify these aspects.

We also observed variability in the hatching response of rotifer species to salinity concentration, which has been observed previously (Santangelo et al., 2014). However, no previous studies have shown different responses to salinity as a function of temperature. Salinity showed a positive effect on hatching success of *B. plicalilis* at 15 ºC and a quadratic effect at 25 ºC, while the hatching success of *B. angularis* and *B. calyciflorus* were, to some extent, negatively related with salinity at 15 ºC (Tables 11 and 12). *B. plicatilis* use to inhabit salt waters (Campillo et al., 2011), and Santangelo et al., (2014), although not cited, observed a more or less quadratic pattern between hatchlings of this species and salinity. Nevertheless, their salinity range was much higher (0.1 – 32 g l^{1}) than ours. García-Roger et al. (2006) considered as successful for hatching of *B. plicatilis* a medium salinity level of 6 g l^{1}. Finally, *Hexarthra fennica* showed a quadratic relationship with salinity at 15 ºC in our study. Santangelo et al., (2014) observed that *Hexarthra* spp. reduced the hatching of resting eggs as salinity increased at 24 ºC, while we did not find a significant effect of salinity on hatchlings of *Hexarthra fennica* at 25 ºC. Nevertheless, experimental procedures were different and, probably, *H. fennica* was not present in the study of Santangelo et al. (2014).

According to our results, increases in salinity may negatively affect to the hatchlings of rotifer populations, with some exceptions if those increases are not very high. In other invertebrates (anostracans), it seems that increasing salinity may also inhibit hatching performance (Atashbar et al., 2014). As a consequence of climate change, lakes may shift to oligosaline or mesosaline conditions. Changes to oligosaline conditions would not be relevant for these rotifer populations (according to their hatching success), while changes to mesosaline conditions could be dramatic for the rotifer populations. Nevertheless, rotifers may show a bet-hedging strategy for the optimal timing of leaving dormancy in response to habitat unpredictability (García Roger et al., 2014), which may be related with climate change.

Several studies have shown that the germination of aquatic plant seeds may increase after gut passage (Espinar et al., 2004), although the seed coat structure may influence on the effect of gut passage (Soons et al., 2008). In this sense, the response variation of gut passage is higher between plant species than between waterbird species (García-Álavarez et al., 2015). In invertebrates, Sánchez et al. (2012) showed no significant differences in hatching between ingested and non-ingested cysts of two Artemia species, while Rogers (2014) observed a positive effect of gut passage on hatchlings of several anostracan eggs. In our study, there were no overall effect of waterbird ingestion on hatching success, but the rotifer *B. angularis* showed higher hatching success from faecal eggs than from sedimentary eggs. In consequence, at least for this rotifer species, the consumption of resting eggs by waterbirds may increase the chance of colonization.

While for plant seeds it has been observed that the effect of gut passage on germination rates may change as a function of salinity (Espinar et al., 2004; Espinar et al, 2006), we did not observe a significant effect of salinity on *B. angularis* nor a significant effect of the interaction between salinity and origin of the eggs. Espinar et al. (2004) observed that germination rates may increase by gut passage at low salinities, while a negative effect of the gut passage may occur with an increase of salinity concentration (Espinar et al., 2004; Espinar et al., 2006). Nevertheless, Espinar et al. (2004) found a quadratic pattern in the response of the percentage germination to salinity in two helophyte species. Although no significant effect of gut passage on percentage germination was observed for these species. Ingestion did not also affect germination speed, although it was reduced by salinity. The same seems to be applied to our results, because most of the rotifer species seems to be not affected by waterbird ingestion, while the overall hatching success was negatively related with salinity.

An aspect to consider is that proportion of propagules surviving digestion may be depended on the diet of waterbirds (Green et al., 2002). Charalambidou & Santamaria (2002) suggest plant seeds mixed with animal diet is better for their survivorship that a purely seed diet. Variations in the effects of ingestion by waterbirds could be also related with differences in the egg age and structure, as suggested for germination patterns of plant seed after ingestion by vertebrates (Traveset, 1998). Anyway, it has been argued that increases in germination rate of aquatic plant seeds after ingestion are not necessarily beneficial for the plants, because it would depend on complex interactions with other factors such as herbivore abundance (Figuerola &

Green, 2004). In the case of rotifers, further studies are needed to clarify if the increase of hatching rates by gut passage would be benefiting.

Other striking result that we obtained is that we only observed hatchlings from faecal droppings belonging to two waterbird species (*Anas platyrhynchos* y Anas clypeata). The number of resting eggs in the faecal samples of the other species was low, but in *Fulica atra* and in *Sterna hirundo* was abundant. It is difficult to explain why there were no hatchlings from these waterbird species. Higher hatchability in *Artemia* species have been related to smaller gizzard size of waterbird species (Sánchez et al., 2012), although maybe the gizzard size would not be so relevant for the small rotifer resting eggs. Frisch et al. (2007) showed cladoceran hatchlings from duck faeces but not from coot faeces, although, as propagules were not isolated from faecal samples, it is quite possible that cladoceran propagules were not present in just 10 coot faecal droppings of their study. Nevertheless, they found rotifers from coot faecal droppings. In a previous study in that area, Figuerola et al. (2003) only found 12 cladoceran ephippia in 61 coot faecal droppings. Green et al. (2008) observed higher abundance and hatchlings of invertebrate propagules in coot than in teal, and rotifer hatchlings were observed in coot but not in teal. They argue that differences may be explained, at least in part, by a higher digestive efficiency of coot (herbivorous) compared with teal (omnivorous). However, in our case, we did not observe hatchlings in coot. Nevertheless, Figuerola et al. (2010) suggested that the reduction of germination percentage of seeds by red-crested pochard (predominatly herbivorous) was possible due to the harsher treatment in the gut of this waterbird species. Other reason could be related with the collection of faecal samples from different lakes (Green et al., 2008). However, in our study, lake location had no significant effect on the hatching success nor on the day of hatching. In consequence, further and specific studies are needed to test different hatching capability of rotifer resting eggs as a function of waterbird species.

As dispersal by waterbirds may occur at ecological time scales, this mechanism responds to rapid environmental changes as those associated with climate change (Viana et al., 2016). Changes in the desiccation pattern could affect to the rotifer dispersal by waterbirds, while changes in salinity concentration due to climate change could favour local adaptation and evolutionary responses of rotifers. Rapid evolution has been described for zooplankton (Hairstone et al., 1999), and genetic changes may have a measurable impact on simultaneous ecological changes (Hairstone et al. 2005). In addition to genetic diversity, the ability of populations to tolerate stress will depend on previous and current environmental conditions (Liao et al., 2015). Recently, Declerck et al. (2015) showed micro-evolutionary responses of a rotifer species to changes in the availability of essential elements. Smith & Snell (2012) demonstrated, in a laboratory study, rapid evolution of higher propensity to sex and dormancy in ephemeral cultures in comparison with permanent cultures. In the case of salinity, the adaptability of zooplankton as a response to salinity is also important to understand the resilience of freshwaters to salinity increase. Some studies showed local adaptations to salinity in *Daphnia* (Liao et al., 2015) and *Brachionus plicatilis* (Alcantara et al., 2012). Alcántara et al. (2012) showed local adaptation to different salinity levels in *Brachionus plicatilis* species complex with different origin, although they did not study evolutionary responses to salinity.

Further studies should focus on this topic. We hypothesize that, at least in a lake with salinity fluctuations, local adaptation and evolutionary change of rotifers will occur as a response to increases in salinity levels, contributing to the resilience of the systems to climate change.

Chapter 5

Zooplankton diversity and community assemblages in Mediterranean ponds with different hydroregimes

Abstract

Mediterranean water bodies are biodiversity hotspots. Zooplankton play a critical role in aquatic ecosystems and its assessment is important. In addition, the study of zooplankton can be particularly useful to assess environmental changes. Freshwater biodiversity in Mediterranean habitats is in increasing threatened due to the highly demands on freshwater resources, strongly seasonal hydroregime changes and the impact of alien species. The number of zooplankton species that inhabit an aquatic system is particularly difficult to assess due to their short life-cycles, especially in rotifers species. In order to understand the environmental framework of our study areas, we characterized the most important abiotic factors which determine the zooplankton community. To do so, we monitored during three years four lakes providing a detailed description of the main limnological variables and zooplankton composition of the four lakes which are object of this thesis. Zooplankton community structure in a freshwater habitat is determined by the proximity of other water bodies that may link and allow the transport of individuals and diapausing eggs. In order to study the possible water flow dispersal we take into account the cyclically connection between Doñana lakes, and additionally sampling a water body connection between the Ruidera lakes. We also relate the results of previous chapters with the biodiversity and dynamics of the zooplankton inhabiting the studied lakes.

Introduction

Mediterranean ponds are biodiversity hotspots (Myers *et al.*, 2000). Freshwater biodiversity in Mediterranean habitats is in increasing threatened due to the highly demands on freshwater resources, strongly seasonal hydroregime changes and the impact of alien species (Jenkins, 2003; García-Berthou *et al.*, 2007; Moss *et al.*, 2009). Therefore, biodiversity assessments are important.

Zooplankton play a critical role in aquatic ecosystems, as they are the main grazers of phytoplankton and are also the primary food source for species in higher trophic levels (Wallace, 2002). Biotic and abiotic factors exert strong influences on the distribution and abundance of zooplankton species. Therefore, zooplankton communities are important indicators of water quality and biodiversity (Pinel-Alloul et al. 1990; Patoine et al. 2000; Dodson et al. 2005). In lakes where abiotic conditions are relatively predictable, biotic interactions such as predation and competition have stronger influence on zooplankton community. However, in freshwater bodies with unpredictable environmental conditions abiotic factors are powerful driving forces. Additionally, in temporary ponds, habitat permanence and hydroperiod length can also affect both zooplankton species richness and community composition (Serrano and Fahd 2005; Boven and Brendonck 2009; Drenner et al. 2009).

The production of diapausing eggs can help since to overcome the period of adverse environmental conditions with the diapause stage as the probability of being dispersed to another point that may have more favorable environmental conditions. In **Chapter 1** we characterize the diapausing egg bank of rotifers from Dulce, Santa Olalla and Tinaja lakes, so we would have to find a similar species richness of rotifers in the active community of the water column.

The differencies in the potential of diapausing eggs dispersal by the main dispersal agents has been verified and the rates and effectiveness, as the capability to survive to dispersal and hatch, depend on different factors as the characteristics of the location in which the aquatic system of study is. In **Chapter 2** and **Chapter 3** we showed higher disperal rates of diapausing eggs dispersed by waterbirds than by wind or rainfall.

The aim of this study was to record the zooplankton diversity and species composition in *Dulce* and *Santa Olalla* lakes in Doñana and in *Morenilla* and *Tinaja* lakes in Ruidera. We also assess regional richness and its relation with diapausing eggs dispersal and egg bank dynamics. We take into account the possible influence of link connection between each pair of study lakes.

Materials and methods

Field sampling and laboratory analyses

Sampling (zooplankton samples collection and physical-chemical measurements) was carried out over a period of 11 seasons from Spring 2008 to Autumn 2010 in *Dulce* and *Santa Olalla* lakes in Doñana, and *Morenilla* and *Tinaja* lakes in Ruidera (Figures 2 and 3). Additionally, zooplankton samples were collected from *El Hundimiento*, the discharge water body from *Del Rey* lake waterfall which, despite the distance, links Tinaja and Morenilla lakes (Figure 4). Sampling procedures are described in detail in the general methodology section.

Zooplankton density and biomass assessment

In the laboratory, to estimate the zooplankton abundance we counted the entire sample when the number of individuals were relatively small. In the contrary, when the number of individuals were very large we took subsamples to facilitate counting. To do so, samples were diluted to 100 ml with distilled water, mixed and the subsamples were counted using an inverted microscope Zooplankton were counted and identified to the lowest feasible taxonomic level, usually genus using the identification keys: Koste (1978) for rotifers, Alonso (1996) for cladocerans, and Dussart (1969) for copepods. Zooplankton abundance were expressed as number per volume units. Bdelloids and ostracods were not taken into account in the calculations of the zooplankton abundance, biomass and diversity. Copepods were divided in two main groups, Cyclopoids and Calanoids, and counted. In addition, as the organisms of the water column varied considerably in size, the biomass of each taxon at each sampling point and date was estimated. For this purpose, at least 20 organisms, or the total amount if their number was lower, were measured to calculate the dry weight using length–weight relationships (length-weight regressions LWR). Body size of crustaceans was converted to dry weight according to the formulae given in Bottrell et al. (1976). Rotifer biomass was calculated using biovolume measurements described by Ruttner-Kolisko (1977) and dry mass conversions assuming a specific density of 1.0 (Dumont et al. 1975) (1 µm3 = 10-6 µg) and a dry/fresh weight relationship of 0.039 for Asplanchna and of 0.1 for the other rotifer species. The total biomass for each sampling site and date was calculated for the whole zooplankton assemblage (total biomass), and also for the three groups: copepods, cladocerans and rotifers.

Zooplankton diversity and community similarity

To account for different sampling sizes, a rarefaction analysis was performed on the number of all individuals counted. Rarefaction was performed with the *vegan* package (Oksanen et al., 2015) in R version 3.1.3 (R Core Team, 2015) to accommodate differences in the count of all zooplankton samples collected. Rarefaction curves calculate the expected number of species in a random subsample of individuals and compare it to the number of species observed in the less-counted samples. *Alpha* diversity was estimated by two measures: local species richness as the number of all identified taxa per lake and year, and the Simpson index, which takes into account the relative abundances of the identified taxa. *Gamma* diversity was estimated as the cumulative species richness (Gaston and Spicer 2004) of all sampled sites in Doñana and

Ruidera. *Beta* diversity was expressed as the variance of the Hellinger-transformed community data table across all samples (Legendre et al. 2005). Diversity indexes were quantified for the whole zooplankton community, as well as for rotifer, cladoceran and copepod assemblages separately. All the diversity indexes caltulations were performed using the *vegan* package.

Statistical analysis

Data were analyzed using R software (http://www.r-project.org). Analysis of variance using distance matrices were used to model multivariate, distance-based differences in species composition between sampling sites. These analyses were performed with the function "*adonis*" of the *vegan* package. A non-metric multidimensional scaling (NMDS) was performed a Bray–Curtis similarity matrix using "metaMDS" function in R package *vegan* (Oksanen et al., 2015).

To satisfy the assumptions of normality and homogeneity of variance of the data, all data were logarithmically transformed log10 (x + 1) before the analyses.

Results

Physico-chemical parameters

The environmental and physico-chemical parameters varied spatially and temporally among the different sampling sites during the study period (Figure 21 and 22, and Table 15). Maximum depth for both Doñana lakes was 2.5 m and decreased drastically during Summer to less than 0.5 m in *Dulce* lake. However, during the winter in 2010 both lakes were linked. In Ruidera, *Tinaja* lake showed more fluctuations in water level than *Morenilla* lake during sampling period. Water temperature ranged from 9ºC to 32.46ºC in Doñana and 6.5ºC to 24.80ºC in Ruidera. The highest ranges for pH and conductivity were presented in *Santa Olalla* and *Dulce* lakes (Table 15). The concentration of nutrients, total phosphous (TP), soluble reactive phosphorus (SRP) and total nitrogen (TN) were similar between the lakes of the same location (Table 1). TP concentration varied throught the study areas between 0.116-1.939 mg L^{-1} in *Dulce* lake, 0.343-2.075 mg L^{-1} in *Santa Olalla* lake, and between 0.001-0.031 mg L^{-1} and 0.005-0.018 mg L^{-1} in *Morenilla* and *Tinaja* lakes respectively. Concentrations of SRP ranged from 0.363-10.672 µg L^{-1} and 0.155-6.478 µg L^{-1} in *Dulce* and *Santa Olalla* lakes, and from 0.050-<0.001 and 0.020-<0.001 µg L^{-1} in *Morenilla* and *Tinaja* lakes. Nitrate (NO3) concentration were similar in *Dulce* and *Santa Olalla* lakes (169.719-2424.881 µg L^{-1} and 269.178-2416.213 µg L^{-1}, respectively), since *Tinaja* presented high concentrations compared to *Morenilla*, 88.898-151.632 µg L^{-1} and 81.289-505.681 µg L^{-1}, respectively. Ammonium (NH4) concentration were also very variable between *Morenilla* and *Tinaja* (14.474-151.632 µg L^{-1} and 13.184-505.681 µg L^{-1}, respectively). (Chl *a*) concentratons were higher in Doñana lakes than in Ruidera lakes, and decreased drastically in *Dulce* lake (Table 15). Concentrations ranged between 0.4435-282.527 and 9.627-259.970 mg m^{-3} in *Dulce* and *Santa Olalla*, respectively, and between 1.752-3.823 and 1.654-2.991 mg m^{-3} in *Morenilla* and *Tinaja*, respectively.ee

		Dulce	Santa Olalla	Morenilla	Tinaja
	Mean±SD	1.06±0.78	1.18±0.69	5.00±0.51	9.63±2.03
Depth (m)	Min.-max.	0.10-2.50	0.10-2.50	4.00-6.00	6.00-12.00
	n	13	15	14	15
	Mean±SD	21.63±6.13	23.15±6.00	16.19±5.78	14.56±4.12
Water Temperature (ºC)	Min.-max.	9.00-28.30	9.00-32.46	6.50-24.80	8.30-21.40
	n	13	13	13	15
	Mean±SD	8.74±0.98	9.30±1.11	8.00±3.77	7.74±0.25
pH	Min.-max.	7.15-10.55	7.70-10.55	7.68-8.18	7.51-8.17
	n	8	8	5	6
	Mean±SD	1481.50±1005.95	2587.00±888.23	628.80±385.26	724.66±103.80
Conductivity (µS cm^{-1})	Min.-max.	2700.00-3170.00	1662.00-3830.00	548.00-747.00	625.00-852.00
	n	8	6	5	6
	Mean±SD	0.642±0.480	0.506±0.441	0.015±0.008	0.010±0.004
TP (mg L^{-1})	Min.-max.	0.116-1.939	0.343-2.075	0.001-0.031	0.005-0.018
	n	15	15	12	15
	Mean±SD	2.396±2.701	2.108±1.982	0.011±0.013	0.006±0.007
SRP (µg-at P L^{-1})	Min.-max.	0.363-10.672	0.155-6.478	<0.001-0.050	<0.001-0.020
	n	15	15	12	15
	Mean±SD	11.283±8.303	10.983±9.937	19.529±8.125	18.876±6.394
TN (mg L^{-1})	Min.-max.	3.331-32.718	1.564-38.447	9.851-31.544	11.106-32.938
	n	15	15	12	15
	Mean±SD	643.452±607.713	767.963±577.256	75.985±38.559	106.686±153.662
NO_3^- (µg-at N L^{-1})	Min.-max.	169.719-2424.881	269.178-2416.213	88.898-151.632	81.289-505.681
	n	15	15	12	15
	Mean±SD	163.979±148.902	138.572±143.281	75.985±38.559	106.686±153.662
NH_4 (µg-at N L^{-1})	Min.-max.	7.205-405.079	7.205-380.130	14.474-151.632	13.184-505.681
	n	15	15	12	13
	Mean±SD	51.653±81.026	51.726±72.094	2.795±1.442	2.226±0.480
Chl *a* (µg L^{-1})	Min.-max.	0.435-282.527	9.627-259.970	1.752-3.823	1.654-2.991
	n	15	15	13	14

Table 15. Physical and chemical variables features of the water columns and maximum depth measured in Dulce, Santa Olalla, Morenilla and Tinaja during the study period. Mean and standard deviation (Mean±SD), minimum and maximum values and total number of samples (n). (TP) total phosphorous; (SRP) soluble reactive phosphorous; (TN) total nitrogen; Chlorophyll *a* (Chl *a*).

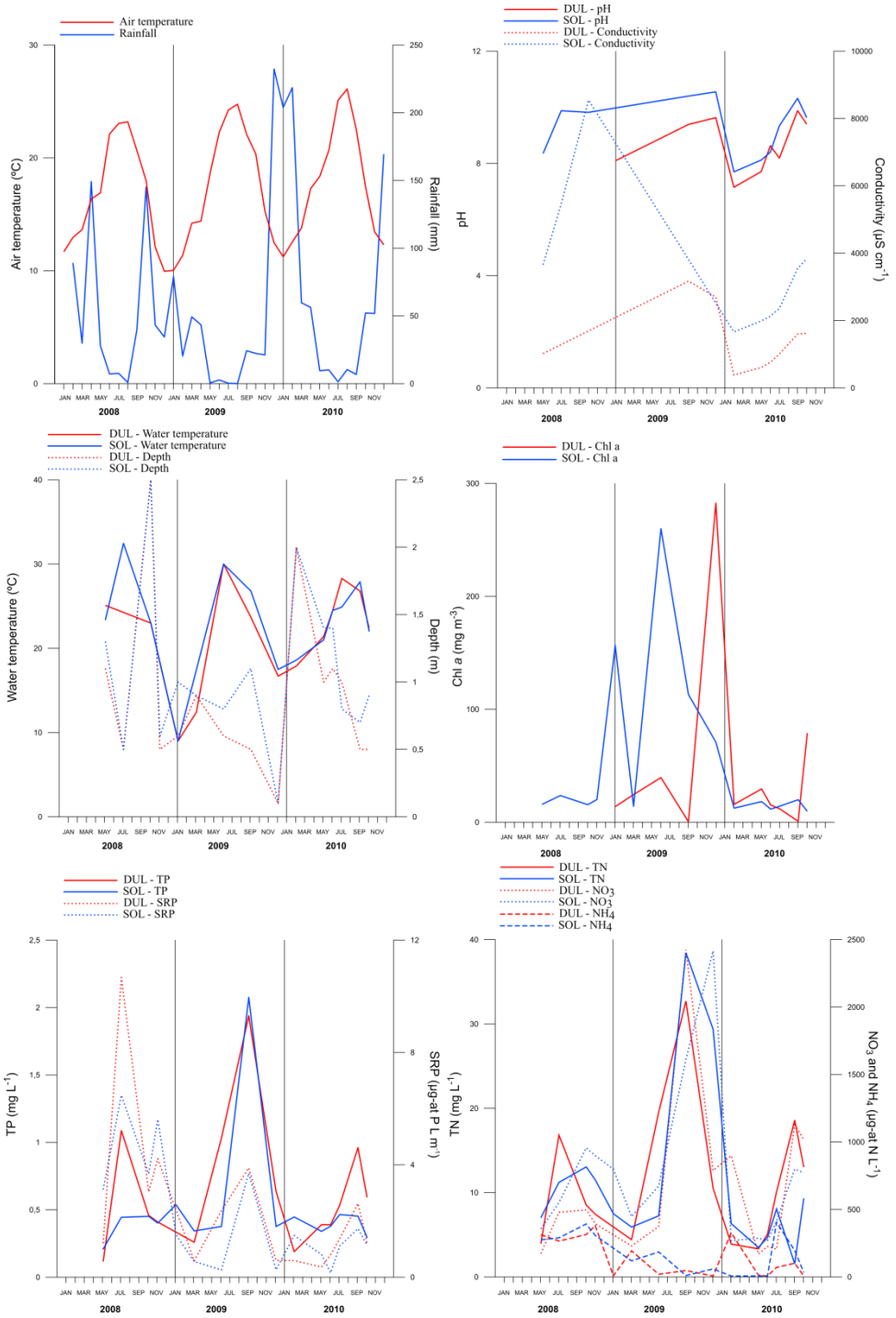

Figure 21. Physical and chemical variables features of the water columns and maximum depth measured in *Dulce* and *Santa Olalla* lakes. Chlorophyll *a* (Chl *a*); (TP) total phosphorous; (SRP) soluble reactive phosphorous; (TN) total nitrogen.

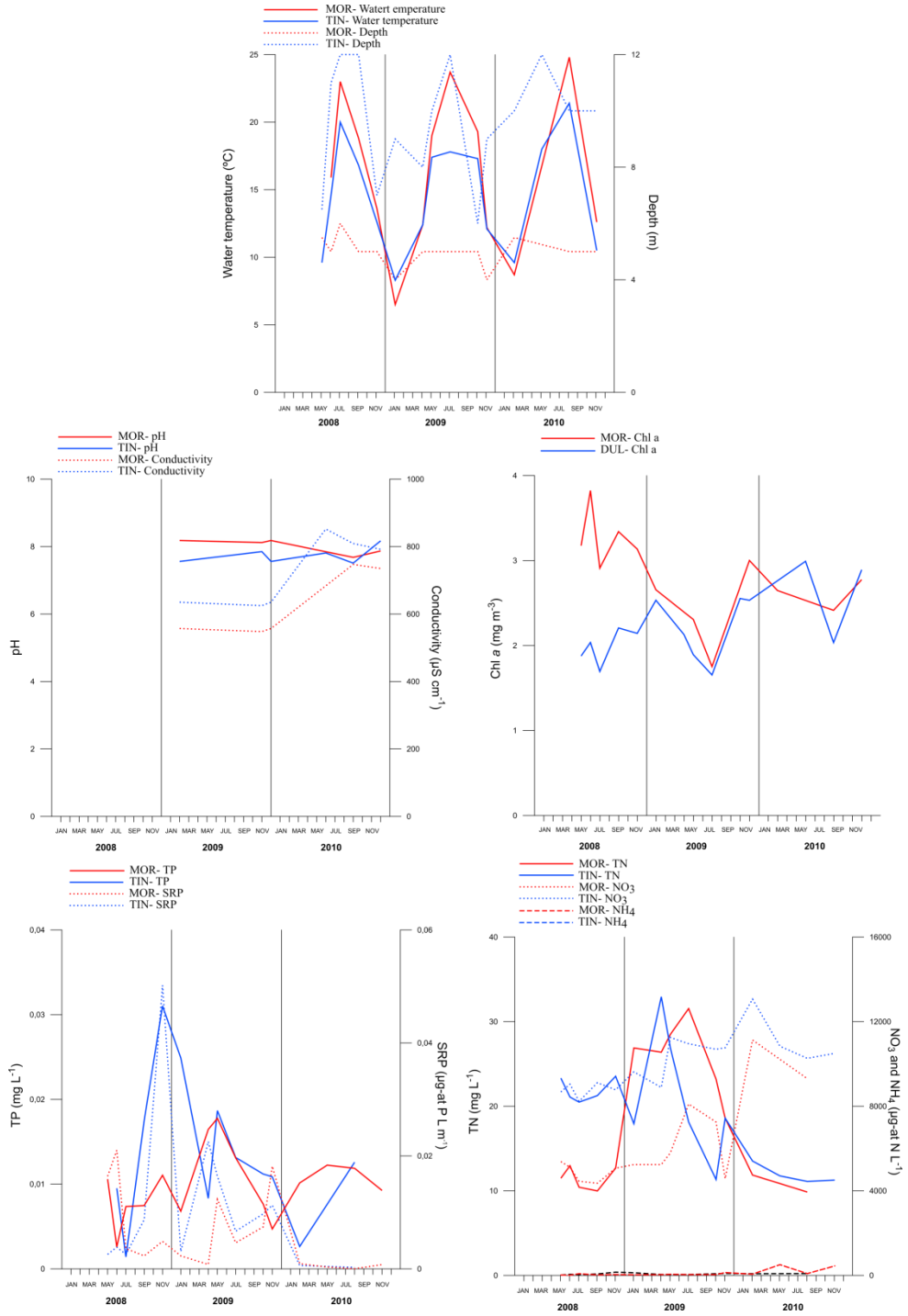

Figure 21. Physical and chemical variables features of the water columns and maximum depth measured in *Morenilla* and *Tinaja* lakes. Chlorophyll *a* (Chl *a*); (TP) total phosphorous; (SRP) soluble reactive phosphorous; (TN) total nitrogen.

Zooplankton species richness and taxonomic assemblages

Zooplankton communities also exhibit differences between lakes from the same location (Table 16 and Table 17). Zooplankton *alpha* diversity and species richness in each lake are showed in Table 18. *Gamma* diversity recorded across the five sampling sites accounted for a total of 56

zooplankton taxa (Table 18). Rotifers were the most diverse, being represented by 35 taxa. Among the crustacean cladocerans were the most diverse, with 16 taxa, and copepods were the least diverse group, being represented by 5 taxa, of which 2 were cyclopoids and 3 calanoids. Zooplankton total species richness varied from 24 to 32 taxa among the five sampling sites (16 to 23 for rotifers, 4 to 8 for cladocerans and 1 to 3 for copepods) (Table 18). Only one taxa were ubiquitous across all 5 waterbodies: *Ceriodaphnia* spp., *Hexarthra* spp., *Polyarthra* spp., *Lecane* spp. and *Keratella* spp (Tables 16 and 17).

	Species richness			
Location	**Rotifers**	**Cladocerans**	**Copepods**	**Total**
Dulce	16	6	2	24
Santa Olalla	22	5	3	30
Tinaja	23	7	1	31
Hundimiento	20	4	1	25
Morenilla	22	8	2	32
Total	35	16	5	56

Table 18. Contributions of the zooplankton community and taxonomic assemblages of each studied waterbody to species richness in Doñana and Ruidera.

In Dulce, zooplankton density and the number of species was higher than in Santa Olalla. Rotifers and cladocera were dominant as Dulce (*B.* angularis, *B. budapestinensis B. plicatilis* and *Daphnia magna*) as Santa Olalla (*B. angularis*, *B. plicatilis*, *Keratella tropica* and *Daphnia magna*). In Ruidera, the zooplankton density was predominantly represented by rotifers and its proportion in species richness was also higher (Figure 22). In terms of biomass, the copepod *Tropocyclops prasinus*, followed by the cladoceran *Bosmina longirostris* and the rotifers *Asplanchna* sp. in *Morenilla* and *Tinaja* lakes and *Keratella cochlearis* in *Hundimiento* (Tables 18, 19 and 20).

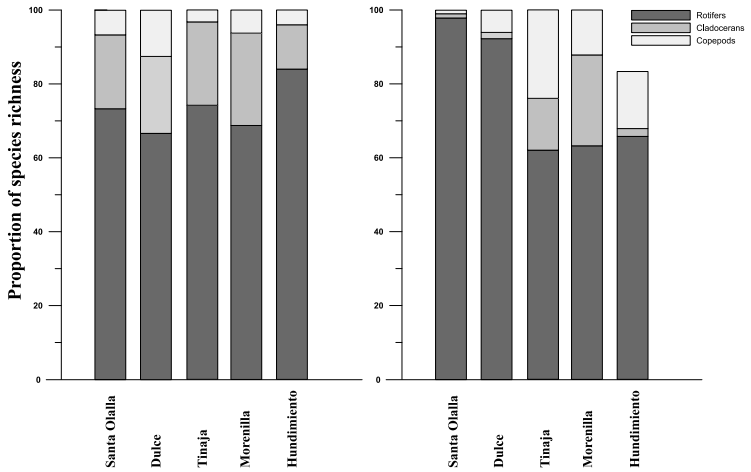

Figure 22. Barplots showing the proportional species richness (left) and species abundance (right) between the zooplankton assemblages (Cladocerans, Copepods and Rotifers) for the five sampling sites.

	T	T*	M	M*	H
Rotifera					
Monogononta					
Anuraeopsis fissa (Gosse, 1851)			X		
Ascomorpha ecaudis (Perty, 1850)		X	X		
Ascomorpha ovalis (Bergendahl, 1892)		X		X	
Ascomorpha spp.	X				X
Asplanchna giroldi				X	
Asplanchna priodonta (Gosse, 1850)		X		X	
Asplanchnna spp.	X	X	X		X
Brachionus calyciflorus (Pallas, 1776)	X		X	X	
Brachionus quadridentatus f.*cluniorbicularis* (Skorikov,1894)	X				
Brachionus urceolaris (Müller, 1773)	X		X		X
Dicranophorus grandis (Ehrenberg, 1832)		X			
Euchlanis dilatata (Ehrenberg, 1832)		X		X	
Euchlanis trichetra (Myers, 1930)		X			
Gastropus stylifer (Imhof, 1891)				X	
Hexarthra fennica (Levander, 1892)	X		X		X
Hexarthra mira (Hudson, 1871)		X			
Itura aurita (Ehrenberg, 1830)				X	
Keratella cochlearis (Gosse, 1851)	X	X	X	X	X
Keratella quadrata (Müller, 1786)	X	X	X		X
Keratella tropica (Apstein, 1907)	X		X		X
Lecane bulla (Gosse, 1851)	X	X			
Lecane closterocerca (Schmarda, 1859)	X	X		X	
Lecane flexis (Gosse, 1886)		X		X	
Lecane furcata (Murray,1913)				X	
Lecane hamata (Stokes, 1896)	X	X			
Lecane ludwigi (Eckstein, 1883)				X	
Lecane luna (Muller,1776)		X			
Lecane lunaris (Ehrenberg, 1832)		X	X	X	
Lecane nana (Murray, 1913)		X			
Lecane quadridentata (Ehrenberg,1832)		X			
Lecane thalera (Harring & Myers,1924)				X	
Lepadella minuta (Montet,1918)				X	
Lepadella sp.	X		X		X
Macrochaetus sericus (Thorpe,1893)	X	X	X	X	
Notholca acuminata (Ehrenberg, 1832)	X	X	X	X	X
Notommata cf. *cerberus* (Gosse,1886)		X			
Notommata codonella (Haning & Myers, 1924)			X	X	
Polyarthra vulgaris (Carlin, 1943)	X	X	X	X	X
Synchaeta oblonga (Ehrenberg, 1832)				X	
Synchaeta pectinata(Ehrenberg, 1832)	X	X	X		X
Testudinella sp.					X
Trichocerca similis (Wierzejski, 1893)	X	X	X	X	X
Trichotria tetractis (Ehrenberg, 1830)	X	X	X	X	X
Bdelloidea	X	X	X	X	X
Cladocera					
Acroperus neglectus (Lilljeborg, 1900)		X			
Alona costata (G.O. Sars, 1862)					
Alona guttata (Sars, 1862)				X	
Alona quadrangularis (O.F. Müller, 1776)	X	X	X	X	X
Alonella excisa (Fischer, 1854)		X		X	
Bosmina longirostris (O. F. Müller, 1785)	X	X	X	X	X
Ceriodaphnia dubia (Richard, 1894)	X	X	X	X	X
Chydorus sphaericus (O.F. Müller, 1776)			X	X	
Daphnia sp.			X		X
Diaphanosoma brachyurum (Liévin, 1848)	X	X	X		X
Pleuroxus aduncus (Jurine, 1820)			X	X	
Pleuroxus truncatus (O.F. Müller,1785)		X			
Sida crystalina (O.F. Müller,1776)				X	
Copepoda					
Acanthocyclops robustus (G.O.Sars, 1863)			X	X	
Cyclops vicinus (Ulyanin, 1875)		X			
Tropocyclops prasinus (Fischer, 1860)	X		X		X

Table 16. Species presence in sampling sites from Ruidera. Tinaja (T) and Morenilla (M) lakes, and Hundimiento (H). *Presence of taxonomic species from previous work in our sampling sites (Álvarez- Cobelas *et al.*, 2007).

	D	D*	D**	D***	S	S*	S**	S***
Rotifera								
Monogononta								
Anchistestudinella sp.			X					
Asplanchnabrightwelli(Gosse, 1850)	X	X	X		X		X	
Brachionus angularis(Gosse, 1851)	X	X	X		X	X	X	X
Brachionusbidentatus(Anderson, 1889)	X			X				
Brachionus budapestinensis(Daday, 1885)	X	X						
Brachionus calyciflorus(Pallas, 1776)	X	X	X	X	X	X	X	X
Brachionus falcatus(Zacharias 1898)	X			X				
Brachionus leydigi(Cohn, 1862)	X				X			
Brachionus plicatilis(Müller, 1786)	X	X	X		X	X	X	X
Brachionus quadridentatus f.*cluniorbicularis* (Skorikov,1894)	X		X		X			X
Brachionus urceolaris(Müller, 1773)	X	X	X				X	
Brachionus variabilis(Hempel, 1896)	X				X			
Cephalodella sp.					X			
Colurella sp.			X					
Filiniaterminalis(Plate, 1886)	X	X		X	X			
Hexarthra fennica(Levander, 1892)	X				X			
Hexarthra mira(Hudson, 1871)	X				X			
Hexarthra quadrata		X						
Hexarthra sp.				X				
Keratella quadrata(Müller, 1786)		X				X		
Keratella tropica(Apstein, 1907)	X		X	X	X		X	X
Lecane luna(Muller,1776)		X						
Lecane lunaris(Ehrenberg, 1832)				X				
Lecane spp.	X				X			
Polyarthra sp.				X				
Polyarthra vulgaris(Carlin, 1943)	X		X		X			
Testudinella patina(Hermann, 1783)	X	X	X				X	
Cladocera								
Alona rectangula(Sars, 1861)			X				X	
Alona sp.					X			
Bosmina longirostris(O. F. Müller, 1785)					X			
Ceriodaphnia reticulata(Jurine 1820)			X				X	
Ceriodaphnia sp.	X				X			
Chydorus sphaericus(O.F. Müller, 1776)			X				X	
Daphnia longispina (O.F. Müller, 1776)		X	X		X		X	
Daphnia magna(Straus, 1820)	X	X	X		X	X	X	
Daphnia similis(Claus 1876)			X					
Diaphanosomabrachyurum(Liévin, 1848)						X		
Diaphanosoma sp.					X			
Leydigia acanthocercoides(Fischer, 1854)		X						
Macrothrix hirsuticornis(Norman & Brady 1867)			X				X	
Macrothrix sp.	X							
Moina brachiata(Jurine, 1820)		X						
Moina sp.	X				X			
Simocephalus vetulus(O. F. Müller, 1776)		X						
Copepoda								
Acanthocyclops sp.			X				X	
Acanthocyclops robustus(G. O. Sars, 1863)	X	X			X	X		
Acanthocyclops wierzejskii		X				X		
*Copidodiaptomus*sp.	X				X			

Table 17. Species presence in sampling sites from Doñana. Dulce (D) and Santa Olalla (S) lakes. *Presence of taxonomic species from previous work in our sampling sites * López *et al.*, 1991; **Galindo *et al.*, 1994; ***Guisande *et al.*, 2008.

	Mean	Max	Min
Bosmina longirostris			
Abundance (ind L^{-1})	5.879 ± 9.586	31.840	0
Biomass (dry weight, µg L^{-1})	131.125 ±	408.070	0
Ceriodaphnia dubia			
Abundance (ind L^{-1})	0.224 ± 0.581	1.860	0
Biomass (dry weight, µg L^{-1})	12.117 ± 36.521	134.850	0
Keratella cochlearis			
Abundance (ind L^{-1})	4.129 ± 7.654	28.680	0
Biomass (dry weight, µg L^{-1})	2.650 ± 4.485	16.730	0
Asplanchna spp.			
Abundance (ind L^{-1})	3.030 ± 6.041	21.440	0
Biomass (dry weight, µg L^{-1})	11.912 ± 19.700	68.340	0
Tropocyclops prasinus			
Abundance (ind L^{-1})	3.112 ± 3.125	9.280	0
Biomass (dry weight, µg L^{-1})	23.273 ± 33.562	115.59	0

Table 18. Average densities and biomass of dominant zooplankton species from X Morenilla lake.

	Mean	Max	Min
Bosmina longirostris			
Abundance (ind L^{-1})	1.812 ± 3.355	12.160	0
Biomass (dry weight, µg L^{-1})	21.276 ± 33.553	119.88	0
Ceriodaphnia dubia			
Abundance (ind L^{-1})	0.590 ± 1.345	5.120	0
Biomass (dry weight, µg L^{-1})	22.620 ± 42.690	139.200	0
Keratella cochlearis			
Abundance (ind L^{-1})	2.440 ± 8.700	33.840	0
Biomass (dry weight, µg L^{-1})	2.720 ± 10.171	39.480	0
Asplanchna spp.			
Abundance (ind L^{-1})	2.893 ± 8.599	33.240	0
Biomass (dry weight, µg L^{-1})	21.420 ± 72.484	282.540	0
Tropocyclops prasinus			
Abundance (ind L^{-1})	9.116 ± 7.403	21.840	0.440
Biomass (dry weight, µg L^{-1})	45.674 ± 77.079	240.630	0.540

Table 19. Average densities and biomass of dominant zooplankton species from lake.

	Mean	Max	Min
Bosmina longirostris			
Abundance (ind L^{-1})	0.270 ± 0.413	0.990	
Biomass (dry weight, µg L^{-1})	1.659 ± 2.576	6.950	
Ceriodaphnia dubia			
Abundance (ind L^{-1})	0.016 ± 0.057	0.200	
Biomass (dry weight, µg L^{-1})	0.362 ± 1.255	4.350	
Keratella cochlearis			
Abundance (ind L^{-1})	3.247 ± 4.875	16.400	
Biomass (dry weight, µg L^{-1})	0.985 ± 1.671	5.740	
Polyarthra spp.			
Abundance (ind L^{-1})	1.833 ± 2.849	5.610	
Biomass (dry weight, µg L^{-1})	0.296 ± 0.535	1.880	
Tropocyclops prasinus			
Abundance (ind L^{-1})	5.845 ± 7.009	19.800	
Biomass (dry weight, µg L^{-1})	4.487 ± 5.478	14.850	

Table 20. Average densities and biomass of dominant zooplankton species of from Hundimiento.

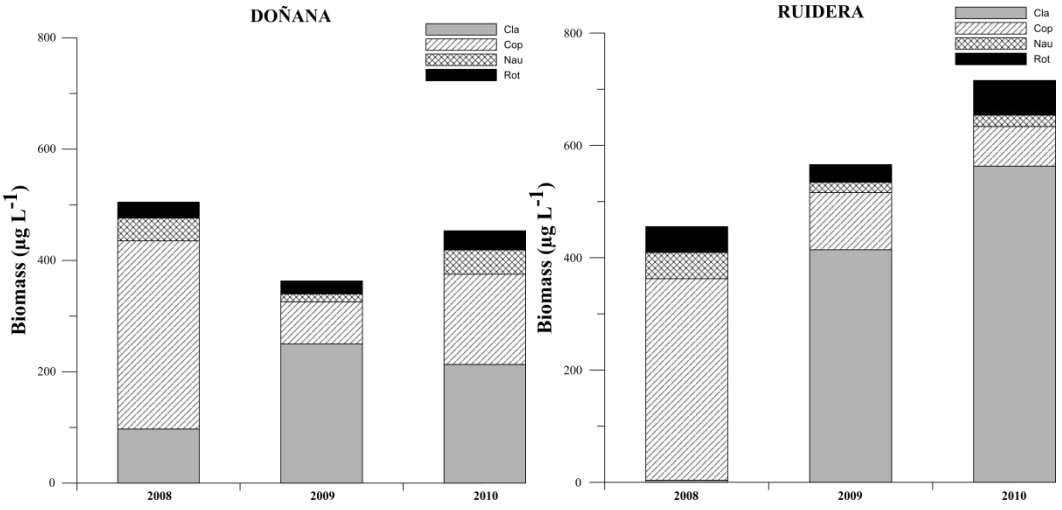

Figure 23. Histograms showing the biomass

Contribution of sites, zooplankton groups and taxa to beta diversity

The analyses of variance showed significant interactions between the factor "lake" for *beta* biodiversity when all the lakes are included (p value < 0.05; 200 permutations). The analyses which included only the two lakes of each study site gave the same result (p value < 0.05; 200 permutations). This difference in species composition between Doñana and Ruidera was also revealed by the NMDS ordination (Figure 24). Adonis results confirm that sampling sites appear to significantly differ from each other based in their *beta* diversity (R^2=0.386; p value $<$ 0.05; 200 permutations). Comparing the lake pairs *Santa Olalla* and *Dulce*, *beta* diversity appear to significantly differ from each other (R^2=0.122; p value < 0.05; 200 permutations). However the pair *Tijana* and *Morenilla* not showed differences (R^2=0.116; p value > 0.05; 200 permutations). However, comparing the pairs of sampling sites in Ruidera there are differences in species richness between *Tinaja-Hundimiento* and *Morenilla-Hundimiento* (p value < 0.05; 200 permutations).

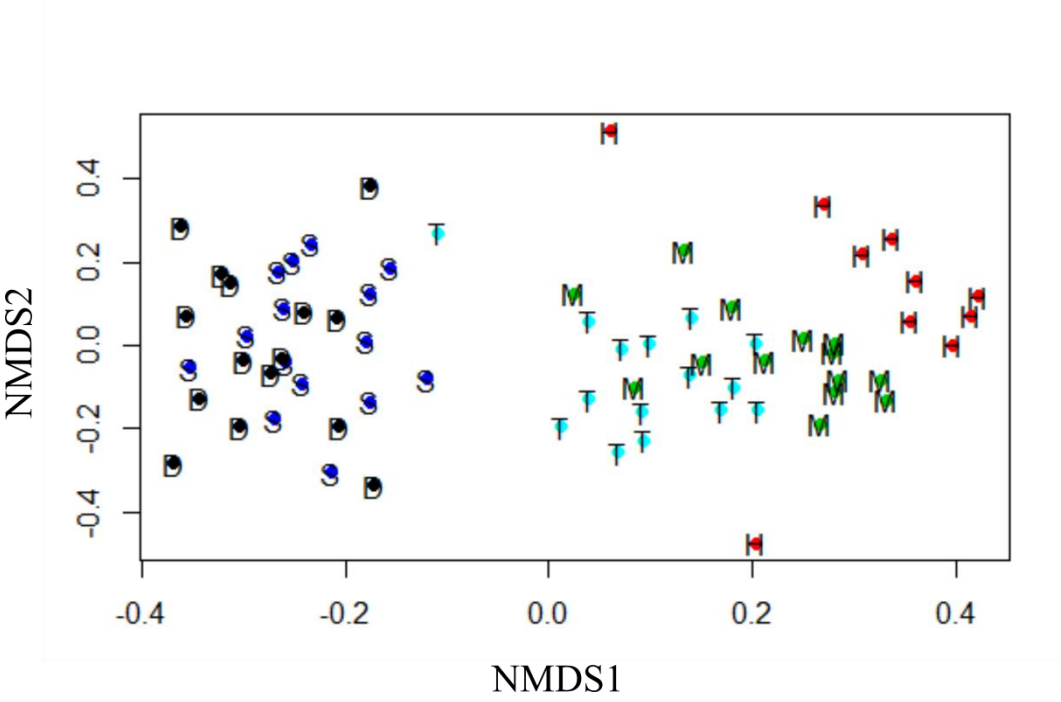

Figure 24. NMDS ordination of zooplankton community based on species richness during all the sampling campaign. Sampling sites are represented by their respective names: Dulce (D), Hundimiento (H), Morenilla (M), Santa Olalla (S) and Tinaja (T). Stress = 0.17

Discussion

The main differences between ponds were in the concentration of NO_3^- and Chl *a* (Table 15). During the sampling period of 2008 and 2009, the volume of *Santa Olalla* and *Dulce* lakes decreased steadily, which is reflected by conductivity values showing an increase (Figure 21).

A large and cumulative sampling survey is important to assess the biodiversity of zooplankton in Mediterranean waterbodies, especially in Doñana ponds where are particularly unpredictable and have highly variable hydroregime. Spatio-temporal assessments of zooplankton biodiversity require large-scale sampling surveys. An alternative that offers many advantages over traditional morphological identification for describing community composition can be DNA barcoding approach applied to diapausing egg banks, as we showed in **Chapter 1**.

Hence, it is likely that the similarities between the zooplankton species occurrence found between Dulce and Santa Olalla lakes and between Morenilla and Tinaja lakes may be due to different dispersion agents. In the case of Dulce and Santa Olalla the presence of waterbirds is very high, mainly during the migratory periods. The number of diapausing eggs found in faeces samples in **Chapter 3** and **Chapter 4** is substantially larger, especially for rotifer group, and their colonization ability would be favored by not losing hatchability.

Diapausing eggs dispersal by waterflow depends on the water link connections. Dulce and Santa Olalla lakes are separated by 300 metres and there is no other water body between them which might act like a linker. However, during high rainfall season both ponds can be linked. Previous references assert that when rainfall surpassed 400 mm Dulce and Santa Olalla are linked and

that phenomenon happened in 1976 and January 1988 (López and Toja, 1991). This phenomenon happened again during the winter 2010 (personal observation). Differences persisted even after periods when superficial water of the ponds were linked together (Serrano and Toja, 1998). The species compostion were similar between *Dulce* and *Santa Olalla* before and after both lakes were linked. Therefore, the higher dispersal rates by waterbirds might be the responsable of the similar species composition of both lakes. Although there might be an evolution of physical-chemical parameters separately by both ponds iniciated after the union of the ponds, this evolution migh be slow and the rainfall break this succession process and starting another. In the case of Ruidera might be slightly different, since *Tinaja* and *Morenilla* are separated by 7 kilometres and between them there are 5 lakes. However, during high rainfall season the upper lakes discharge to the lower lakes by surface drainage acting like a river. This phenomenon might be the cause of the dipausing eggs disperal by water flows, and might explain the similarities that we find between Hundimiento and Morenilla.

Species number inhabiting water bodies is particularly difficult to assess because is affected by the spatio-temporal scale of observation, which might increases the sampling effort. Mediterranean region has been considered as a sensitive area to climate change (Sánchez-Fernández et al., 2004). Therefore, the monitoring of zooplankton biodiversity and the development of tools for assessing are needed.

In summary, environmental parameters and hydrology are important factors determining species richness and composition of zooplankton (cladoceran and rotifers) in Mediterranean water bodies. Evaluate the influence of the connections between water bodies and their potential as dispersal agent by water flow is important for zooplankton communities and their structure. The continuous water links in Ruidera lakes and the intermittent links in Doñana lakes may play a key role in the composition of species at a local scale, and just like it happens with wind dispersal, water flow may influence colonization over long time intervals.

Synthesis and future perspectives

The role of diapausing eggs in the maintenance of zooplankton populations of freshwater systems from the diapausing egg banks and their potential to disperse and to colonize new habitats are fundamental questions in aquatic ecology. Zooplankton biodiversity in Mediterranean freshwater systems is maintained by local populations which are adapted to a fluctuating and unpredictable environment (Jiménez-Melero *et al.* 2007; Moscatelo & Belmonte, 2004). Under a climate change scenario diapausing eggs not only offers an advantage to face the unfavourable conditions but also may be transported by several vectors to other waterbodies, which might offer better conditions. To assess the zooplankton species richness of freshwater systems is necessary to collect samples from different locations in the lake and at multiple occasions throughout the year so as to cover the spatial and temporal heterogeneity of the active community. The accumulation of diapausing eggs of different zooplankton species, generations and genotypes results in a mixed egg bank with greater potential biodiversity than the active community sampled at any one moment (Brendonck & DeMeester, 2003). For this reason, the development of tools applied to diapausing egg banks are need since they may store the past and present zooplankton biodiversity. The assessment of zooplankton species richness using diapausing eggs isolated from sediments is precluded by the lack of identification keys which offer an overview of diapausing egg morphotypes. The application of hatching experiments to diapausing egg banks from sediments offers the advantage to know the species richness from a single sediment sample without the needed of diapausing eggs identification (May, 1986; Vandekerkhove et al., 2004). Using the identified hatched species assignation to their diapausing egg morphotype might enable the development of a user-friendly key allowing the species level identification of diapausing eggs from sediments. However, some zooplankton species have low number of available diagnostic characters and present high intra-specific morphological variability of diapausing eggs making impossible their identification to species level (Vandekerkhove et al., 2004; Piscia et al., 2012). Additionally, hatching method limitations related with the hatching cues (light, temperature or salinity), the latency period variability and the potential biases introduced by bet-hedging highlight the needed of alternatives methods (Gilbert & Walsh, 2005; Schröder, 2005; Vandekerkhove *et al.*, 2005; García- Roger *et al.*, 2014). An alternative to morphological identification and hatching approaches is using molecular techniques, which can be applied on individual diapausing eggs or adults. DNA barcoding applied to a one single sediment sample is a powerful tool for assessing rotifer diapausing egg bank biodiversity.

Chapter 1 shows the characterization of rotifer communities from *Santa Olalla* and *Dulce* lakes in Doñana and *Tinaja* lake in Ruidera applying DNA barcoding to one single sample of sediment gave the identification of 91% of diapausing egg morphotypes to likely genus level. Despite the conservative issues of rotifer individual samples from the water column, we created a reference collection of DNA barcodes for taxonomically diagnosed adult individuals sampled from the water column for comparing with the sequences obtained from individual eggs from the diapausing egg banks. Our approach, based on a single sediment sample, gave higher or similar estimates of rotifer biodiversity than previous studies based on a number of seasonal samples, reducing time and sampling effort.A total number of 22 and 16 rotifers where found in zooplankton samples from *Santa Olalla* and *Dulce* lakes, respectively. In *Tinaja* lake a total number of 23 rotifers where collected. Therefore, the biodiversity reached with DNA barcoding were similar to the biodiversity reached during the three year sampling campaing.

Once we could morphologically identify properly the majority of the rotifer resting eggs morphotypes in **Chapter 1**, we were able to evaluate zooplankton dispersal not only for rotifers but also for cladocerans whose ephippia has been described (Vandekerkhove et al., 2004) or we identified using the hatching method. As the efficiency of zooplankton dormant propagules dispersal by different vectors is still under debate, we evaluate different dispersal mechanisms according to the characteristics of the study systems. In Doñana, wind and waterbirds may be relevant dispersal mechanisms, while in Ruidera wind and water currents should be important ones.

In **Chapter 2**, studying the dispersal by wind, we showed that the collection of diapausing eggs by the automated wet/dry collectors was higher in Doñana than in Ruidera. Both Doñana lakes, *Dulce* and *Santa Olalla*, exhibit a higher water level reduction compared with the lakes surrounded the automated wet/dry collector, which increase the dried area of the lakes and leaving diapausing eggs exposed to the action of the wind. Therefore, the relevance of diapausing eggs dispersal by wind is dependent of the waterbody characteristics and seasonally, may be different between zooplankton taxa and operates as a local-scale influencing the colonization over long time intervals. However, we support the idea despite the fact that overland movement of zooplankton dormant propagules by wind appears to be infrequent, their dispersal may influence colonization over long time intervals. Therefore, despite the low dispersal rates by wind new waterbodies and disturbed aquatic systems could be colonized by air currents.

In **Chapter 3**, the zooplankton dispersal by waterbirds in Doñana was studied, and the results show that dispersal by waterbirds should be considered a relevant mechanism of rotifer dispersal. Green & Figuerola (2005) suggest that rotifer resting eggs should be dispersed in such abundance by air currents and rain than other vectors, like waterbirds, are not relevant. However, according to our results, dispersal of rotifers by waterbirds should be considered, at least, as relevant as that by rain and air currents. In **Chapter 2**, we showed that passive deposition rates of rotifers from the air were low and infrequent in the same geographical area of Doñana National Park, although may influence colonization over long time intervals. We estimate with a rough extrapolation that more than 20 000 diapausing eggs per day may rain down to *Santa Olalla* and *Dulce* lakes. If we use those calculations just for rotifers, the total area of both lakes would potentially receive less than 6723 rotifer resting eggs per day, because most of the passively deposited propagules in Santa Olalla lake were bryozoan statoblasts. In **Chapter 3**, we found a mean of 0.8 rotifer resting eggs per faecal dropping of waterbirds in total (regardless of waterbird species). In the global peridunal lakes of Doñana, mean annual waterbird densities were aproximately between 800 and 1700 individuals (Equipo de Seguimiento de Doñana. ICTS – Reserva Biológica de Doñana (EBD-CSIC), 2009; 2010). Most of those waterbirds were present in the lakes of *Santa Olalla* and *Dulce*, because they are the highest peridunal lakes and they serve as a refuge for waterbirds during summer in Doñana, when other water bodies are dry. Assuming a daily excretion of 12.2 faecal drops per bird, as observed for mallards (Marion et al., 1994), 300 waterbirds in each lake could potentially release almost 3000 rotifer resting eggs per day, so considering both lakes as a whole, around 6000 rotifer resting eggs per day could be released. This value is quite similar to that obtained for rotifer dispersal by air currents, although it could be also higher. Nevertheless, this type of extrapolation may be contentious, although it may indicates the relevance of waterbirds as vectors of rotifer species, and similar extrapolations have been made in other studies on dispersal by waterbirds (Soons et al., 2016). In **Chapter 2**, we observed that dispersal of rotifers by air currents was 5 times higher than for cladocerans, while in **Chapter 3** the dispersal of rotifers by waterbirds was 10 times higher than for cladocerans. No previous studies have compared dispersal by air currents with that by waterbirds. Further studies are needed to evaluate the relative importance of the different zooplankton dispersal mechanisms in different types of aquatic systems. Due to the different physical-chemical parameters differences between *Santa Olalla* and *Dulce,* and *Tinaja* and *Morenilla*, the zooplankton species composition were similar during the study period, speciallay for some rotifer species. This can be related with the potential high diapausing eggs dispersal rates by waterbirds in

Doñana region which maintain a constant transport of diapausing eggs between water bodies. In addition, it is also important to highlightthe dispersion by wind and water flow described in **Chapter 2** and **Chapter 5**, although they might be acting by lower rates.

Nevertheless, in **Chapter 4**, the results on the hatching response of rotifer resting eggs from the sediments and from the faecal droppings of waterbirs show that the capacity of colonization of rotifer resting eggs is dependent of the salinity concentrations and of the dispersal by waterbirds. Moreover, the hatching response of rotifers to salinity was temperature dependent, but, in general, high salinities (>8 g l-1) would decrease the colonization efficiency of rotifer populations. Therefore, the expected increase in salinity and temperature of Mediterranean lakes due to climate change would have consequences on the colonization patterns of rotifer species. In addition to this, only rotifer resting eggs from faeces of *Anas platyrhynchos* and *Anas clypeata* hatched, although in **Chapter 3** rotifer hatchlings were also observed from faeces of *Phalacrocorax carbo* and *Netta rufina.* However, in **Chapter 4** there were no resting eggs from faeces of these latter species. Anyway, there were no hatchlings of resting eggs from faeces of *Fulica atra* in both studies (**Chapters 3** and **4**), although in other studies have been described zooplankton hatchlings from faeces of *Fulica atra* (Green et al., 2008). Moreover, **Chapter 4** shows that waterbird ingestion may favour the hatching success in rotifers. In summary, differences in hatchlings of faecal eggs between waterbird species should be specifically tested under different conditions. This thesis did not properly study the hatching response of zooplankton dispersed by air currents because the study design in **Chapter 2** was not appropriate to determine egg viability, although viable rotifers were observed. In consequence, future studies should also analyse the hatching response of zooplankton dispersed by air currents in order to compare them with the hatching responses of diapausing propagules dispersed by waterbirds.

Overall, this thesis has demonstrated the importance of diapausing eggs for zooplankton biodiversity and their dispersal, especially for rotifers species. As a whole, this thesis presents a complete description of the main dispersal vectors (wind, which taking into account the passive and rainfall deposition, and waterbirds) that have an important role for dispersal.

Conclusions

The main conclusions derived from this thesis are enumerated below:

1. DNA barcoding of diapausing eggs from a single sediment sample is an efficient method to characterize rotifer communities from lentic aquatic systems. The combination of DNA barcoding with integrative taxonomic approaches for delimiting species might be a powerful complementary tool for revealing hidden diversity. Diapausing egg banks provide an useful and cost-effective tool in combination with DNA barcoding and DNA taxonomy approaches for studying biodiversity in freshwater systems.

2. The passive deposition of diapausing eggs dispersed by the wind is more frequent during dry periods in Doñana. Overall, rotifers were the most dispersed group of zooplankton. Wind dispersal appears to be infrequent but may influence colonization over long time intervals.

3. Rotifers are also the main zooplankton group dispersed by waterbirds in the studied lakes. Diapausing eggs dispersal by waterbirds seems to be the most plausible and effective dispersal agent for cladoceran and rotifers in Doñana.

4. The hatching response of sedimentary and dispersed rotifer resting eggs to salinity was dependent of temperature. Globally, that response is negative at 15 ^{o}C and quadratic at 25 ^{o}C, although results are different between rotifer species. Under a scenario of a moderate increase in salinity levels, the capacity of resilience and colonization of rotifers may be negatively affected.

5. The hatching success of the rotifer *Brachionus angularis* increases after ingestion by mallards. Other rotifer species remain unaffected after ingestion.

6. The hatching success of dispersed rotifer diapausing eggs is dependent of the waterbird species, at least for the studied conditions. Higher viabilities of dispersed diapausing eggs are related to *Anas platyrhynchos.*

Conclusiones

Las principales conclusiones derivadas de esta tesis se enumeran a continuación:

1. La aplicación de la técnica DNA barcoding a los huevos diapáusicos de una sola muestra de sedimento es un método eficiente para caracterizar las comunidades de rotíferos de sistemas acuáticos lénticos. La combinación del DNA barcoding con herramientas taxonómicas integrales para delimitar especies podría ser una potente y complementaria herramienta para revelar la diversidad oculta. Los bancos de huevos diapáusicos proporcionan una poderosa herramienta en combinación con el DNA barcoding y DNA taxonomy para estudiar la biodiversidad en sistemas de agua dulce.

2. La deposición pasiva de los huevos diapáusicos dispersados por el viento es más frecuente durante los periodos secos en Doñana. En general, los rotíferos fueron el grupo más dispersado de zooplancton. La dispersión por el viento parece infrecuente, pero podría influir en la colonización durante largos intervalos de tiempo.

3. Los rotíferos también son el principal grupo de zooplancton dispersado por aves acuáticas en los lagos estudiados. La dispersión de los huevos diapáusicos por las aves acuáticas parece ser el agente de dispersión más plausible y eficaz para cladóceros y rotíferos en Doñana.

4. La respuesta de eclosión a la salinidad de los huevos diapáusicos localizados en el sedimento y de los dispersados fue dependiente de la temperatura. Globalmente, esa respuesta es negativa a 15 ^{o}C y cuadrática a 25 ^{o}C, aunque los resultados son diferentes entre las especies de rotíferos. Bajo un escenario de un aumento moderado en los niveles de salinidad, la capacidad de resiliencia y colonización de rotíferos podría verse afectada negativamente.

5. El éxito de eclosión del rotífero *Brachionus angularis* aumenta tras la ingestión por los patos silvestres. Otras especies de rotíferos no se ven afectadas después de la ingestión.

6. El éxito de eclosión de los huevos diapáusicos dispersados depende de las especies de aves acuáticas, al menos para las condiciones estudiadas. Las mayor viabilidad de los huevos diapáusicos está relacionada con la especie *Anas platyrhynchos*.

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Appendices

OTU	Sample ID	Haplotype	GMYC entity	ABGD group	Lake	Type	GenBank
Asplachna brightwelli	SOL12DE	H64	GMYC15	34	SOL	DE8	KY749540
Asplachna brightwelli	SOL9AR	H64	GMYC15	34	SOL	AR6	KY749541
Asplachna brightwelli	SOL10AR	H64	GMYC15	34	SOL	AR6	KY749542
Asplachna brightwelli	SOL1AR	H64	GMYC15	34	SOL	AR6	KY749543
Asplachna brightwelli	SOL12AR	H64	GMYC15	34	SOL	AR6	KY749544
Asplachna brightwelli	SOL20AR	H64	GMYC15	34	SOL	AR6	KY749545
Asplachna brightwelli	SOL22AR	H64	GMYC15	34	SOL	AR6	KY749546
Asplachna brightwelli	SOL24AR	H64	GMYC15	34	SOL	AR6	KY749547
Asplachna brightwelli	SOL13AR	H65	GMYC15	34	SOL	AR6	KY749548
Brachionus "Almenara"	DUL121DE	H5	GMYC5	5	DUL	DE14	KY749347
Brachionus "Almenara"	SOL105DE	H5	GMYC5	5	SOL	DE14	KY749366
Brachionus "Almenara"	SOL3AR	H5	GMYC5	5	SOL	AR12	KY749373
Brachionus "Almenara"	SOL9DE	H5	GMYC5	5	SOL	DE14	KY749344
Brachionus "Almenara"	SOL7AR	H5	GMYC5	5	SOL	AR12	KY749345
Brachionus "Almenara"	DUL120DE	H5	GMYC5	5	DUL	DE14	KY749346
Brachionus "Almenara"	DUL128DE	H5	GMYC5	5	DUL	DE14	KY749348
Brachionus "Almenara"	DUL130DE	H5	GMYC5	5	DUL	DE14	KY749349
Brachionus "Almenara"	SOL123AR	H5	GMYC5	5	SOL	AR12	KY749350
Brachionus "Almenara"	DUL131AR	H5	GMYC5	5	DUL	AR12	KY749351
Brachionus "Almenara"	DUL132AR	H5	GMYC5	5	DUL	AR12	KY749352
Brachionus "Almenara"	DUL133AR	H5	GMYC5	5	DUL	AR12	KY749353
Brachionus "Almenara"	DUL137AR	H5	GMYC5	5	DUL	AR12	KY749354
Brachionus "Almenara"	DUL138AR	H5	GMYC5	5	DUL	AR12	KY749355
Brachionus "Almenara"	DUL140AR	H5	GMYC5	5	DUL	AR12	KY749356
Brachionus "Almenara"	SOL8AR	H5	GMYC5	5	SOL	AR12	KY749357
Brachionus "Almenara"	DUL1DE	H5	GMYC5	5	DUL	AR12	KY749358
Brachionus "Almenara"	DUL52DE	H5	GMYC5	5	DUL	AR12	KY749359
Brachionus "Almenara"	SOL53AR	H5	GMYC5	5	SOL	AR12	KY749360
Brachionus "Almenara"	SOL40DE	H5	GMYC5	5	SOL	DE14	KY749361
Brachionus "Almenara"	SOL100DE	H5	GMYC5	5	SOL	DE14	KY749362
Brachionus "Almenara"	SOL102DE	H5	GMYC5	5	SOL	DE14	KY749363
Brachionus "Almenara"	SOL103DE	H5	GMYC5	5	SOL	DE14	KY749364
Brachionus "Almenara"	SOL104DE	H5	GMYC5	5	SOL	DE14	KY749365
Brachionus "Almenara"	SOL99DE	H5	GMYC5	5	SOL	DE14	KY749367

Brachionus "Almenara"	DUL84DE	H5	GMYC5	5	DUL	DE14	KY749368
Brachionus "Almenara"	DUL85AR	H5	GMYC5	5	DUL	AR12	KY749369
Brachionus "Almenara"	DUL86AR	H5	GMYC5	5	DUL	AR12	KY749370
Brachionus "Almenara"	DUL88AR	H5	GMYC5	5	DUL	AR12	KY749371
Brachionus "Almenara"	SOL17AR	H5	GMYC5	5	SOL	AR12	KY749372
Brachionus "Almenara"	DUL103DE	H5	GMYC5	5	DUL	DE14	KY749374
Brachionus "Almenara"	DUL87DE	H5	GMYC5	5	SOL	DE14	KY749375
Brachionus "Almenara"	SOL101DE	H7	GMYC5	5	SOL	DE14	KY749376
Brachionus angularis	DUL9DE	H6	GMYC17	6	DUL	DE18	KY749377
Brachionus angularis	DUL10DE	H6	GMYC17	6	DUL	DE18	KY749378
Brachionus angularis	DUL16AR	H6	GMYC17	6	DUL	AR15	KY749379
Brachionus angularis	DUL20AR	H6	GMYC17	6	DUL	AR15	KY749380
Brachionus angularis	DUL30DE	H6	GMYC17	6	DUL	DE18	KY749381
Brachionus angularis	DUL53DE	H6	GMYC17	6	DUL	DE18	KY749382
Brachionus angularis	DUL35DE	H6	GMYC17	6	DUL	DE18	KY749383
Brachionus angularis	SOL98AR	H6	GMYC17	6	SOL	AR15	KY749384
Brachionus angularis	SOL85DE	H6	GMYC17	6	SOL	DE18	KY749385
Brachionus budapestinensis	DUL71DE	H25	GMYC18	14	DUL	DE13	KY749439
Brachionus budapestinensis	DUL75AR	H25	GMYC18	14	DUL	AR11	KY749440
Brachionus calyciflorus	DUL70DE	H18	GMYC3	12	DUL	DE15	KY749422
Brachionus calyciflorus	DUL109DE	H18	GMYC3	12	DUL	DE15	KY749415
Brachionus calyciflorus	DUL38DE	H18	GMYC3	12	DUL	DE15	KY749416
Brachionus calyciflorus	DUL46DE	H18	GMYC3	12	DUL	DE15	KY749417
Brachionus calyciflorus	SOL55DE	H18	GMYC3	12	SOL	DE15	KY749418
Brachionus calyciflorus	DUL7AR	H18	GMYC3	12	DUL	AR13	KY749419
Brachionus calyciflorus	DUL80AR	H18	GMYC3	12	DUL	AR13	KY749420
Brachionus calyciflorus	DUL9AR	H18	GMYC3	12	DUL	AR13	KY749421
Brachionus calyciflorus	DUL77DE	H18	GMYC3	12	DUL	DE15	KY749423
Brachionus calyciflorus	DUL97DE	H18	GMYC3	12	DUL	DE15	KY749424
Brachionus calyciflorus	DUL101DE	H18	GMYC3	12	DUL	DE15	KY749425
Brachionus calyciflorus	DUL99DE	H18	GMYC3	12	DUL	DE15	KY749426
Brachionus calyciflorus	SOL19AR	H18	GMYC3	12	SOL	AR13	KY749427
Brachionus calyciflorus	DUL10AR	H19	GMYC3	12	DUL	AR13	KY749428
Brachionus calyciflorus	DUL93DE	H19	GMYC3	12	DUL	DE15	KY749429
Brachionus calyciflorus	DUL96DE	H20	GMYC3	12	DUL	DE15	KY749430
Brachionus calyciflorus	SOL14AR	H21	GMYC3	12	SOL	AR13	KY749431
Brachionus leydigi	SOL94DE	H10	GMYC19	8	SOL	DE19	KY749399

Brachionus leydigi	SOL95AR	H10	GMYC19	8	SOL	AR16	KY749400
Brachionus leydigi	SOL96DE	H10	GMYC19	8	SOL	DE19	KY749401
Brachionus leydigi	SOL70DE	H10	GMYC19	8	SOL	DE19	KY749402
Brachionus quadridentatus	SOL38DE	H16	GMYC8	11	SOL	DE16	KY749411
Brachionus quadridentatus	DUL4AR	H16	GMYC8	11	DUL	AR14	KY749412
Brachionus quadridentatus	DUL50AR	H17	GMYC8	11	DUL	AR14	KY749413
Brachionus quadridentatus	DUL62AR	H17	GMYC8	11	DUL	AR14	KY749414
Brachionus quadridentatus	DUL107AR	H11	GMYC6	9	DUL	AR14	KY749403
Brachionus quadridentatus	DUL108DE	H11	GMYC6	9	DUL	DE16	KY749404
Brachionus quadridentatus	SOL5AR	H12	GMYC6	9	SOL	AR14	KY749405
Brachionus quadridentatus cluniorbicularis	DUL94DE	H13	GMYC7	10	DUL	DE16	KY749406
Brachionus quadridentatus cluniorbicularis	SOL6AR	H13	GMYC7	10	SOL	AR14	KY749407
Brachionus quadridentatus cluniorbicularis	DUL1AR	H14	GMYC7	10	DUL	AR14	KY749408
Brachionus quadridentatus cluniorbicularis	DUL23AR	H15	GMYC7	10	DUL	AR14	KY749409
Brachionus quadridentatus cluniorbicularis	TIN16DE	H15	GMYC7	10	TIN	DE16	KY749410
Brachionus spp.	DUL81DE	H26	GMYC21	15	DUL	DE17	KY749441
Brachionus spp.	DUL18DE	H27	GMYC20	16	DUL	DE17	KY749442
Brachionus spp.	SOL37DE	H28	GMYC22	17	SOL	DE17	KY749443
Brachionus spp.	SOL53DE	H28	GMYC22	17	SOL	DE17	KY749444
Brachionus spp.	SOL78DE	H29	GMYC23	18	SOL	DE17	KY749445
Brachionus "Tiscar"	SOL87DE	H8	GMYC4	7	SOL	DE14	KY749391
Brachionus "Tiscar"	DUL110DE	H8	GMYC4	7	DUL	DE14	KY749386
Brachionus "Tiscar"	DUL111DE	H8	GMYC4	7	DUL	DE14	KY749387
Brachionus "Tiscar"	DUL112AR	H8	GMYC4	7	DUL	AR12	KY749388
Brachionus "Tiscar"	DUL113DE	H8	GMYC4	7	DUL	DE14	KY749389
Brachionus "Tiscar"	DUL114AR	H8	GMYC4	7	SOL	AR12	KY749390
Brachionus "Tiscar"	SOL68DE	H8	GMYC4	7	SOL	DE14	KY749392
Brachionus "Tiscar"	SOL81DE	H8	GMYC4	7	SOL	DE14	KY749393
Brachionus "Tiscar"	SOL80AR	H8	GMYC4	7	SOL	AR12	KY749394
Brachionus "Tiscar"	DUL115DE	H9	GMYC4	7	DUL	DE14	KY749395
Brachionus "Tiscar"	DUL116AR	H9	GMYC4	7	DUL	AR12	KY749396
Brachionus "Tiscar"	SOL92DE	H9	GMYC4	7	SOL	DE14	KY749397
Brachionus "Tiscar"	SOL93DE	H9	GMYC4	7	SOL	DE14	KY749398
Brachionus variabilis	DUL76DE	H22	GMYC9	13	DUL	DE20	KY749432
Brachionus variabilis	DUL95DE	H22	GMYC9	13	DUL	DE20	KY749433
Brachionus variabilis	DUL104DE	H22	GMYC9	13	DUL	DE20	KY749434
Brachionus variabilis	SOL111DE	H22	GMYC9	13	SOL	DE20	KY749435

Brachionus variabilis	DUL80DE	H23	GMYC9	13	DUL	DE20	KY749436
Brachionus variabilis	DUL90DE	H23	GMYC9	13	DUL	DE20	KY749437
Brachionus variabilis	DUL3AR	H24	GMYC9	13	DUL	AR17	KY749438
Family *Collothecaceae*	TIN40DE	H3	GMYC24	3	TIN	DE3	KY749342
Family *Flosculariidae*	TIN62DE	H4	GMYC25	4	TIN	DE1	KY749343
Family *Notommatidae*	TIN25DE	H1	GMYC26	1	TIN	DE7	KY749339
Filinia longiseta	DUL26DE	H40	GMYC16	24	DUL	DE2	KY749490
Filinia longiseta	DUL49AR	H40	GMYC16	24	DUL	AR1	KY749484
Filinia longiseta	DUL5AR	H40	GMYC16	24	DUL	AR1	KY749485
Filinia longiseta	DUL17DE	H40	GMYC16	24	DUL	DE2	KY749486
Filinia longiseta	DUL19DE	H40	GMYC16	24	DUL	DE2	KY749487
Filinia longiseta	DUL23DE	H40	GMYC16	24	DUL	DE2	KY749488
Filinia longiseta	DUL25DE	H40	GMYC16	24	DUL	DE2	KY749489
Filinia longiseta	DUL32DE	H40	GMYC16	24	DUL	DE2	KY749491
Filinia longiseta	DUL39DE	H40	GMYC16	24	DUL	DE2	KY749492
Filinia longiseta	DUL45DE	H40	GMYC16	24	DUL	DE2	KY749493
Filinia longiseta	DUL56DE	H40	GMYC16	24	DUL	DE2	KY749494
Filinia longiseta	DUL59DE	H40	GMYC16	24	DUL	DE2	KY749495
Filinia longiseta	SOL32AR	H40	GMYC16	24	SOL	AR1	KY749496
Filinia longiseta	SOL36DE	H40	GMYC16	24	SOL	DE2	KY749497
Filinia longiseta	DUL98DE	H41	GMYC16	24	DUL	DE2	KY749498
Hexarthra fennica	SOL1DE	H30	GMYC1	19	SOL	DE4	KY749446
Hexarthra fennica	SOL17DE	H31	GMYC1	19	SOL	DE4	KY749447
Hexarthra fennica	SOL77DE	H31	GMYC1	19	SOL	DE4	KY749448
Hexarthra fennica	SOL54AR	H32	GMYC1	19	SOL	AR2	KY749449
Hexarthra fennica	SOL72AR	H33	GMYC1	19	SOL	AR2	KY749450
Hexarthra fennica	TIN27DE	H34	GMYC27	20	TIN	DE4	KY749451
Hexarthra fennica	TIN46DE	H34	GMYC27	20	TIN	DE4	KY749452
Hexarthra fennica	TIN57AR	H34	GMYC27	20	TIN	AR2	KY749453
Hexarthra fennica	TIN58DE	H34	GMYC27	20	TIN	DE4	KY749454
Hexarthra mira	SOL23AR	H35	GMYC28	21	SOL	AR3	KY749455
Hexarthra mira	DUL6AR	H35	GMYC28	21	DUL	AR3	KY749456
Hexarthra mira	DUL66DE	H35	GMYC28	21	DUL	DE5	KY749457
Hexarthra mira	DUL100DE	H35	GMYC28	21	DUL	DE5	KY749458
Hexarthra mira	DUL91DE	H35	GMYC28	21	DUL	DE5	KY749459
Hexarthra mira	DUL69DE	H35	GMYC28	21	DUL	DE5	KY749460
Hexarthra mira	DUL79DE	H35	GMYC28	21	DUL	DE5	KY749461

Hexarthra mira	SOL2AR	H35	GMYC28	21	SOL	AR3	KY749462
Keratella cochlearis	TIN1AR	H39	GMYC29	23	TIN	AR18	KY749480
Keratella cochlearis	TIN2AR	H39	GMYC29	23	TIN	AR18	KY749481
Keratella cochlearis	TIN4AR	H39	GMYC29	23	TIN	AR18	KY749482
Keratella cochlearis	TIN10AR	H39	GMYC29	23	TIN	AR18	KY749483
Keratella tropica	SOL46DE	H38	GMYC2	22	SOL	DE11	KY749468
Keratella tropica	DUL57DE	H38	GMYC2	22	DUL	DE11	KY749474
Keratella tropica	SOL93AR	H36	GMYC2	22	SOL	AR9	KY749463
Keratella tropica	SOL3DE	H38	GMYC2	22	SOL	DE11	KY749464
Keratella tropica	SOL8DE	H38	GMYC2	22	SOL	DE11	KY749465
Keratella tropica	SOL20DE	H38	GMYC2	22	SOL	DE11	KY749466
Keratella tropica	SOL35DE	H38	GMYC2	22	SOL	DE11	KY749467
Keratella tropica	SOL60DE	H38	GMYC2	22	SOL	DE11	KY749469
Keratella tropica	DUL2AR	H38	GMYC2	22	DUL	AR9	KY749470
Keratella tropica	DUL8AR	H38	GMYC2	22	DUL	AR9	KY749471
Keratella tropica	SOL21DE	H38	GMYC2	22	SOL	DE11	KY749472
Keratella tropica	DUL48DE	H38	GMYC2	22	DUL	DE11	KY749473
Keratella tropica	SOL102AR	H37	GMYC30	35	SOL	AR9	KY749475
Keratella tropica	SOL113AR	H37	GMYC30	35	SOL	AR11	KY749476
Keratella tropica	DUL12DE	H37	GMYC30	35	DUL	DE11	KY749477
Keratella tropica	DUL29DE	H37	GMYC30	35	DUL	DE11	KY749478
Keratella tropica	DUL47DE	H37	GMYC30	35	DUL	DE11	KY749479
Lecane closterocerca	TIN3DE	H61	GMYC31	31	TIN	DE6	KY749536
Lecane closterocerca	TIN45AR	H61	GMYC31	31	TIN	AR5	KY749535
Lecane hamata	TIN39AR	H59	GMYC10	30	TIN	AR4	KY749533
Lecane hamata	TIN60DE	H60	GMYC10	30	TIN	DE6	KY749534
Lecane spp.	TIN53DE	H62	GMYC33	32	TIN	DE6	KY749537
Lecane spp.	DUL24DE	H62	GMYC33	32	DUL	DE6	KY749538
Lecane spp.	DUL11DE	H63	GMYC32	33	DUL	DE6	KY749539
Polyarthra sp.	DUL72DE	H52	GMYC34	27	DUL	DE9	KY749516
Polyarthra vulgaris	SOL50AR	H53	GMYC13	28	SOL	AR7	KY749521
Polyarthra vulgaris	SOL73DE	H53	GMYC13	28	SOL	DE9	KY749523
Polyarthra vulgaris	TIN50DE	H55	GMYC14	29	TIN	DE9	KY749529
Polyarthra vulgaris	SOL49AR	H52	GMYC13	28	SOL	AR7	KY749517
Polyarthra vulgaris	SOL61DE	H52	GMYC13	28	SOL	DE9	KY749518
Polyarthra vulgaris	SOL90DE	H52	GMYC13	28	SOL	DE9	KY749519
Polyarthra vulgaris	SOL91DE	H53	GMYC13	28	SOL	DE9	KY749520

Polyarthra vulgaris	SOL51DE	H53	GMYC13	28	SOL	DE9	KY749522
Polyarthra vulgaris	SOL83DE	H53	GMYC13	28	SOL	DE9	KY749524
Polyarthra vulgaris	SOL62DE	H53	GMYC13	28	SOL	DE9	KY749525
Polyarthra vulgaris	SOL4AR	H54	GMYC13	28	SOL	AR7	KY749526
Polyarthra vulgaris	TIN22AR	H55	GMYC14	29	TIN	AR7	KY749527
Polyarthra vulgaris	TIN35DE	H55	GMYC14	29	TIN	DE9	KY749528
Polyarthra vulgaris	TIN63DE	H56	GMYC14	29	TIN	DE9	KY749530
Polyarthra vulgaris	TIN8DE	H58	GMYC14	29	TIN	DE9	KY749531
Polyarthra vulgaris	DUL106DE	H57	GMYC35	29	DUL	DE9	KY749532
Proales sp.	TIN37DE	H2	GMYC36	2	TIN	DE12	KY749340
Proales sp.	TIN37AR	H2	GMYC36	2	TIN	AR10	KY749341
Trichocerca similis	TIN28AR	H43	GMYC12	25	TIN	AR8	KY749500
Trichocerca similis	TIN30DE	H42	GMYC12	25	TIN	DE10	KY749499
Trichocerca similis	TIN31AR	H44	GMYC12	25	TIN	AR8	KY749501
Trichocerca similis	TIN32DE	H45	GMYC12	25	TIN	DE10	KY749502
Trichocerca similis	TIN43DE	H46	GMYC12	25	TIN	DE10	KY749503
Trichocerca similis	TIN52DE	H47	GMYC12	25	TIN	DE10	KY749504
Trichocerca similis	TIN42DE	H48	GMYC12	25	TIN	DE10	KY749505
Trichocerca sp.	TIN23DE	H49	GMYC11	26	TIN	DE10	KY749506
Trichocerca sp.	TIN38DE	H49	GMYC11	26	TIN	DE10	KY749507
Trichocerca sp.	TIN21DE	H49	GMYC11	26	TIN	DE10	KY749508
Trichocerca sp.	TIN44DE	H49	GMYC11	26	TIN	DE10	KY749509
Trichocerca sp.	TIN41DE	H49	GMYC11	26	TIN	DE10	KY749510
Trichocerca sp.	TIN48DE	H49	GMYC11	26	TIN	DE10	KY749511
Trichocerca sp.	TIN54DE	H49	GMYC11	26	TIN	DE10	KY749512
Trichocerca sp.	TIN61DE	H49	GMYC11	26	TIN	DE10	KY749513
Trichocerca sp.	TIN56DE	H50	GMYC11	26	TIN	DE10	KY749514
Trichocerca sp.	TIN14DE	H51	GMYC11	26	TIN	DE10	KY749515

TIN Tinaja

SOL Santa Olalla

DUL Dulce

DE Diapausing egg morphotype

AR Adult rotifer morphotype

AR1 *Filinia longiseta*

AR2 *Hexarthra fennica*

AR3 *Hexarthra mira*

AR4	*Lecane hamata*
AR5	*Lecane chlosterocerca*
AR6	*Asplachna brightwelli*
AR7	*Polyarthra vulgaris*
AR8	*Trichocerca similis*
AR9	*Keratella tropica*
AR10	*Proales sp.*
AR11	*Brachionus budapestinensis*
AR12	*Brachionus plicatilis*
AR13	*Brachionus calyciflorus*
AR14	*Brachionus quadridentatus*
AR15	*Brachionus angularis*
AR16	*Brachionus leydigi*
AR17	*Brachionus variabilis*
AR18	*Keratella cochlearis*

Table A1.

Appendix2

MCMC model specifications and diagnostics

The MCMC models were run for 7000000 iterations with a burn-in of 40000 and a thinning interval of 700. For hatching rates (binary response variable), the residual variance was fixed to 1 (Hadfield, 2010). For that reason, we used a χ^2 distribution with one degree of freedom as the prior distribution rather than the inverse-Gamma distribution (Villemereuil et al., 2013), while in the case of the analyses of the day of hatching, a flat noninformative prior was used.

For hatching rates models, we used the family *categorical*, which uses a logit link function, while the *Poisson* family was used for studying the day of hatching. We evaluate autocorrelation between posterior samples (Wilson et al., 2010) to validate the models. Autocorrelation was always less than 0.1 for the first lag, while the effective sample sizes for all intercepts and variance terms were above 1000. We ensured that each model properly

converged by using the Gelman-Rubin statistic. For this purpose, we ran each analysis 5 times. Models have converged if the Gelman-Rubin statistic (PSRF) is lower than 1.1 (Fisher et al., 2013).

We used the deviance information criterion (DIC) for comparing models with and without random factors. A difference in the DIC of five is considered substantial, and a difference of 10 rules out the model with the larger DIC (Spiegelhalter et al. 2007, Barnett et al., 2010).

Priors and model codes

Prior for hatching rates models (4 random factors)

prior1.1 <- list(G = list(G1 = list(V=1, nu=1000, alpha.mu=0,alpha.V=1), G2 = list(V=1, nu=1000, alpha.mu=0,alpha.V=1), G3 = list(V=1, nu=1000, alpha.mu=0,alpha.V=1), G4 = list(V=1, nu=1000, alpha.mu=0,alpha.V=1)), R = list(V=1, fix=1))

Prior for hatching time models (4 random factors)

prior2.1 <- list(G = list(G1 = list(V = 1, n = 0.002), G2 = list(V = 1, n = 0.002), G3 = list(V = 1, n = 0.002), G4 = list(V = 1, n = 0.002)), R = list(V = 1, n = 0.002))

Example of model code for hatching rates models

MCMCmodel <-MCMCglmm(Eclo ~ Salinity * Origin + Lake, random = ~ Date + Experiment + Rotiferspecies + Waterfowlspecies, data = subset(Dataset, Temp==15), family = "categorical", prior = prior1.1, burnin=40000, nitt = 7000000, thin = 700, verbose = FALSE)

summary(MCMCmodel)

Example of model code for hatching time models

MCMCmodel <-MCMCglmm(Days ~ Salinity * Origin + Lago, random = ~ Date + Experiment + Rotiferspecies + Waterfowlspecies, data = subset(Dataset, Temp==15), family = "poisson", prior = prior2.1, burnin=40000, nitt = 7000000, thin = 700, verbose = FALSE)

summary(MCMCmodel)