Ecological and evolutionary impact of diapause on zooplankton

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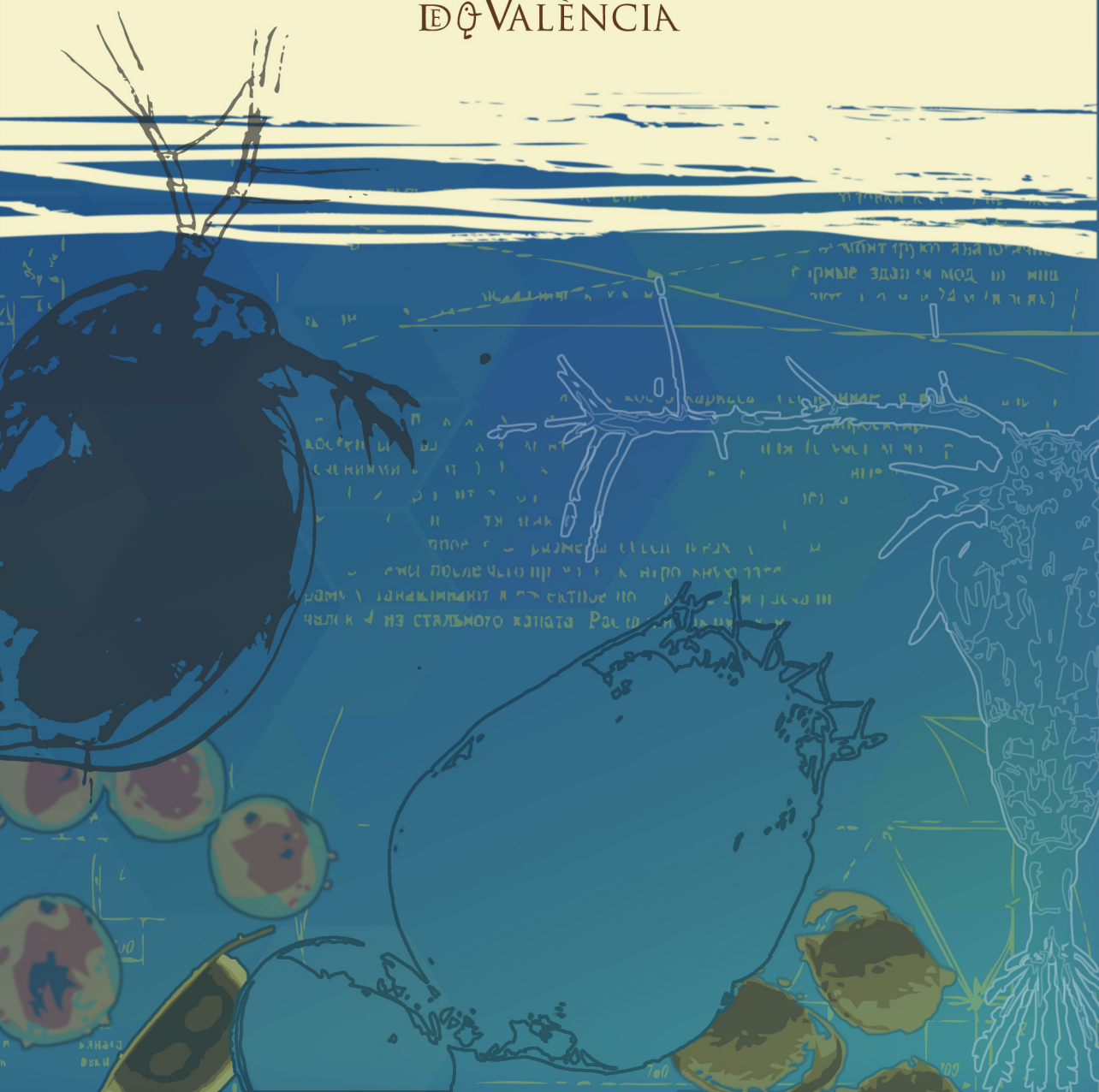
PhD Thesis 2012

VNIVERSITAT DE VALÈNCIA



Facultat de Ciències Biològiques

Institut Cavanilles de Biodiversitat i Biologia Evolutiva

Programa de doctorado de *Biodiversidad y Biología Evolutiva*

Ecological and evolutionary impact of diapause on zooplankton

2012

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Tesis presentada por JAVIER MONTERO PAU para optar al grado de Doctor en Ciencias Biológicas por la Universitat de València.

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Este Trabajo ha sido financiado por una Beca-Contrato del programa para la Formación del Personal Investigador concedida a JAVIER MONTERO PAU, y por el proyecto de investigación BOS2003-00755 del Ministerio de Ciencia y Tecnologia del Gobierno de España.

A mis padres,
por todo lo que me hicieron aprender
sin darse cuenta.

A mi hermano.

A Paula, por ser hogar.

Agradecimientos

Esta tesis empezó con mal pie; en lugar de un interesantísimo proyecto de investigación EN África, resultó ser un proyecto CON Africa (para descanso de mi madre). Pero además resultó que Africa en lugar de trabajar en Hull, Canadá, un sitio alucinante en mitad de los bosques canadienses, trabajaba en Hull, Inglaterra, una ciudad que ni los propios nativos tienen en demasiada alta estima. Sin embargo, pese al desengaño inicial, esta tesis ha sido una gran aventura. Durante estos años he disfrutado investigando y discutiendo ideas, pero también viajando y conociendo a muchísimas personas interesantes. Y ahora que esto toca a su fin, quiero agradecer a todas esas personas la compañía, el apoyo y el cariño recibido en el discurrir de estos años.

En primer lugar quisiera agradecerles a Manuel Serra y Africa Gómez su confianza al depositar en mis manos este proyecto de tesis. A su lado he ido aprendiendo a responder interrogantes, pero sobre todo, me han enseñado a hacer preguntas. Me han transmitido su curiosidad y entusiasmo por el conocimiento y gracias a ellos he ido creciendo y formándome como científico. A ambos además me une una gran amistad. A Africa, además, agradecerle que me abriera las puertas de su casa y me acogiera en su familia. Estos agradecimientos son por tanto extensibles a Dave, Calima, Pablo y Aitana que me hicieron sentir como en casa todo el tiempo que estuve en Hull.

Una parte muy importante de esta tesis se la debo a los miembros del grupo de Ecología Evolutiva. Agradecer a Mª José Carmona, directora en la sombra, su apoyo, cariño y consejos durante todos estos años. A Edu, Sergi, Ana, Carmen, Raquel, Nohemí, Elo, Natalia y Marta, agradeceros todos los buenos momentos, toda la ayuda y las discusiones interesantes (sobre rotíferos, ciencia o lo que fuera), pero sobre todo, vuestra amistad.

También agradecer a los miembros del Cavanilles su acogida, mucha gente se ha prestado a echarme una mano durante estos años, David Martínez, Eladio Barrio, Ana González, Silvia Ramos. También a muchos amigos que han pasado por aquí: Benja, Teresa, Araceli…

En Hull (mi segunda casa) recibí la ayuda de mucha gente. Agradecer el consejo científico a Dave Lunt, Bernd Hänfling y Cock van Oosterhout. De todo el tiempo que pasé en Hull, además de las largas conversaciones con Boris mis primeros meses allí, recuerdo con especial cariño a Thorid, compañera de laboratorio, casa, pintas y fatigas y a Mark y Richard. También a Quini, Andy, Helen, Laura, Javier, Nora...

A todos ellos les debo los muchos buenos momentos pasados allí. Pero por encima de todos destaca Isabel, una gallega con alma de cerillera que me acogió y sin la que aquellas estancias no habrían sido lo mismo.

A Rosa, Ángel, Darío, Cristina, Javivi, Mónica, Irene, Nacho… por tantas tardes de parque y cavernita, por las risas al final del día y por todo el apoyo que me dais. A María, Mª Ángeles, Arantxa, Mabel, Mireia, Laura, Amador por tantos cafés y cervecitas cuando todavía no sabíamos para qué servía un biólogo. Y especialmente a Sara y Jorge, porque, desde que lo descubrimos, siempre están ahí supliéndolo con humor y cariño.

Dar las gracias también a todos los andaneros, especialmente a Josemi (Jose Miguel Mayo) por la *peazo* portada que me ha regalado.

A Paula por estar ahí todo el tiempo, apoyándome, empujándome, recogiéndome y haciéndolo todo más fácil. Esto desde luego no habría sido lo mismo sin su cariño.

En este tiempo, además, he ganado una familia, que al igual que la mía propia, me arropa, comprende y se preocupa. Muchas gracias.

A mi hermano porque es una alegría constante. A Noe. Y por supuesto, a la nueva adquisición familiar.

A mis padres, por tanto esfuerzo, cariño, apoyo, desvelos y amor. Porque no se me ocurre cómo lo podían haber hecho mejor.

¡Va por ustedes!

Ecological and evolutionary impact of diapause on zooplankton

"Seek simplicity, but distrust it"

L.C. Birch

Contents

Resumen

La diapausa es un rasgo fascinante del ciclo vital, una especie de "máquina del tiempo" que permite a los organismos sobrevivir a periodos ambientales desfavorables y lidiar con la impredicibilidad del hábitat. Se trata de un tipo de dormición que se puede presentar en diferentes estadios del ciclo vital dependiendo de la especie o el hábitat y se encuentra ampliamente extendido en el reino animal. La producción de huevos diapáusicos es especialmente común en especies de invertebrados acuáticos hololimnéticos (i.e., sin una fase terrestre) como ostrácodos, anostráceos, notostráceos, copépodos, cladóceros o rotíferos. Esto es debido a que, frecuentemente, las masas de aguas continentales donde habitan experimentan cambios que imposibilitan la continuidad de la vida activa a lo largo de todo el año. En muchos casos, además, esta variación es impredecible. Un ejemplo paradigmático de hábitats con este carácter discontinuo y errático en sus condiciones ambientales son las lagunas saladas de la región mediterránea. Estas

especies de invertebrados además han desarrollado una diapausa de larga duración, lo que permite sobrevivir a sus huevos durante largos periodos de tiempo enterrados en el sedimento de los lagos y lagunas. Así pues, los huevos se acumulan en el sedimento, lo que da lugar a la formación de enormes bancos de huevos. En lagunas de tamaño mediano dichos bancos de huevos pueden alcanzar valores de miles de millones de huevos.

Esta tesis se propone profundizar en el estudio de las implicaciones ecológicas y evolutivas de la diapausa tanto a nivel poblacional, como a nivel de comunidad, un aspecto este último que tradicionalmente ha recibido una menor atención. La tesis se centra en organismos acuáticos zooplanctónicos productores de huevos diapáusicos y específicamente trata de esclarecer (1) su efecto sobre la estructuración genética de las poblaciones y (2) sus implicaciones para la coexistencia de especies ecológicamente similares.

Los objetivos concretos son (1) estudiar el impacto evolutivo de los bancos de huevos diapáusicos en la estructura genética de la poblaciones de organismos acuáticos zooplanctónicos productores de huevos diapáusicos, (2) analizar las implicaciones de los huevos diapáusicos para la coexistencia de especies crípticas a través del efecto de almacenamiento en ambientes fluctuantes y (3) examinar los compromisos entre diapausa y crecimiento poblacional en un escenario competitivo.

El gran tamaño de los bancos de huevos diapáusicos tiene un profundo impacto en la estructura genética de las poblaciones zooplanctónicas. La eclosión masiva y sincrónica desde el banco de huevos, junto con las altas tasas de crecimiento del zooplancton dan

lugar a que se alcancen altas densidades poblacionales poco tiempo después de que la columna de agua vuelva a ser habitable. Esto crea un "efecto de bloqueo por alta densidad" (*high-density blocking*) que evita que los migrantes puedan establecerse y puede resultar en la formación de efectos fundadores persistentes. La combinación de ambos procesos explicaría un patrón paradójico encontrado en muchos organismos zooplanctónicos continentales. El patrón consiste en la existencia de una alta diferenciación poblacional en marcadores genéticos neutros pese a la alta capacidad de dispersión que poseen estas especies. Esta explicación fue posteriormente ampliada para incluir la adaptación local como una fuerza adicional que reduciría el flujo génico efectivo y que por tanto, ayudaría a mantener una marcada estructuración genética de estos organismos. La suma de estos tres factores se conoce como la Hipótesis de Monopolización y aunque se ha convertido en un marco teórico muy popular en la literatura de organismos zooplanctónicos continentales, la importancia relativa y las interacciones entre los procesos demográficos, neutrales y selectivos que actúan en la estructuración genética de estas poblaciones no han sido estudiadas con detalle. En el Capítulo 2 de esta tesis se aborda este problema. Haciendo uso de modelización, se evalúa el impacto de los bancos de huevos en la diferenciación genética poblacional y su relación con el flujo génico, la deriva genética y los efectos fundadores persistentes. También se exploran otras características demográficas de los organismos acuáticos, como las altas tasas de crecimiento o las altas densidades poblacionales, así como la presencia de fuerzas selectivas para la adaptación local. Los resultados muestran que los efectos fundadores persistentes son los que dirigen la diferenciación genética en estos organismos. Dichos efectos

fundadores surgen fundamentalmente de la formación de enormes bancos de huevos diapáusicos, altas densidades poblaciones y rápidas tasas de crecimiento poblacional (i.e., efectos demográficos). Los efectos demográficos son capaces de contrarrestar el impacto de la migración, y la adaptación local coadyuva marginalmente en la diferenciación. Sólo cuando los tamaños poblacionales son pequeños o no existe un banco de huevos diapáusicos, la migración, la adaptación local o el arrastre de los genes neutros ligados a genes bajo selección tienen un efecto sobre la estructuración genética de las poblaciones. Los resultados de esta tesis muestran la importancia del marco ecológico a la hora de discriminar la importancia relativa de las fuerzas que actúan en la estructuración genética de las poblaciones de organismos acuáticos de dispersión pasiva. Aspectos como la continuidad temporal del hábitat o la disponibilidad de recursos o el tamaño de los individuos pueden ser primordiales para entender la importancia de los efectos demográficos, así como su relación con las fuerzas selectivas y neutrales.

En el Capítulo 3 se describe un protocolo de laboratorio para obtener ADN de huevos diapáusicos de un amplio rango de especies de invertebrados acuáticos. Se trata de un método de extracción sencillo y barato basado en un protocolo de lisis celular alcalina. Este método mejora el ampliamente usado método Chelex ya que mejora su almacenamiento a largo plazo y evita los problemas de inhibición de la PCR causados por la quelación de los cationes Mg^{+2} por los grupos de ácido iminodiacético.

La diapausa no sólo permite la supervivencia de los linajes y la persistencia de las especies ante la adversidad del medio, sino que además tiene implicaciones en el régimen competitivo de especies bajo

regímenes fluctuantes mediante el efecto de almacenamiento (*storage effect*), donde la exclusión competitiva es evitada por la inversión en una fase insensible a la competencia. En el Capítulo 4 se explora el régimen ecológico y las dinámicas poblacionales de dos especies de rotífero ecológicamente similares y se analiza la implicación de la diapausa como mediador de su coexistencia en el contexto del efecto de almacenamiento. El estudio se llevó a cabo mediante *barcoding* molecular de las poblaciones planctónicas, el análisis paleogenético de las dinámicas de huevos diapáusicos y realizando experimentos de laboratorio para comprobar la respuesta a la salinidad de las especies. Los resultados proporcionan por primera vez evidencia cuantitativa de la coexistencia histórica de *B. plicatilis* y *B. manjavacas*, dos especies críticas de rotíferos que habitan aguas saladas y que poseen un amplio solapamiento potencial de nicho. Ambas especies presentan una respuesta parcialmente diferenciada con respecto a la salinidad; *B. plicatilis* crece mejor que *B. manjavacas* a salinidades bajas. Sin embargo, dado que sus rangos de salinidad solapan, este factor no debe de limitar directamente la presencia de las especies, sino que afectaría su eficacia relativa. Los resultados sugieren que las fluctuaciones de salinidad median la coexistencia de estas especies, lo explicaría las fluctuaciones poblacionales observadas en el registro histórico. Esto apoya la hipótesis de que el efecto de almacenamiento (*storage effect*) sea el mecanismo subyacente en la coexistencia de estas dos especies.

Además del efecto de almacenamiento causado por los huevos diapáusicos que se ha apuntado en el Capítulo 4, en esta tesis se demuestra que la desviación de recursos para la producción de estos huevos (la inversión en diapausa) puede favorecer la coexistencia

estable de especies competidoras. En este caso, el efecto no es debido a la protección del competidor inferior, como cuando actúa el efecto de almacenamiento (Capítulo 4), sino porque la capacidad competitiva del competidor superior se ve moderada. La existencia de diapausa implica un compromiso entre crecimiento poblacional actual e inversión en crecimiento demorado (diapausa); es decir, parte de los recursos, en lugar de destinarse íntegramente a crecimiento poblacional presente, se destinan a la formación de formas de diapausa. Al reducirse el crecimiento poblacional actual, se crean oportunidades para que una especie competitivamente inferior pero con una menor inversión pueda competir con otra especie competitivamente superior. Este compromiso entre crecimiento poblacional presente e inversión en diapausa es explorado en los capítulos 5 y 6. En el Capítulo 5 se desarrolla un método para integrar la inversión en diapausa y el crecimiento poblacional presente en una medida conjunta. Como en ciclos vitales con inversión en diapausa los recursos se reparten entre un componente a corto plazo (crecimiento poblacional presente) y otro a largo plazo (supervivencia de la población a través de periodos ambientales desfavorables), el crecimiento a corto plazo no es una medida adecuada del potencial de un linaje o población para proliferar. Esta división del uso de recursos dificulta el uso de la tasa intrínseca de crecimiento poblacional (*r*) como medida de rendimiento (*performance*), que es un uso común en muchos estudios ecológicos y evolutivos. Así pues, una *r* baja puede deberse a una mayor inversión en diapausa y no a un peor rendimiento del individuo. Este problema tiene una especial importancia cuando se comparan genotipos, poblaciones o especies con una diferente inversión en diapausa o bien se comparan tratamientos que

pudieran dar lugar a una inversión variable. En esta tesis se ha derivado una medida análoga a *r*, la tasa intrínseca potencial de crecimiento (r_{pot}), la cual es la tasa de incremento que una población/genotipo tendría si no ocurriera ninguna inversión en estadios de diapausa. A pesar de que el cálculo de la r_{pot} requiere de algunos supuestos y simplificaciones, se propone que esta nueva medida de rendimiento demográfico superior a *r*, y debería ser usada cuando se espera o se observa una inversión variable en diapausa. Junto a esta propuesta, se ilustra cómo calcular r_{pot} según algunos ciclos vitales y tipos de experimentos.

En el Capítulo 6, se proporciona una demostración teórica de que el coste de la inversión en diapausa o de la reproducción sexual es capaz de mediar la coexistencia de especies ecológicamente similares, incluso cuando hay un solapamiento completo de sus nichos. El estudio centra el análisis en dos grupos de organismos acuáticos partenogenéticos cíclicos (cladóceros y rotíferos). En estos organismos la producción de huevos diapáusicos está ligada a la reproducción sexual, por lo que al coste de la diapausa se añade el coste de la reproducción sexual. Mediante modelización, análisis simbólico del modelo y simulación se demuestra que la inversión denso-dependiente en diapausa y/o reproducción sexual crea oportunidades que permiten que especies competitivamente inferiores puedan coexistir con especies competitivamente superiores. Así se identifica un nuevo mecanismo de coexistencia (*life-cycle switching*) capaz de actuar sobre especies ecológicamente equivalentes. Esta coexistencia es posible incluso cuando las señales denso-dependientes que inician los costes son parcialmente compartidas entre especies competidoras. Se propone que este mecanismo, puede generalizarse a otras especies en las que existan

costes derivados de un cambio denso-dependiente en el ciclo vital, aunque el cambio no implique la diapausa o la inversión sexual. El *life-cycle switching* es un mecanismo general de coexistencia que se inserta en el grupo de mecanismos de coexistencia no basados en diferenciación de nicho. Solo se han identificado unos pocos de estos mecanismos, pero pueden ser de gran importancia para explicar, por ejemplo, la coexistencia de especies crípticas o especies filogenéticamente próximas, las cuales suelen presentar un fuerte solapamiento de nicho.

Finalmente, en el Capítulo 7 se presentan y discuten en un contexto global la diapausa y su importancia ecológica y evolutiva. De manera global, esta tesis representa un esfuerzo para mostrar las implicaciones de la diapausa tanto a nivel poblacional como a nivel de comunidad, y presta una especial atención a la interrelación entre ecología y evolución. Se anticipa que esta interrelación irá cobrando mayor importancia en investigaciones futuras, en las que, por un lado, no se ignorarán las características de ciclos vitales complejos, y en las que, por otro lado, se incrementará el esfuerzo por aproximar el enfoque evolutivo a la ecología de comunidades.

1

Introduction

Ecological and evolutionary interplay

"Nothing in biology makes sense, except in the light of evolution…"

T.H. Dobzhansky

"… but very little in evolution makes sense except in the light of ecology"

M. Begon, J.L Harper and C.R. Townsend

Although somewhat exaggerated, Begon, Harper and Townsend's quote holds a capital idea; evolution and ecology are tightly linked. Many evolutionary changes cannot be understood without taking into account the environment and the interactions of the organisms. Classical examples of this are, for instance, the adaptation of the beaks of Darwin's finches to different ecological niches (Darwin 1859) or the change of frequencies of melanism of peppered moths due to the

increase of pollution after the Industrial Revolution (Kettlewell 1956). Meanwhile, many ecological aspects are the result of selective forces, as for example, trade-offs on life-history traits (Stearns 1992). The tight relationship between ecology and evolution has been recognized since long time ago, and is in the core of the modern evolutionary biology scientific program (McIntosh 1986). However, it has not been until more recently that ecologist and evolutionary biologists are increasingly stressing that both ecological and evolutionary processes actually occur contemporarily, interacting and modulating each other, and giving rise to the so-called "eco-evo dynamics" (e.g., Thompson 1998; Fussmann et al. 2007; Pelletier et al. 2009; Post & Palkovacs 2009; Schoener 2011). Traditionally, a clear-cut distinction between ecological and evolutionary time scales had been postulated; Slobodkin (1961) defined the "ecological time" as *circa* 10 generations, while he suggested "evolutionary time" to be on the order of half million years. The discovery of rapid evolution processes has challenged this idea (Thompson 1998 and references therein; Gingerich 2001; Yoshida et al. 2003; Hairston et al. 2005), making clear that both scales overlap. This interplay between evolution and ecology occurs at all levels of ecological organization, and eco-evo dynamics have been described from the population to the ecosystem level (see for instance the special volumes *Functional Ecology* 21 and *Philosophical transactions of the Royal Society of London. Series B* 364).

Ecological and evolutionary impact of diapause

Diapause is a type of dormancy characterized by a hypometabolic state and/or development arrestment (Hand & Podrabsky 2000), which can

occur at different life-cycle stages (e.g., egg, larvae, pupae or adult in insects) depending on species and habitat. The main difference from quiescence – the other form of dormancy in animals – is the way this dormancy is maintained; whereas in quiescence it is controlled by external factors (e.g., temperature or daylight), in diapause the regulation occurs by internal physiological factors. Diapause is present in a wide variety of animal taxa, for instance, sponges, flatworms, rotifers, crustaceans, insects, arachnids, worms (see a review in Cáceres 1997a), fishes (Wourms 1972), reptiles (Fordham *et al*. 2006) or mammals (Lopes *et al*. 2004).

Production of diapausing stages is a life history trait with manifold ecological and evolutionary implications. Diapause, through delayed reproduction or survival along time, extends generation time, which increases the overlap between the ecological and evolutionary scales. Moreover, diapause is of importance not only at a population level – for example, to avoid adverse environmental periods –, but also at a community level, as diapausing stages are insensitive to interspecific competition. From an ecological perspective, diapause works as a sort of "time machine", allowing organisms to survive unfavorable environmental periods, and to cope with the unpredictability of their habitats (Hairston 1998). Besides this time dispersal, diapausing stages often act also as spatial dispersing stages. That is the case, for example, of many aquatic invertebrates (e.g., Havel & Shurin 2004). Thus, diapause can be considered as a strategy to spread risks through time and space. Other ecologically important roles of diapause are the seasonal synchronization of life cycles (Chippendale 1982), and the mediating effect on coexistence (Chesson 1983; Cáceres 1997b).

Competitive outcome in fluctuating environments can be affected by diapause; as dormant stages are insensitive to resource competition, so switching or investing in diapause creates a way of avoiding competitive exclusion in the long term (i.e., storage effect) (Chesson 1983).

As any other life-history trait, diapause is evolutionary constrained by trade-offs. In temporary habitats, production of viable diapausing stages should be maximized by natural selection given that it is necessary for the future survival of a genotype. However, diapause investment involves costs to the organism; resources are allocated in the production of long-lived forms that will not contribute to current performance. This leads to non-trivial problems related to the timing of diapause and betting strategies in fluctuating environments (Ellner 1997). For instance, if an organism starts producing diapausing stages too early, before the end of the suitable period for growing, it will miss opportunities for its descendants to proliferate, while if too late, it will incur in the risk of dying before producing any diapausing stages. Analogously, a similar compromise will happen with the timing of diapause termination. In some cases, when the duration of diapause can be variable, bet-hedging strategies can evolve (i.e., only a fraction of the dormant stages leave diapause) as a way of spreading risks over time (Cohen & Levin 1987).

Diapausing egg banks in continental zooplankton

Diapause is particularly widespread among continental species of aquatic invertebrates (Cáceres 1997a) such as ostracods, anostracans, notostracans, copepods, cladocerans or rotifers (Fryer 1996; Gyllström & Hansson 2004; Schröder 2005). This is due to the fact that continental

aquatic habitats are usually highly variable, so zooplanktonic species rely on diapause to cope with this variability. In addition, as mentioned before, it is common that diapausing stages act as a dispersing phase in these organisms (Frisch et al. 2007; Allen 2007; Vanschoenwinkel et al. 2011). Many of these species produce diapausing eggs able to remain viable in that dormant state for extended periods (De Stasio 1989); duration of this long-term diapause may vary from several years to centuries (see examples in Hairston 1996). These eggs, thus, accumulate in the sediment of lakes and ponds forming extensive diapausing egg banks (Hairston 1996; Brendonck & De Meester 2003) similar to plant's seed banks. Reported densities for diapausing egg banks for these organisms range from 10^3 to 10^7 eggs m^{-2}, which is equivalent to billions of eggs in a moderately sized pond (De Meester *et al*. 2004). As noticed by several authors (e.g., De Stasio 1990; Gómez & Carvalho 2000; Berg 2005; De Meester et al. 2007; Mergeay et al. 2007) and as it will be shown in this thesis, the evolutionary and ecological dynamics of continental zooplankton cannot be thoroughly understood without considering these banks.

Egg banks, due their large size, are able to buffer genotypes and species against environmental and demographic stochasticity, and to prevent local extinction (Hairston 1996). Also, as eggs in the banks were produced at different times, and under different biotic and abiotic selective pressures, the banks harbor a wide genetic reservoir of genotypes adapted to multiple environments (Brendonck & De Meester 2003). As a result, rapid adaptation to novel conditions is promoted. However, the contrary may be also true. If populations are under

directional selection, recruitment of genotypes adapted to different environment could slow down the rate of evolution.

Diapausing egg banks and genetic differentiation

The large size of diapausing egg banks has also a profound impact on the genetic structure of zooplanktonic populations. Synchronic massive hatching events from such huge diapausing egg banks, associated with the high growth rates of zooplanktonic organisms (Allan 1976), result in the rapid achievement of high population densities shortly after the habitat becomes suitable again. This can create a "high-density blocking" effect (Hewitt 1993) that could prevent the establishment of migrants. Boileau *et al.* (1992) suggested that the high levels of neutral genetic differentiation observed in many aquatic invertebrates (e.g., Freeland et al. 2000; Zierold et al. 2007; Muñoz et al. 2008; Xu et al. 2009; Makino & Tanabe 2009; Escudero et al. 2010; Xiang et al. 2011), despite their high migration capabilities, could be due to such blocking effect and persistent founder effects. This explanation was later expanded by De Meester et al. (2002) into the so-called "Monopolization Hypothesis" to include local adaptation as an important force contributing to reduce effective gene flow and maintain the genetic structure of passively dispersed aquatic organisms. According to this hypothesis, the high neutral genetic differentiation in a scenario of potential high migration could be explained by a combination of (1) persistent founder effects, (2) selection against immigrants due to local adaptation and (3) associations arising randomly between neutral markers and genes under selection. Local adaptation has been described in many zooplanktonic systems (e.g., Decaestecker et al. 2007; Campillo et al. 2010; Costanzo &

Taylor 2010; Alcántara-Rodríguez et al. 2012; Orsini et al. 2012), and it is known to be able to promote genetic differentiation in neutral markers (i.e., "isolation by adaptation") (Nosil *et al*. 2007) and not only reinforce it. However, the effect of local adaptation on genetic structure could not be so direct and general in continental aquatic invertebrates (Campillo et al. 2009; Allen et al. 2010), and neutral and demographic factors could be key to explain the high differentiation. Although the Monopolization Hypothesis has become a popular theoretical framework in continental zooplankton literature, the relative importance and interactions between the demographic, neutral and selective processes acting on the genetic structuration of populations remains poorly understood.

Diapausing egg banks and species coexistence

At the community level, diapausing egg banks have also a role on the coexistence dynamics of competing species. Continental aquatic habitats are usually variable and under fluctuating regimes, and fluctuations have been considered traditionally as an important element to explain the high biodiversity of planktonic communities (i.e., "The paradox of the plankton") (Hutchinson 1961). In the last decades theoretical refinements of this idea have been developed, and it is now clear that fluctuation-mediated coexistence needs special conditions. One of the two possible mechanisms for coexistence based on fluctuations (Chesson 2000) is the storage effect (Chesson 1983). This coexistence mechanism requires some conditions to be met. One of them is the existence of a stage relatively insensitive to the competition, such as diapausing eggs. In fact, diapausing egg banks have been hypothesized

many times as important mediators of the coexistence in continental zooplankton through this mechanism (e.g., Hairston 1996; Brendonck & De Meester 2003), and one study has demonstrated the importance of the storage effect in these organisms (Cáceres 1997b). However, coexistence in many zooplanktonic species can be challenging; cryptic species (i.e., species without noticeable morphological differentiation), which are common among aquatic invertebrates (Knowlton 1993; Gómez et al. 2002), are likely to possess largely overlapping niches. This is expected on one hand, due to the high degree of morphological resemblance, and on the other, to the close phylogenetic relationship that can lead to retention of the niche (Webb et al. 2002; Wiens & Graham 2005; Losos 2008). If there is a wide niche overlap between species, the differences allowing coexistence might be subtle (Chesson 2000). Examples of coexistence of cryptic species are known (e.g., in the rotifer cryptic species complex *Brachionus plicatilis*) (Gómez et al. 1997; Ciros-Pérez et al. 2001). However the study of the mechanisms mediating coexistence of such species through the storage effect, and the effect of fluctuations and subtle ecological differences, is still a field that has received little attention.

An additional and especially interesting feature of diapausing egg banks is that they represent ecological and evolutionary archives of populations (Brendonck & De Meester 2003). These banks hold a complete record of the past ecological conditions and selective forces that have operated on the populations. Hence, these records are useful tools for paleolimnological studies. Its study has allowed, for example, the reconstruction of predatory pressures (Hairston et al. 1999; Cousyn et al. 2001), population dynamics (Jankowski & Straile 2003), ecological

invasions (Mergeay et al. 2005; Mergeay *et al*. 2006), or "Red Queen" dynamics (Decaestecker et al. 2007). Surprisingly, despite its potential, its use has been mainly restricted to cladocerans (mainly *Daphnia*) (De Meester et al. 2007). For instance, in rotifers only one study has used so far the diapausing egg banks to study past dynamics (Epp et al. 2009).

Diapause in cyclical parthenogens

The most common groups of planktonic animals of continental waters, cladocerans and monogonont rotifers, are cyclical parthenogens (i.e., they combine clonal reproduction with occasional bouts of sexual reproduction during their life cycle), and in both cases, the production of diapausing stages is the result of the sexual reproduction. Other than differences in, for example, the mechanism of sex determination, or the sexually reproducing stage, the life cycle of these two groups is quite similar (reviewed in De Meester et al. 2004). Monogonont rotifer's cycle starts with diploid asexual females hatched from diapausing eggs from the sediment of the pond or lake. These females asexually reproduce by ameiotic (apomictic) parthenogenesis resulting in daughters genetically identical to them. This clonal propagation phase usually comprises several generations until sexual reproduction is induced by changes in environmental conditions. Cues inducing sexual reproduction often include crowding, photoperiod and dietary compounds (e.g. Nogrady et al. 1993). In the rotifer genus *Brachionus*, one of the most studied, population density is the factor triggering sexual reproduction (Snell & Boyer 1988; Carmona et al. 1993). Specifically, sex is induced by a protein released into the environment by the rotifers (Snell et al. 2006). As population density increases, this protein accumulates, and at a

threshold concentration it triggers sexual reproduction, in a process akin to quorum sensing in bacteria (Kubanek & Snell 2008). Once sex is induced, asexual females start producing sexual (mictic) females, which produce haploid eggs that develop into either haploid males or, if fertilized, into diapausing eggs. Diapausing eggs settle in the sediment, where they remain until the suitable conditions resume in the water column and hatching is induced. Some relevant differences between the life cycle of monogonont rotifers and cladocerans exist. In the latter, sex determination is not haplodiploid, so all individuals are diploid. Moreover, a single cladoceran female may be able to produce both meiotic and ameiotic eggs, being so involved in parthenogenetic proliferation and in diapausing stage production.

As it has been shown, sexual reproduction is required for the production of diapausing stages in monogonot rotifers and cladocerans. Thus, in these species, besides the general cost over growth rate of producing diapausing eggs (see above), the commonly known "two-fold cost of sex" (Maynard Smith 1978) is added. This cost, compared to asexual reproduction, emerges from the need of producing two kinds of organisms (i.e., females and males) to reproduce, whereas asexual organisms do not. Aparici *et al.* (1998) analyzed sex-ratio in cyclically parthenogenetic rotifers, and showed that this cost acts despite the complexities of the cycle. The selective pressures driving sex investment and its effects on population dynamics of cladocerans and rotifer populations has been thoroughly studied (Serra & King 1999; Serra et al. 2005; Carmona et al. 2009). The cost of diapause and sexual investment has been analyzed extensively in terms of the optimal investment in sex and diapause in relation to the habitat characteristics (see review in

Serra et al. 2004; Serra et al. 2008). Moreover, timing of sex affects not only the number of diapausing eggs produced, but also the amount of genetic diversity harbored in these eggs, as during asexual growth clonal erosion of genetic diversity can occur (Gómez & Carvalho 2000; Vanoverbeke & De Meester 2010); the longer this period, the lower the population genetic diversity (Ortells *et al*. 2006). Thus, a long phase of clonal proliferation can lead to deleterious effects on the populations (e.g., inbreeding depression) (Cáceres *et al*. 2009). Contrasting with the attention drawn by the implications of sexual and diapause investment over population growth, studies in a framework of species competition – and its effect in the community diversity – are scarce, as they are mostly limited to the effects of diapausing eggs, rather than the effects of diapausing egg production, as a refuge against competition.

Tools for the study of ecology and evolution

Biology is fundamentally an experimental science; however, experimentation is not always feasible, as critically illustrated when dealing with endangered species, or biological processes lasting more than human life. Additionally, as any other science, biology pursues generality; that is, to formulate general statements that work as premises to predict previously unobserved phenomena. Contrasting with other natural sciences, biological problems are often complex and multifactorial, requiring the integration of many parameters and processes, which can hinder the design of experiences (Levins 1966). In those cases, the use of theoretical abstractions or models has proven to be a fruitful tool for researches. Given the nature of ecological and evolutionary processes, whose dynamics usually require long time

frames and include multiple factors, theoretical models have been extensively used in those two fields for a long time (Lynch & Walsh 1997). Helping in the use of models is the fact that many evolutionary and population features of interest are quantitative, e.g., population size, or can be projected in a quantitative space, as kinship. Famous examples are the population genetic models by R.A. Fisher (1930), J.B.S. Haldane (1932) or S. Wright (1931), or the models for population growth by T.R. Malthus (1798), P.F. Verhulst (1838) or A.J. Lotka (1920) and V. Volterra (1926). In fact, probably, one of the first mathematical models applied to biology was to study the population growth of rabbits by Fibonacci in the 13th century (Gillman 2009).

Mathematical models can arise either from inductive or deductive processes; they can be the result of a symbolic abstraction from a set of observations, or they can be the result of a theoretical idea whose biological derivations need to be tested, or whose plausibility needs to be contrasted with other ideas. Models have a double role; descriptive and predictive (Levins 1966; Begon et al. 1996; Hastings 1997). On one hand, they can be used to explore the dynamics or behavior of complex processes and help to find experimentally testable situations. On the other hand, they serve to define and clarify a conceptual framework, problem or hypothesis. For instance, in their textbook of population ecology, Begon and his co-workers (1996) state as a function of modeling to be 'an aid to enlightenment on aspects of population dynamics previously been unclear'. Modeling is, thus, a useful tool to understand biological phenomena.

Ideally, mathematical model analysis should be performed symbolically (also said, analytically), so that general theoretical results

are achieved, not depending on specific assumptions on parameter values. However, given the complexity and the large number of parameters of many biological models, desirable way is not always feasible. In such cases, numerical approaches are needed, and it is frequent that the models are simulated using computer programing (Wilson 2000). Different approaches (i.e., theoretical analysis, laboratory and field work, and modeling and computing analysis) have been used in this thesis to answer the raised objectives.

Objectives and outline of this thesis

The aim of this thesis is to explore the evolutionary and ecological implications of diapause and sex investment in zooplankton. In the previous sections we have identified several important questions regarding the phenomenon of diapause that are unanswered or require further analysis. These questions are (1) the effect of diapause on population genetic differentiation, (2) whether diapausing egg banks allow the coexistence of ecologically similar competing species and (3) the impact of diapause and sex investment on population growth.

Thus, the objectives of this thesis are (1) to study the evolutionary impact of diapause egg banks for the genetic structure of populations of passively dispersed aquatic organisms, (2) to test the role of the diapausing eggs as mediators of the coexistence of cryptic species through the storage effect in fluctuating environments, and (3) examine the trade-off between diapause and sexual investment and current population growth in a competitive scenario.

The impact of diapausing egg banks on the genetic structure of populations of passively dispersed aquatic organisms is presented in

Chapter 2. Using a modeling approach, the effect of diapausing egg banks on the genetic differentiation of populations and its interplay with gene flow, genetic drift and persistent founder effects are studied. Other demographic characteristics of aquatic organisms, such as fast growth rates or high population densities, as well as the presence of selective forces for local adaptation are also explored in this chapter.

A laboratory protocol to obtain DNA from diapausing eggs from a range of aquatic taxa is presented in Chapter 3. This new methodology allows the application of high throughput techniques for DNA screening when dealing with large amounts of eggs. This method is applied in Chapter 4.

Diapause creates favorable conditions for survival and persistence of species, but also has implications on the coexistence regime between species under a fluctuating environment. This ecological advantage of diapause is addressed in Chapter 4. The ecological regime and population dynamics of two ecologically similar competing and closely related rotifer species were analyzed by molecular barcoding surveys of planktonic populations, palaeogenetic analysis of diapausing eggs dynamics, and laboratory experiments to test their response to salinity. The role of diapause as mediator of their coexistence is discussed in the framework of the storage effect.

The trade-off between diapause and sex investment and current population growth is explored in Chapter 5 and 6. In Chapter 5 we develop a methodological technique to estimate an intrinsic population growth rate where this investment is included. This results in a new performance measure that enables to account for the cost of the investment when comparing species, population or clones with a

differential diapause and sex investment. In Chapter 6, we theoretically demonstrate that a density-dependent diapause or sexual investment is able to allow the coexistence of ecologically similar species, even when there is a complete overlap of their niches.

Finally, in Chapter 7, the main results of this thesis are summarized and discussed in a global context about diapause and its ecological and evolutionary importance.

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2

Founder effects drive the genetic structure of passively dispersed aquatic invertebrates

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Abstract

Populations of passively dispersed organisms in continental aquatic habitats often show high levels of neutral genetic differentiation, despite their high dispersal capabilities. Several evolutionary factors, including founder events and local adaptation, and life cycle features such as high population growth rates and the presence of propagule banks, have been proposed to explain this paradox. Here, we have modeled the population colonization process in these organisms to assess the impact of migration rate, growth rate, population size, local adaptation and life-cycle features on their population genetic structure. Our simulation results show that the strongest effect on population structure is that of persistent founder effects, resulting from the interaction of a small number of population founders, high population growth rates, large population sizes and the build up of diapausing egg banks. In contrast, the role of local adaptation, genetic hitchhiking and migration is limited to small populations, which could result in a different impact of local adaptation on genetic structure of different groups of zooplankters.

Introduction

Successful dispersal and colonization are essential for population establishment and persistence of species, and an understanding of these processes is crucial in the face of changing climate and habitat destruction, which is rapidly affecting the abundance and distribution patterns of many species (Parmesan & Yohe 2003; Chen et al. 2011). The evolutionary outcome of dispersal and colonization results from a potentially complex interplay of neutral and selective factors, including local adaptation, founder effects or bottlenecks causing genetic drift during the first stages of colonization, inbreeding depression, or high gene flow that could erode local adaptation (Lenormand 2002; Kliber & Eckert 2005; Rosenblum et al. 2007; Keller & Taylor 2008; Verhoeven et al. 2011). These factors shape the genetic structure of populations and the evolutionary history of species. In addition, life-cycle features and demographic characteristics may act as modulators and lead to different evolutionary outcomes (Burton et al. 2010). For example, species with high population growth rates after a bottleneck are more likely to maintain their genetic variability ("founder-flush" model) (Carson 1968; Templeton 2008), and populations of organisms with resistant life stages (e.g., diapausing eggs) are more likely to be connected by migration even at long distances (Frisch et al. 2007). Predicting the outcome of these factors is a major question in evolutionary and conservation ecology, and requires an understanding of the effect of each factor and their interactions.

Populations of passively dispersing aquatic invertebrates (e.g., freshwater bryozoans, rotifers, cladocerans, copepods, anostracans, notostracans) and macrophytes inhabiting lentic habitats often present a

high level of neutral genetic differentiation (e.g., Freeland et al. 2000; Zierold et al. 2007; Muñoz et al. 2008; Xu et al. 2009; Makino & Tanabe 2009; Escudero et al. 2010; Xiang et al. 2011), despite their high dispersal capabilities through diapausing propagules (Frisch et al. 2007; Allen 2007; Vanschoenwinkel et al. 2011). This has been termed the "migration-gene flow paradox". Regardless of their taxonomic disparity, these organisms share biological features promoting a rapid monopolization of resources in the new environment: high population growth rates, large population sizes and the production of resistant stages in their life cycle. The latter can accumulate in sediments and form diapausing propagule banks (Hairston 1996;Brendonck & De Meester 2003) and constitute the main dispersal stage. As a result, once a habitat becomes available and is colonized, the population can grow in size very quickly creating a numerical advantage that dilutes the genetic impact of further immigrants ("high-density blocking"; Hewitt 1993), resulting in a persistent founder effect (Boileau et al. 1992). This explanation was expanded by De Meester et al. (2002) into the so-called "Monopolization Hypothesis" (MH hereafter) to include local adaptation as an important force contributing to reduce effective gene flow and therefore maintaining the genetic structure of passively dispersed aquatic organisms. The MH postulates that the migration-gene flow paradox could be explained by a combination of three factors: (1) persistent founder effects, (2) selection against immigrants due to local adaptation and (3) buildup of linkage disequilibrium between neutral markers and genes under selection.

There is no doubt that local adaptation is an important and rapid process in many zooplanktonic organisms (e.g., Cousyn et al. 2001;

Decaestecker et al. 2007; Costanzo & Taylor 2010). However, the impact of local adaptation on population genetic structure is diverse as it is dependent on the impact of other evolutionary forces, not only selection (Kawecki & Ebert 2004). For instance, it can promote genetic differentiation ("isolation-by-adaptation"; Nosil et al. 2007) or reinforce the existing genetic differentiation (De Meester et al. 2002) by reducing effective gene flow. Irrespective of local adaptation, populations recently founded by a small number of propagules can be highly inbred and show inbreeding depression, and therefore, low fitness (De Meester 1993; Tortajada et al. 2009). This could give migrants a fitness advantage and favor gene flow into the population (Ebert et al. 2002; Haag et al. 2006). The accumulation of large numbers of resistant stages as seed or diapausing egg banks (i.e., propagule banks) in sediments is also a characteristic of many aquatic species inhabiting temporary habitats. These propagule banks have an important role in ecological (Chesson 1983; Cáceres 1997) and evolutionary processes (Brendonck & De Meester 2003). They increase the effective population size due to postponed reproduction in the bank, and thus reduce genetic drift (Kaj et al. 2001). However, this effect may be indirect, as gene flow is also postponed in the bank (Kaj et al. 2001; Berg 2005).

The relative importance of and the interactions between the demographic, neutral and selective processes acting during colonization have remained poorly understood. Therefore, an explicit analysis of the effects of local adaptation, persistent founder effects, and their interplay on the differentiation of populations of aquatic organisms is due, especially during the first stages of colonization when populations are still small and, thus, more sensitive to stochastic effects.

Here, we have modeled the colonization process of zooplanktonic organisms to shed light on how migration rate, growth rate, population size, local adaptation and the existence of a propagule bank shape the population genetic structure during the first stages of colonization. Our primary interest is to gain insights into the relative importance of (1) persistent founder effects, (2) selection against immigrants as a consequence of local adaptation, and (3) random associations between neutral genes and genes under selection (linkage disequilibrium).

Materials and methods

We developed a genetic and demographic model to analyze the effects of population growth rate, population size, presence of a diapausing egg bank and local adaptation on the population genetic structure of aquatic organisms. We assumed a geographic scenario with two habitats connected through migration, which are founded simultaneously after a single event of migration from a source population.

The model was based on the life cycle of rotifers and cladocerans (i.e., cyclical parthenogenesis), which are major taxonomic groups in the zooplankton. Cyclical parthenogenesis combines parthenogenesis with episodic sexual reproduction and typically consists of several asexual generations followed by a sexual generation, generally associated with habitat degradation. The sexual generation produces diapausing eggs that hatch into asexual individuals once the habitat becomes suitable again. As all eggs do not hatch from one planktonic growth period to the next, they may accumulate in the sediment and may form extensive diapausing egg banks (Brendonck & De Meester 2003).

The demographic submodel is outlined in Fig. 2.1. Briefly, it consists in six steps:

step 1. Hatching of diapausing eggs (resident and immigrant)

step 2. Asexual proliferation

step 3. Sexual reproduction and production of diapausing eggs

step 4. Diapausing eggs survival in the sediment

step 5. Migration of diapausing eggs

step 6. Back to step 1

Note that this demography implies two time scales: (1) a within-planktonic growth period (often within-year; index, *t*), and (2) an among-sexual generations scale (often among-years; index, *y*).

Migration, either from the source population or between habitats, is assumed to occur via diapausing eggs, which are passively transferred between habitats, and they are assumed to hatch synchronously with the locally produced diapausing eggs.

Genetic submodel

All individuals are considered to have *n* neutral loci and *s* loci under selection. All loci are biallelic and no mutation is assumed. The model accounts for physical linkage between selected and neutral loci with a variable recombination level. Loci under selection act additively on growth rate. Consequently, no dominance and no epistatic effects are assumed. Local adaptation requires a genotype-environment interaction on fitness. This is modeled through $\delta_{i,j,l}$, which is the effect on the intrinsic growth rate (see below) of allele *i* (*i*: 1, 2) at locus *j* (*j*: 1,…, *s*) in locality *l* (*l*: 1, 2). The assumptions are (1) $\delta_{1,j,1} = \delta_{2,j,2}$, and (2) $\delta_{i,j,l} = -\delta_{j\neq i,j,l}$; so, homozygotes will experience an increase or decrease of their

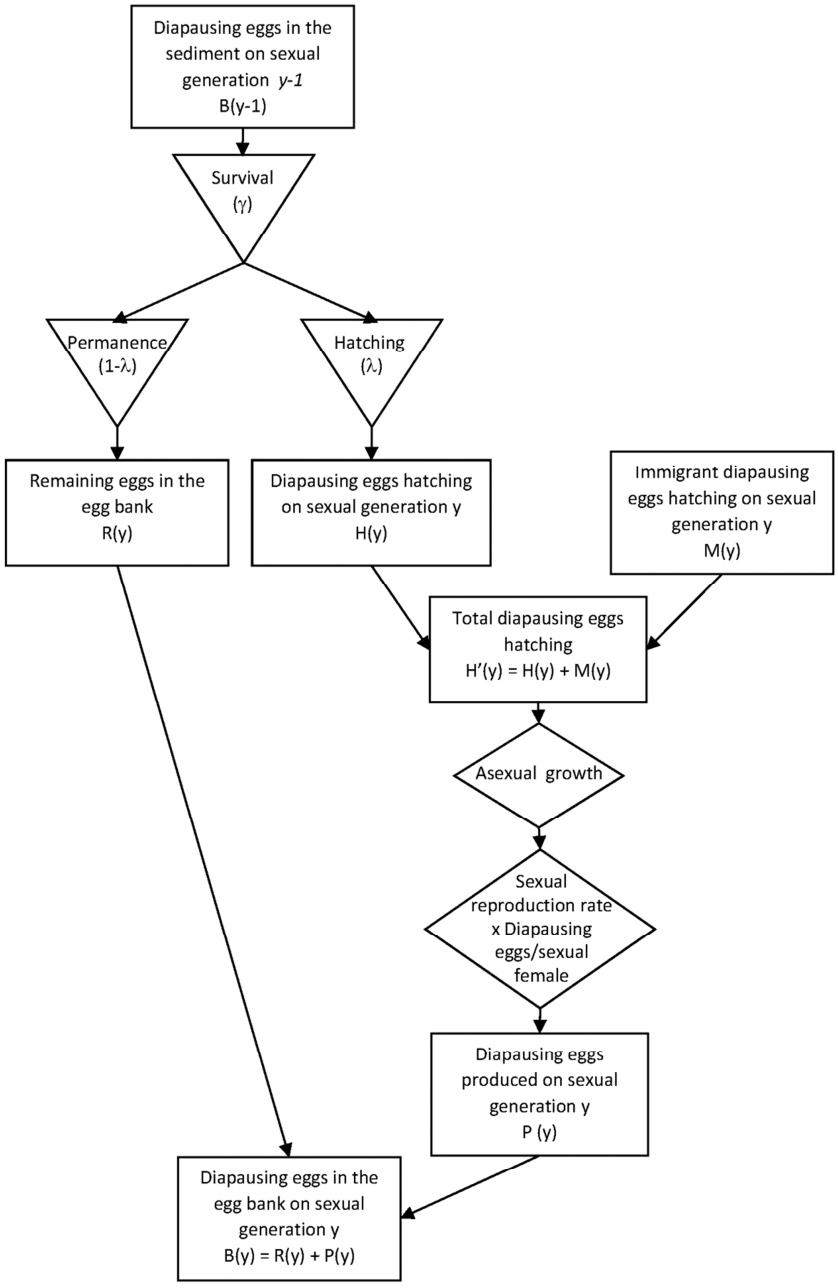

Figure 1. Demographic submodel.

growth rate by $|2\delta|$ depending on the locality, whereas heterozygotes do not. Hence, the growth rate for each genotype *g* in each locality *l* ($r_{g,l}$) can be decomposed into *r* (basal growth rate) and θ (deviation of each genotype), so that

$$r_{g,l} = r + \theta_{g,l}$$

where *g* is the genotype, *l* is the locality, and $\theta_{g,l}$ is the summation of the fitness components (δ) in locality *l* of the alleles carried by a genotype *g* in the *s* loci. Thus, in any given locality, the growth rate during the asexual reproduction will vary between the limits $r \pm 2s\delta$.

Sexual reproduction is assumed to be panmictic and, for simplicity, is considered to be synchronic and at the end of the growing season ($t = \tau$). As linkage disequilibrium can occur due to selection and genetic drift, gametic frequencies are computed. Gametes are then drawn to produce the diapausing eggs.

Genetic distance between populations was estimated based on neutral loci as

$$F_{ST} = \frac{\overline{H}_T - \overline{H}_S}{\overline{H}_T}$$

where $\overline{H}_T$ is the average expected heterozygosity for the two populations considered as a single one for the neutral loci, and $\overline{H}_S$ the average of the mean expected heterozygosity within each population for the neutral loci. For the loci under selection, genetic distance (F_{STQ}) was computed analogously to the neutral loci (Le Corre & Kremer 2012). F_{ST} and F_{STQ} values were obtained just after hatching of diapausing eggs.

Population growth

The asexual phase spans from time $t = 0$ to τ, which is the moment when sexual reproduction takes place. During the asexual phase the population grows deterministically according to a logistic growth model:

$$\frac{dN_{l,g}}{dt} = N_{l,g} r_{l,g} \left[1 - \frac{\sum_g N_{l,g}}{K}\right],$$

where $N_{l,g}$ is the population density, $r_{l,g}$ the intrinsic population growth rate during the asexual phase, and K the carrying capacity (l and g as above). Note that K is the same regardless of genotype. At the onset of each asexual growth season ($t = 0$), $N_{l,g}$ is the sum of the hatched diapausing eggs, a fraction of them having been locally produced $H_{l,g}$, and the rest being immigrants $M_{l,g}$.

At $t = \tau$ of the sexual generation y, the number of diapausing eggs produced $P_{l,g}$ (y) is calculated from $N_{l,g}$ (τ, y) assuming a sexual proportion m (fraction of the females that becomes sexual), a sex ratio sr and an effective fecundity e (number of diapausing eggs produced per sexual female).

Mortality of diapausing eggs in the sediment is assumed to be age-independent (annual survival rate γ). When a new planktonic growing season starts ($t = 0$) a fraction λ of the diapausing eggs in the sediment hatches.

Source population and local population founding

The two populations are founded at time $y = t = 0$ by F diapausing eggs randomly drawn from a single source population. The source population is assumed to be in Hardy-Weinberg equilibrium and of infinite size, so that extraction of migrants does not change genotype frequencies. All

loci are considered neutral in the source population, so no preadaptation to any of the localities exists.

Model implementation

The impact of carrying capacity (*K*), growth rate (*r*), migration (*M*), selection pressure (δ) and recombination rate on F_{ST}'s were analyzed by exploring a range of realistic values for zooplanktonic organisms. *K* was varied from $2 \cdot 10^2$ to $2 \cdot 10^7$ individuals, which is equivalent to densities from 0.001 to 100 individuals/L in a small pond of 200 m^2 and 1 m depth, in good agreement with reported average densities of cladocerans and rotifers (Carmona et al. 1995; Ortells et al. 2003; Tavernini 2008). *r* was explored from 0.05 to 1 $days^{-1}$. Cladocerans show maximum *r* of 0.2-0.6 $days^{-1}$ and rotifers 0.2-1.5 $days^{-1}$ (Allan 1976). The number of population founders (*F*) was set to 1 diapausing egg across most simulations, that is, foundation is considered a rare event. Note that as the model assumes cyclical parthenogenesis, a single diapausing egg is enough for population foundations. The effect of numbers of founders (*F*) was also explored (1, 2, 5, 50 diapausing eggs). Other parameter values used in the simulations are shown in Table 2.1.

Simulations considered two scenarios regarding diapausing egg banks: (1) an annual, age-independent, diapausing egg survival rate on the sediment (γ = 0.763) (i.e., existence of a diapausing egg bank); and (2) γ = 0.763 for eggs of age 1 and a γ = 0 for older eggs (i.e., absence of diapausing egg bank). Parameters for the diapausing egg bank (γ and λ, the annual hatching rate) were estimated from rotifer diapausing egg

Table 2.1. Summary of model parameters and explored range of values.

Parameter	Definition	Value
F	Number of founders (individuals)	1 - 50
M	Number of immigrants per sexual generation (individuals)	0 - 10^5
γ	Egg annual survival proportion in the bank egg	0.763 *
λ	Annual hatching proportion of diapausing eggs	0.046 *
Y	Sexual generations	1000/4000
τ	Duration of the asexual growth period (days)	60
r	Clonal growth rate of each genotype (days^{-1})	0.05 - 1.00
K	Carrying capacity (individuals)	$2{\cdot}10^2$ - $2{\cdot}10^7$
m	Sexual proportion	0.7 †
sr	Sex ratio	0.5 ‡
e	Diapausing egg production per sexual female	3
n	Number of neutral loci	5
s	Number of loci under selection	5
δ	Additive value on r (days $^{-1}$)	10^{-5} - 10^{-1}

* Calculated from García-Roger et al. (2006b) † (Alver & Hagiwara 2007) ‡ (Aparici et al. 1998)

banks (García-Roger et al. 2006b) by adjusting them to the model described by García-Roger et al. (García-Roger et al. 2006a).

The simulation model was implemented in C++ and based on Monte-Carlo procedures. The Mersenne Twister algorithm (Matsumoto & Nishimura 1998) was used as random number generator. 50 replicates for each parameter combination were performed, except for δ and recombination rate, where 100 replicates were carried out. For each replicate, a source population was randomly created by drawing the

allelic frequencies of the *n* and *s* loci from a uniform distribution. After foundation of the two populations, 1000 sexual generations (4000 generations for some scenarios) were simulated.

Sampling effects were taken into account for hatching and survival of diapausing eggs if the total number of eggs in the population was lower than 1000. Selection of immigrants and gametes for mating were performed randomly regardless of the number of eggs/individuals involved.

Statistical differences between F_{ST} values under a neutral scenario and scenarios with selective pressure and different recombination rates were assesed with an ANOVA and *a priori* contrasts. Correlations between F_{ST} and F_{STQ} at different combinations of population size, recombination rates and selective pressure were also tested using Kendall's Tau and Sperman's Rho. All statistical analyses were performed using SPSS v. 17 (SPSS Inc., Chicago, USA).

Results

The population dynamics of a newly founded population, using the parameters shown in Table 2.1, with τ = 60 days and r = 0.3 days^{-1} – realistic values for both the length of the growth season (Tavernini 2008) and the intrinsic growth rate of many aquatic invertebrates (Allan 1976) – show that carrying capacity (K) is reached in less than two sexual generations, even in the case of the highest K (i.e. $K = 2 \cdot 10^7$ individuals). Thus, K is a good proxy of population size and we will use both terms interchangeably hereafter.

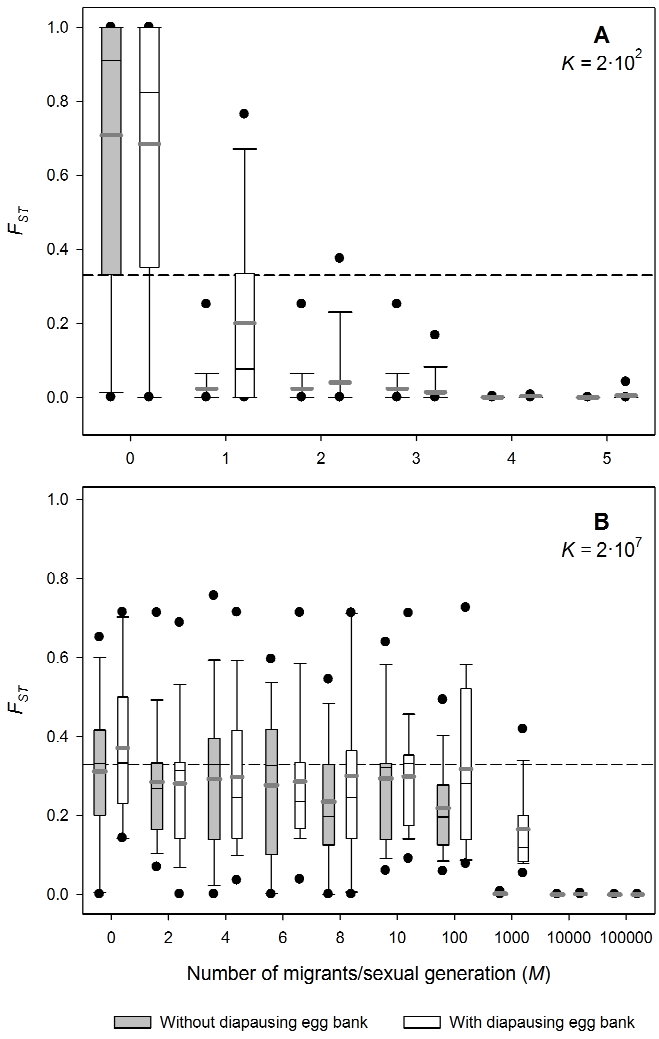

Figure 2.2. Population differentiation (F_{ST}) after 1000 sexual generations plotted against migration (M) with and without a diapausing egg bank for (A) $K = 2{\cdot}10^2$, and (B) $K = 2{\cdot}10^7$ individuals. The rest of parameters were r = 0.3 d^{-1}, n = 5, s = 0 and F =1. Box plots are based on 50 replicate simulations. Boxes represent 25th /75th percentile and black dots the 5th/95th percentile. Thin black lines and thick gray lines in each bar represent the median and the mean, respectively. Dashed, horizontal lines show the initial value of F_{ST} after foundation.

Effect of migration

The effect of the number of immigrants on genetic differentiation strongly depends on *K* (i.e., population size; Fig. 2.2). In both the small and the large populations, F_{ST} decreases with increasing migration rates, as expected under a neutral scenario (Wright 1931). For the lowest carrying capacity tested (K=$2 \cdot 10^2$ individuals; Fig. 2.2.A), F_{ST} decreased rapidly down to very low levels with increasing migration. By contrast, for the highest *K* tested (*K* = $2 \cdot 10^7$ individuals; Fig. 2.2.B), F_{ST} was rather insensitive to the effect of migration, and populations remained highly differentiated ($F_{ST} > 0.2$) even at high levels of migration. The number of migrants needed to cause a considerable decrease of genetic differentiation is in the order of 100 and 1000 individuals/sexual generation for the situation without and with diapausing eggs respectively.

Effect of population size

Carrying capacity (i.e., population size) had strong effects on F_{ST} (Fig. 2.3). At samall population sizes (i.e., low *K*) populations did not differ genetically, while at large population sizes, F_{ST} remained as high as it was just after population foundation. This pattern suggests importance of migration and persistent founder effects respectively. At intermediate values of *K*, genetic differentiation peaked, probably due to higher drift effects. In other words, the highest F_{ST} values are found at intermediate population sizes. The pattern is qualitatively similar with and without diapausing egg bank, but the maximum genetic differentiation occurred at higher population sizes without egg bank, and maximum F_{ST} is higher with diapausing egg bank. These results are robust to changes in the

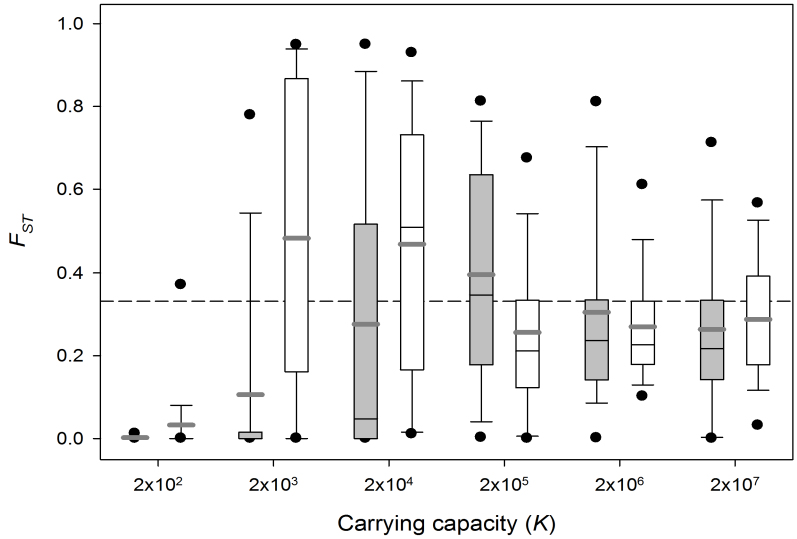

Figure 2.3. Population differentiation (F_{ST}) after 1000 sexual generations plotted against carrying capacity (K) with and without a diapausing egg bank. Simulation values for other parameters were r = 0.3 d^{-1}, n = 5, s = 0, F =1 and M = 2. Data is based on 50 replicate simulations. Boxes represent 25th /75th percentile and black dots the 5th/95th percentile. Thin black lines and thick gray lines in each bar represent the median and the mean, respectively. Dashed line shows the initial value of F_{ST} after foundation.

maximum number of sexual generations explored (results for maximum y = 100, 500, 2000 and 4000 generations, data not shown). However, at 100 and to a lesser extent 500 sexual generations, the peak of F_{ST} at intermediate population sizes was less pronounced than at later sexual generations.

The dynamics and net increment of F_{ST} was explored from the 1st to the 4000th sexual generation (Figure 2.4). In the absence of a diapausing egg bank (Fig. 2.4 A), F_{ST} decreases with time at small population size, and this situation is reversed when K increases, to finally

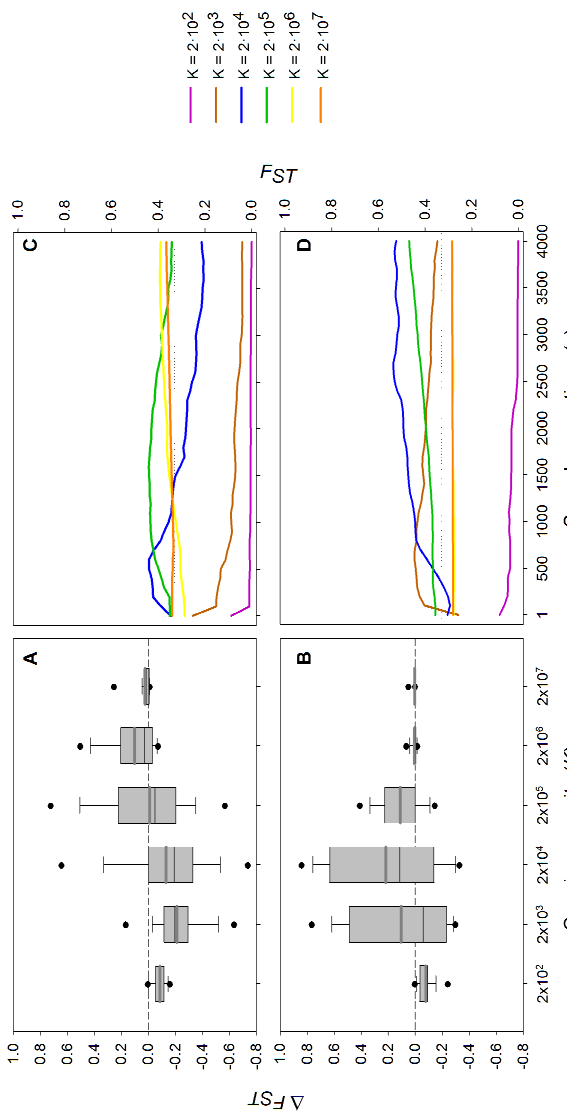

Figure 2.4. Effect of different carrying capacities (K) on the trajectory of F_{ST} along 4000 sexual generations. (A, B) Box plot of the increment of F_{ST} (ΔF_{ST}) after 4000 sexual generations (A) without and (B) with diapausing egg bank is shown. (C, D) Time course of the average F_{ST} values along 4000 generations (C) without and (D) with diapausing egg bank. Simulation conditions were r = 0.3 d^{-1}, n = 5, s = 0, F =1 individual and M = 2 individuals. Data is based on 50 replicates. Boxes represent 25th /75th percentile and black dots the 5th/95th percentile. Thin black lines and thick gray lines in each bar represent the median and the mean, respectively. Doted lines show the initial value of F_{ST} after foundation.

become virtually constant at the largest population size explored (K = $2 \cdot 10^7$). A similar qualitative pattern is found when a diapausing egg bank is present (Fig. 2.4 B), although the shift to an increasing F_{ST} time course occurs at smaller population sizes, and also F_{ST} constancy is achieved at lower K. Note that the small negative change found at $K = 2 \cdot 10^2$ with and without bank is associated to the very low initial F_{ST} values (Fig. 2.4 C, D). Also note that F_{ST} values are calculated after hatching of residents and immigrants; for instance, at y = 1, F_{ST} value is not the value after foundation but after migration. In summary, population size and presence or absence of a diapausing egg bank are key to predict the main force shaping the genetic structure. Decreasing F_{ST} indicates that migration is the dominant factor, while increasing values show that drift becomes dominant. The stability of F_{ST} through time indicates the importance of persistent founder effects on the shaping of the genetic structure of populations.

Population growth rate interacts with population size in determining the level of genetic differentiation (Fig. 2.5). Low growth rates result in low genetic differentiation, regardless of population size, indicating a high impact of migration. In contrast, for population growth rates above 0.1 d^{-1}, which are common for zooplanktonic organisms, genetic differentiation becomes sensitive to variations in population size.

Effects of the number of founders

Increasing the number of population founders, F, results in a dramatic decrease of F_{ST} values just after foundation (Fig. 2.6); for instance, compared to F = 1, F_{ST} is reduced by half for F = 2, and approaches 0 for

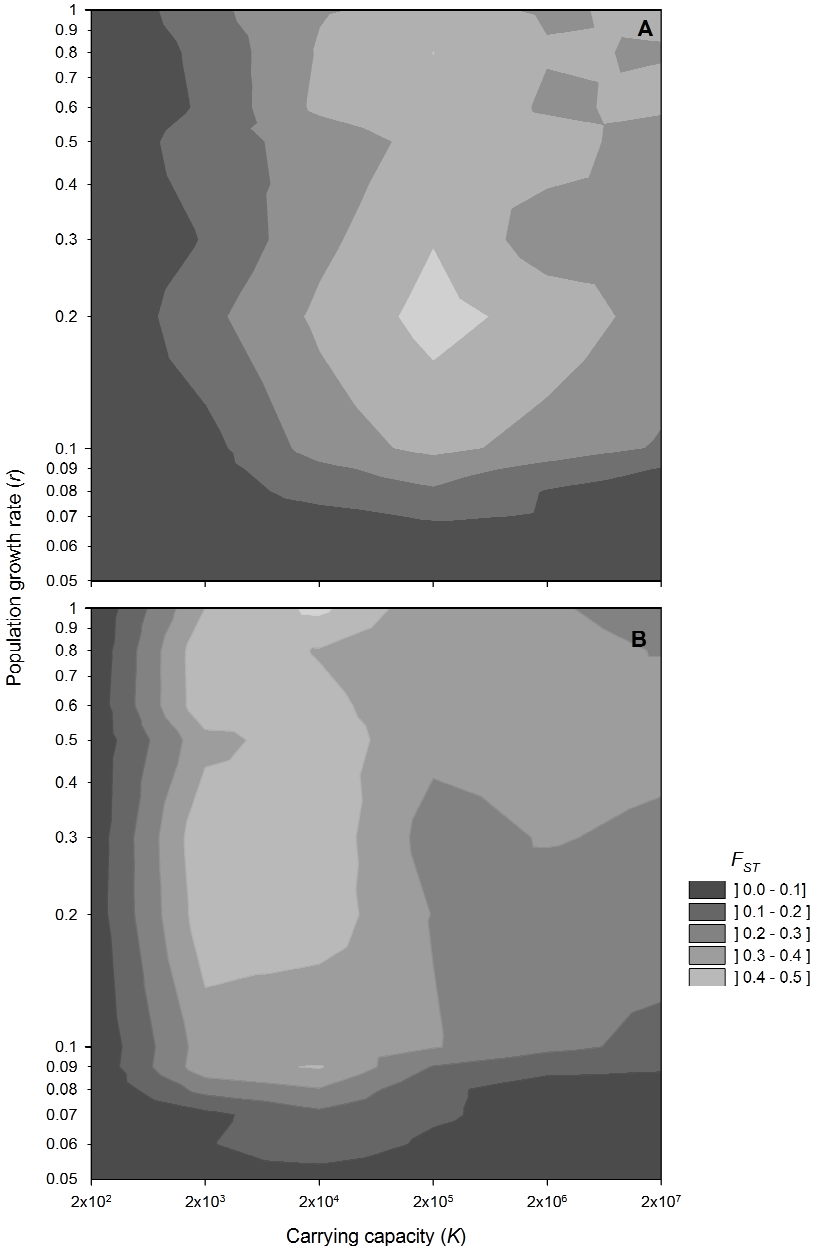

Figure 2.5. Contour plot showing F_{ST} values after 1000 sexual generations at different combinations to population growth rates and carrying capacity (A) without and (B) with diapausing egg bank. Simulation conditions were n = 5, s = 0. F =1 and M = 2. Data is based on 50 replicates.

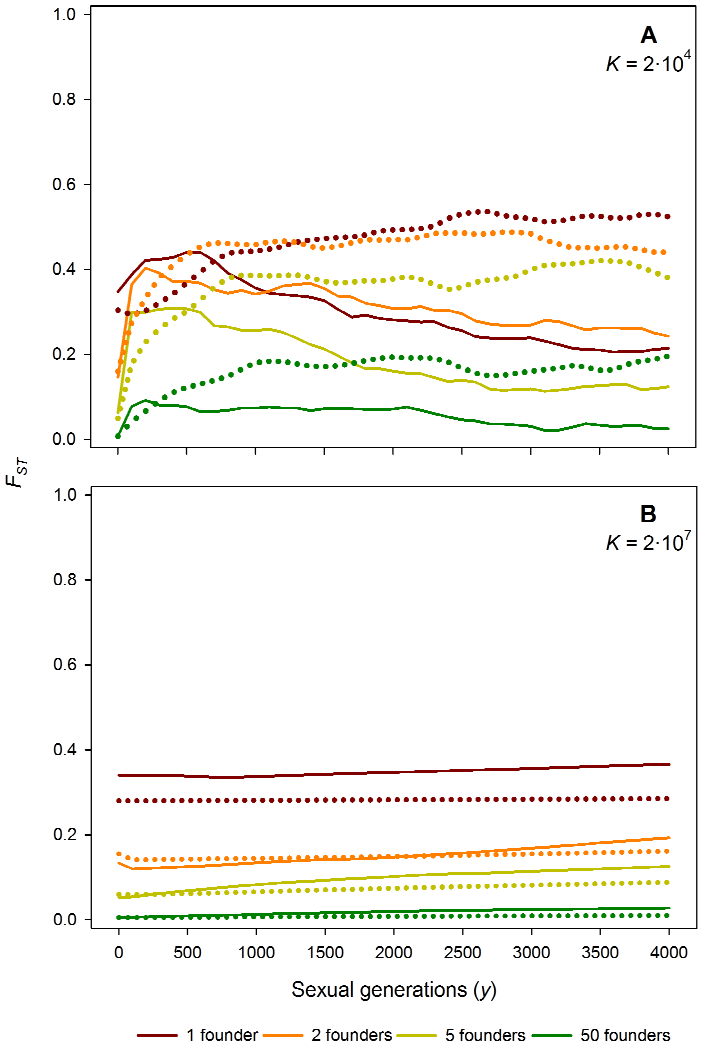

Figure 2.6. Time course of the average F_{ST} value along 4000 generations for different number of founders (F = 1, 2, 5 and 50), for K = $2 \cdot 10^4$ (A) and K = $2 \cdot 10^7$ (B) and M = 2. Solid lines: without diapausing egg bank, dotted lines: with diapausing egg bank. Average F_{ST} values obtained from 50 replicates.

F = 50. After 4000 sexual generations, the level of population differentiation still shows a negative relationship with the number of founders. Given this strong effect, we carried out further simulations to explore how *F* affects the relationships between population differentiation and other factors. Our results suggest that the patterns outlined above are qualitatively maintained for F > 1 (data not shown).

Effect of local adaptation

The effect of local adaptation was explored at two levels of *K* ($2 \cdot 10^4$ and $2 \cdot 10^7$ individuals), which are realistic values for cladocerans and rotifers respectively. Two different selection scenarios ($\delta = 10^{-4}$ days^{-1}, weak selection, and 10^{-2} days^{-1}, strong selection) in the presence/absence of diapausing egg bank, and six recombination rates – from complete linkage to unlinked genes – were tested (Fig. 2.7 summarizes the results for the scenario with diapausing egg bank; see Apendix A, for the equivalent scenario without diapausing egg bank).

With strong selection, F_{STQ} reaches almost maximum values – i.e., populations are almost fixed for the locally adapted alleles – regardless of *K* (Fig. 2.7). In populations with $K=2 \cdot 10^4$, all F_{ST} values are statistically different from those obtained without selection (p-values < 0.05 except at 0.5 recombination rate; p-value = 0.057). However, F_{ST} values are insensitive to recombination rate. In contrast, for high *K* and complete linkage (recombination rate = 0), F_{ST} in the presence of selection is statistically different to that without selection. This indicates that genetic hitchhiking in large populations acts only on neutral loci tightly linked to those under selection. Otherwise, the drag of the genes under

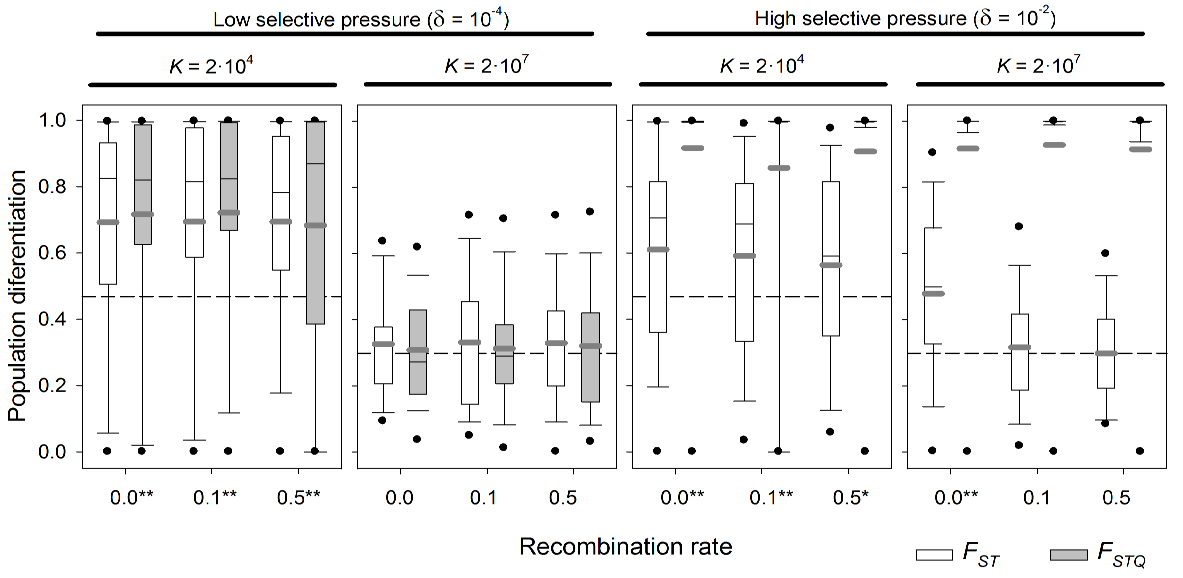

Figure 2.7. Box plot graph of F_{ST} and F_{STQ} values after 1000 sexual generations with different recombination rates for two different values of fitness components ($\delta = 10^{-4}$ and 10^{-2} d^{-1}) and with presence of a diapausing egg bank. For each of the fitness scenario, the left panel refers to $K = 2{\cdot}10^4$ and the right panel to $K = 2{\cdot}10^7$. Other parameters were $r = 0.3$ d^{-1}, $n = 5$, $s = 5$, $F = 1$ and $M = 2$. Data is based on 100 replicates. Boxes represent 25th /75th percentile and black dots the 5th/95th percentile. Thin black lines and thick gray lines in each bar represent the median and the mean, respectively. Dashed lines show the initial value of F_{ST} after foundation. Asterisks indicate F_{ST} statistically different from those without selection ($\delta = 0$) (**, $\alpha = 0.05$; *, $\alpha = 0.1$).

selection does not seem to be able to break the persistence of founder effects.

As expected, F_{STQ} indicates that local adaptation becomes less important with weak selection. With large population size ($K = 2{\cdot}10^7$), F_{ST} values do not statistically differ from the neutral scenario, showing the higher importance of founder effects. Moreover, F_{STQ} values also appear to be affected by persistent founder effects. In contrast to the situation with strong selection, genetic linkage does not alter differentiation at neutral loci. In contrast, local adaptation does play a role at small population sizes ($K = 2{\cdot}10^4$). Mean F_{ST} values statistically differ from the neutral scenario at all recombination rates (from 0.0 to 0.5), and the variance of the distribution of F_{ST} values is decreased (see Fig. 2.3 for comparison). Note that dispite a role for local adaptation, drift is still the dominant factor in relatively small ($K = 2{\cdot}10^4$) populations with diapausing egg bank.

The scenario without diapausing egg bank (see Apendix A) is similar to that with diapausing egg bank in the case of strong selection. However, some differences can be highlighted. If compared to the strong selection scenario, in weak selection conditions: (1) at $K = 2{\cdot}10^7$ genes under selection are less affected by persistent founder effects and populations show a trend to be locally adapted; (2) at $K = 2{\cdot}10^4$, F_{ST} values at recombination rates 0.0 and 0.1 are statistically different from the neutral scenario – unlike at higher recombination rates –, which indicates that genetic hitchhiking could be of some importance; (3) at $K = 2{\cdot}10^4$ F_{ST} and F_{STQ} had higher variance at all recombination rates.

In the absence of a diapausing egg bank, populations reach maximum F_{STQ} values in about 40-50 sexual generations regardless of

population size (data not shown). However, when a diapausing egg bank exists, advantageous alleles need a longer time to reach fixation (about 150 sexual generations for $K = 2 \cdot 10^4$, and about 300 generations for $K = 2 \cdot 10^7$).

We computed F_{STQ} vs. F_{ST} correlations within each tested parameter combination. Significant correlations were found only in the case of the low K ($2 \cdot 10^4$) without diapausing egg bank. Correlation coefficient is always positive, and the ranges are: Kendall's tau = 0.53-0.66 and Spearman's rho = 0.56-0.73 for strong selection; Kendall's tau = 0.32-0.68 and Spearman's rho = 0.38-0.80 for weak selection.

Discussion

The understanding of the evolutionary factors responsible for the strong population structure of passively dispersed aquatic organisms in the face of potentially high gene flow has attracted considerable attention in the last decade. We have presented a specific model and, by simulation, explored the effects of genetic drift (founder effects), gene flow via migration and local adaptation on genetic differentiation. Our results show that the strongest effect are persistent founder effects, resulting largely from the distinctive life history traits of these organisms: few population founders, high rates of population growth, large population sizes and the presence of diapausing egg banks. These results are in agreement with those of Boileau et al. (1992), who proposed that persistent founder effects are an important force shaping the genetic structure of passively dispersed aquatic organisms, although they did not explore the importance of selection or genetic linkage. The most remarkable and novel result of our simulations is that the role of local

adaptation and genetic hitchhiking on shaping genetic structure of these organisms is not significant in large populations, although it plays a significant role in small populations.

In agreement with Boileau et al. (1992), migration has a very limited effect on the population structure of passively dispersed aquatic organisms. For instance, a migration rate of 1000 individuals per sexual generation is needed to cause a noticeable effect on F_{ST} in a large population. Although direct estimates of the number of dispersing stages in these organisms are unavailable, this seems an extremely large value unlikely to occur between non-connected ponds (Cáceres & Soluk 2002; Frisch et al. 2007; Allen 2007), and inconsistent with estimates of the number of founders in populations, which are expected to be correlated with regular immigration rates (Louette et al. 2007, Badosa *personal communication*). However, in small populations, our model recovers the expected pattern for the combined effect of migration and drift under neutral genetic differentiation.

Among the factors studied in our model, population size has been shown to be largely responsible for establishing the levels of genetic differentiation observed in natural populations of aquatic organisms. In addition, this effect is strongly reinforced when a diapausing egg bank is established. Although egg banks could increase gene flow by postponing migration in the bank (Kaj et al. 2001; Berg 2005), they act mainly buffering the effects of migration and reducing genetic drift, which favors the establishment of persistent founder effects. In our model, we assumed a parameter range in agreement with values reported for many aquatic organisms. Nevertheless, due to computational limitations the values used for population sizes and egg bank densities had to be

limited, and could underestimate those attained in many natural populations. Some estimated population sizes and diapausing egg bank densities in rotifers are 1 or 2 orders of magnitude higher than the maximum values considered here (Carmona et al. 1995; Ortells et al. 2003). Diapausing egg bank densities for zooplanktonic organisms are in the order of 10^3-10^7 eggs/m^2 (review in Hairston, 1996 for different zooplanktonic taxa), although densities in the sediment layers that could provide recruits are uncertain. However, modeling larger population sizes is unlikely to change our results qualitatively; if anything, they would make the relative impact of persistent founder effects stronger.

Local adaptation seems to be common and has been well documented in cladocerans (e.g., De Meester 1996; Cousyn et al. 2001; Decaestecker et al. 2007) but, seems to be rarer in rotifers – though study effort in rotifers is much lower and restricted to rather generalist species living in highly fluctuating environments – (Campillo et al. 2011). However, the effect of local adaptation on population genetic structure is limited, as it is weakened by neutral and demographic factors. A limited role for local adaptation in continental aquatic invertebrates has been recently suggested (Campillo et al. 2009; Allen et al. 2010). Our results indicate that local adaptation does occur, but it only has a noticeable effect on population structure when population sizes and diapausing egg banks are relatively small. Given that rotifers tend to have larger population sizes than cladocerans, this would mean that the effects of local adaptation on population structure could differ between these organisms. According to our results, genetic hitchhiking appears to be of limited importance in shaping neutral genetic differentiation. We have only detected signs of its effect at (1) completely linked genes with

high population size and strong selection, and at (2) intermediate population size without egg bank and weak selection. The lack of observed impact does not mean that genetic hitchhiking has no importance, but that other processes are dominating the outcome. We must stress that we do not question that local adaptation occurs, but its impact on genetic differentiation in neutral markers. We acknowledge that, due to computational limitations, our model simplifies the selective scenarios acting on continental aquatic invertebrates. As selection in natural populations of aquatic invertebrates is likely to be multifactorial, and fluctuating, more complex selection scenarios should be further explored.

Genetic analyses in recently established populations indicate that the number of founders is small (Haag et al. 2005; Louette et al. 2007; Ortells et al. 2011; Badosa *personal communication*), and consequently a single founder was assumed in most simulations. When we relaxed the assumption of a single founder, the only remarkable observed effect was a negative one on the final value of F_{ST}.

Globally, our results show that population genetic structure in these organisms is driven by an interaction between persistent founder effects, genetic drift or local adaptation, with population size and the presence of a dipausing egg bank having a strong control on the dominance of each of these factors. In turn, such demographic variables can be linked to ecological features. If so, a habitat classification linking ecological factors, demographic features, and mechanisms acting on genetic structure could be possible. For example, in populations inhabiting permanent ponds and lakes where a low investment in diapause is generally found – as reflected in small diapausing egg banks

in comparison to temporary or ephemeral ponds (Hebert 1974a; Hebert 1974b; García-Roger et al. 2006b; Campillo et al. 2011) –, an increased effect of local adaptation and genetic drift is expected. In contrast, in environmental conditions limiting population sizes, such as small rock pools or nutrient-poor lakes, migration can attain higher importance. If despite small population sizes, populations are highly differentiated genetically, an effect of selective forces can be hypothesized. For instance, genetic hitchhiking has been suggested for a *Daphnia* metapopulation inhabiting temporal rock pools (Haag et al. 2006). Besides ecological features, our results suggest that differences can be expected between taxa differing in body size and so in their typical population sizes, and therefore differences between the smaller rotifers and the larger cladocerans are expected. As far as our results identify a restricted number of factors driving the genetic structure, they provide insights beyond the life cycle assumed (i.e., cyclical parthenogenesis), and could be extended to organisms with similar demographic features (i.e. high growth rates, high population densities or presence of seed or egg banks). For example, populations of sexual species with high growth rates (i.e. *r* strategists) like crustaceans such *Artemia* or copepods, which produce egg banks, are also likely to benefit from a numerical advantage that will reduce the impact of migration on the genetic structure of their populations (Boileau et al. 1992).

As we have shown, the rapid growth rate of colonists acts as a barrier against new migrants, and this is reinforced by the formation of diapausing stage banks and, in some cases, by local adaptation. This process leads to a persistent founder effect, and consequently, to a deviation from the migration-drift equilibrium. This has repercussions

when interpreting phylogeographic signals (Gómez et al. 2002; Waters 2011). For instance patterns of "isolation-by-distance" found in several aquatic organisms, regardless of their reproductive mode, have been suggested to be due to a process of sequential colonizations (Gómez et al. 2007; Gouws & Stewart 2007; Mills et al. 2007; Muñoz et al. 2008). Our results are consistent with these proposals and suggest that caution should be applied when inferring a migration-drift equilibrium mechanism of 'isolation by distance' from such patterns (i.e., correlation between genetic and geographical distances). Also, the establishment of persistent founder effects and competitive exclusion of closely related species can explain the phylogenetic overdispersion in communities, given a phylogenetic limiting similarity between species (Violle et al. 2011).

Our results suggest that the time window from the arrival of first colonizers to the establishment of the founder effects – the period of time when the genetic structure of the population is still sensitive to migration or drift – is short, due to high population growth rates of most aquatic organisms. Nevertheless, we found that with relatively low population growth rates, the numerical advantage is delayed and genetic differentiation is relatively low. In a similar way, inbreeding depression could act favoring gene flow (Tortajada et al. 2009; Tortajada et al. 2010) due to the resulting hybrid vigor of the offspring of migrants (Ebert at el. 2002) Although this factor has not been explicitly modeled here, it will act in a similar way of reducing the growth rate, which will favor effective gene flow. However, severe inbreeding could also reduce the effective population size, and increase genetic drift, which will increase genetic differentiation. A more detailed exploration of this

scenario will be needed and it will depend on the relative magnitude of the purging and migration.

Further factors not implemented in our model, but likely to occur in the wild, could also counteract the high genetic differentiation. For example, processes able to reduce population size during asexual growth phase (e.g., perturbations or environmental fluctuations) could increase the impact of gene flow. In addition, it will be of interest to test the strength of persistent founder effects buffering migrants with a higher fitness than locally adapted residents. These factors – inbreeding depression, environmental fluctuations, and preadapted immigrants – were not invoked in the initial formulation of the Monopolization Hypothesis and should be investigated in future analyses. An additional prospective is to include the effect of metapopulation structure. Recently, Walser & Haag (2012) have shown that population turnover, which is expected to have high rate in small populations, could also explain the high genetic population differentiation.

Concluding remarks

Molecular screening of natural population has uncovered an unexpectedly high genetic diversity in taxa with high dispersal potential. These findings challenged classical views of the evolutionary processes in small multicellular organisms, and when focused on aquatic invertebrates, brought to postulate a combination of processes as causal factors for that genetic differentiation, the Monopolization Hypothesis (De Meester et al. 2002). Our analysis shows that a quantitative elaboration of this multifactorial hypothesis is able to dissect the relative weights of the different factors, and their interactions. Specifically, we

found that founder effects drive the genetic structure of passively dispersed aquatic organisms. We conclude that although selective factors and migration have a role in explaining genetic structure of continental aquatic invertebrates, demographic processes are dominant. By studying which factors are important in what circumstances, our analysis can help understanding relevant differences among the population genetic structure of different species.

Acknowledgements

We thank Guillermo García Franco and José Gargallo Tuzón for their invaluable help with some parts of the code and programming support. We also would like to thank Luc De Meester, Raquel Ortells and Mª José Carmona for helpful comments on previous versions of this manuscript. This work was funded by a grant from the Spanish Ministerio de Ciencia e Innovación (CGL2009-07364) to MS. AG was supported by a National Environment Research Council (NERC) Advanced Fellowship (NE/B501298/1) and JM-P by a fellowship by the Spanish Ministerio de Ciencia y Tecnología (BES2004-5248).

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3

Application of an inexpensive and high-throughput genomic DNA extraction method for the molecular ecology of zooplanktonic diapausing eggs

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Published in Limnology and Oceanography: Methods 2008 **6:**218-222

Abstract

We describe the application of a simple, low-cost, and effective method of DNA extraction (hot sodium hydroxide and Tris, HotSHOT) to the diapausing propagules of continental aquatic invertebrates for its use in PCR amplification. We illustrate the use of the technique in cladocerans, rotifers, anostracans, notostracans and copepod diapausing eggs. We compare the performance of the HotSHOT technique to the currently most widely used method for DNA extraction of zooplankton eggs and individuals, the chelating resin (or Chelex) technique. The HotSHOT technique overcomes several of the problems posed by Chelex and permits easy optimization for its use with 96-well plates for high-throughput DNA extraction and subsequent genetic characterization. We foresee a wide use of this technique in the future from DNA barcoding of diapausing stages to the genetic characterization of the diapausing egg banks of continental aquatic invertebrates.

Introduction

In recent years, an explosive development of high-throughput, relatively cheap, molecular techniques for the scoring of microsatellites, AFLPs, SNPs, and sequencing has taken place. These technological advances have spurred population-level analysis from phylogeography and population structure to barcoding and large-scale multigene sequencing projects, all key areas of ecological and evolutionary research. The development of methods to prepare DNA extractions in a quick, simple, cost-effective and high-throughput way has shown a parallel progress for model organisms including human forensic material (Walsh et al. 1991; Rudbeck & Dissing 1998), mouse (Truett et al. 2000), and *Arabidopsis* and crop plants (Xin et al. 2003), but their wider application for molecular ecology is timely.

Molecular genetic surveys of continental aquatic invertebrates (cladocerans, anostracans, rotifers, copepods, ostracods, etc.) have yielded valuable insights into the evolutionary forces shaping their genetic structure and microevolution (De Meester et al. 2002; Gómez 2005; Ishida & Taylor 2007), and are helping to characterize the cryptic biodiversity of these groups (Gómez et al. 2002; Adamowicz & Purvis 2005). Continental aquatic invertebrates cope with the temporal character and unpredictability of their habitats by producing diapausing stages. Diapausing eggs – which are encysted embryos in arrested state of development, also called cysts or resting eggs – are resistant to a wide range of environmental extremes (Proctor et al. 1967; Carlisle 1968), and are long-lived dormant stages in pond and lake sediments forming diapausing egg banks (Hairston 1996). For many molecular genetic surveys in these organisms, sampling diapausing egg banks in

populations is a useful or even the only way of obtaining population or species level genetic information. Given the ephemeral nature of many aquatic habitats, the year-round availability of diapausing stages – as opposed to often ephemeral planktonic stages – is a great advantage for their use, and when the ponds and lakes are sampled during dry periods, they represent the best sample of the population gene pool (Moorad et al. 1997; Gómez et al. 2000; Ortells et al. 2000). In addition, and conveniently, sediment samples containing living diapausing stages can be maintained in the laboratory for long periods of time. Finally, old diapausing stages from lake and pond sediment cores are a repository of the historical genetic constitution of past populations, and their use for genetic analysis has revealed much valuable information from historical changes in the environment, the dynamics of *Daphnia* communities, adaptive changes in antipredator behavior, alien invasions to the dynamics of co-existence of cryptic species .

Although the importance of sampling diapausing stages was realized a while ago (Carvalho & Wolf 1989; Hairston 1996), the genetic characterization of diapausing egg banks depended on hatchlings obtained in the laboratory to obtain sufficient biomass for genetic analysis, as DNA extractions from such small samples remained a challenge. Ideally, DNA extraction techniques from individual diapausing stages should obtain reliable results from a very small amount of tissue (for example, rotifer diapausing stages are less than 100 µm in length) using as few tube transfers as possible to avoid contamination of samples. A chelating resin method (using Chelex® 100 resin or InstaGene matrix, Bio-Rad) (Walsh et al. 1991), originally developed to extract DNA from forensic material, has been, and remains to this date, the most

widely used method to prepare DNA from diapausing stages and individual zooplankton in rotifers (Gómez & Carvalho 2000; Fontaneto et al. 2007), *Daphnia* (Taylor et al. 1996; Reid et al. 2000), copepods (Edmands 2001; Bohonak et al. 2006), anostracans (Moorad et al. 1997) and ostracods (Yamaguchi 2000). However, various problems become apparent when working with this method. Resin beads inhibit PCR, and therefore, in order to use high-throughput 96-well plates for PCR amplification using multichannel pipettes, centrifugation and transfer of the sample supernatant into fresh tubes or plates is needed after extraction, before preparing PCR reactions, therefore increasing preparation time and cost, and the chances of contamination. In addition, and more importantly, the technique does not produce a stable DNA extract and samples often deteriorate with time and become unusable for PCR (Greenspoon et al. 1998; Hajibabaei et al. 2005) unless buffered (Söller et al. 2000).

Here we describe an optimized alkaline lysis protocol to produce inexpensive, rapid and simple DNA extractions which avoids the above mentioned problems and produces reliable DNA extractions to conduct PCR-based genetic screening (e.g., mitochondrial DNA and microsatellite amplification) to process large samples of diapausing stages of zooplanktonic organisms. The HotSHOT technique (Truett et al. 2000), a modified alkaline lysis method (Sambrook et al. 1989), is a reliable, cheap, and simple DNA extraction technique which can be easily adapted to aquatic invertebrates organisms with diapausing propagules. An additional advantage is that, being a single-tube technique, the likelihood of cross-contamination is reduced. We demonstrate the use of this technique for diapausing stages in zooplankton, and illustrate its

efficiency with cysts or diapausing eggs of cladocerans, rotifers, copepods, anostracans and notostracans, both for fresh and preserved material. In addition, we compare directly the efficiency of the technique with the widely used Chelex resin method.

Materials and Procedures

Biological material

Diapausing eggs were obtained from plankton samples from natural populations. Eggs were extracted from lake sediments using a sucrose-flotation technique (Onbé 1978; Gómez & Carvalho 2000), or taken directly from laboratory cultures (see Table 3.1 for details). We obtained samples of five different groups of aquatic invertebrates: rotifers (*Brachionus plicatilis*), cladocerans (*Daphnia magna*), anostracans (*Artemia salina*), notostracans (*Triops cancriformis*) and copepods (unidentified species). Mature *Daphnia* ephippia were separated from their mothers and diapausing eggs manually decapsulated. The diapausing eggs of these organisms have a range of sizes from under 100 to ca. 400 µm. Some of the diapausing eggs had been preserved in ethanol 100%, some were collected fresh from mature females, and others had been stored dry and in the dark or in sediment samples. A few samples had been kept in the laboratory for several years, either in dormant (live) state or fixed (Table 3.1).

Table 3.1. Details of samples used for Fig. 3.1.

Species	Location	Sampling date	Sample	Preservation
Artemia salina	Cagliari, Sardinia, Italy	May 2004	Plankton	Dry/Ethanol*
Artemia salina	Trapani, Sicily, Italy	May 1985	Plankton	Dry/Ethanol*
Brachionus plicatilis	Laguna de Pétrola, Albacete, Spain	November 2004	Sediment	Live/ salt water**
Daphnia magna	Pearson Park Pond, Hull, United Kingdom	June 2007	Plankton	Live
Daphnia magna	Pearson Park Pond, Hull, United Kingdom	August 2000	Plankton	Ethanol
Triops cancriformis	Königswartha Pond 12, Germany	2005	Culture	Live
Unidentified copepod	Laguna de Fuente de Piedra, Málaga, Spain	June 1999	Sediment	Live

* Samples were obtained as dried resting eggs and transferred to 100% ethanol in 2004.

** Samples were extracted from sediments using a sugar-flotation technique (see text for details) and stored in 60 g/L salt water at 4 ˚C in the dark in 2004.

DNA extraction protocol

Prior to the DNA extraction, fresh and dry preserved diapausing eggs were washed with MilliQ water. Ethanol preserved samples were re-hydrated for two hours in MilliQ water.

To perform the HotSHOT extraction (see Table 3.2 for details), individual diapausing eggs were transferred into a 0.2 mL tube containing 50 µL of alkaline lysis buffer under a stereomicroscope. Once in the buffer, the eggs were crushed against the side of the tube using a sterile pipette tip. Samples were incubated at 95°C for 30 min and stored on ice for 3-4 min. A further 50 µL of neutralizing buffer was added to each tube and the samples were vortexed briefly and spun down. Samples were stored at 4°C to be used directly in PCR reactions, or at –20°C for a long storage period.

For the Chelex extraction, the diapausing eggs were transferred individually to 0.2 mL tubes containing 50 µL of Instagene Matrix (containing 6% Chelex resin, Bio-Rad) and crushed against the side of the tube using a sterile pipette tip. The samples were incubated for 30 min at 60°C and 8 min at 100°C. Finally, the samples were centrifuged at 14000 rpm during 5 min and the supernatant directly used in PCR reaction or stored at -20°C until needed.

Polymerase Chain Reaction (PCR)

To assess the success of the DNA extractions, a fragment of the mitochondrial gene cytochrome *c* oxidase subunit I (COI) (ca. 710 bp) was

Table 3.2. Procedure of the HotSHOT method applied to diapausing stages.

1. Aliquot 50 µL of alkaline lysis buffer (NaOH 25mM, disodium EDTA 0.2mM) into 0.2 mL individual tubes
2. Under a stereomicroscope, transfer individual diapausing eggs to the tubes with a pipette using the minimum amount of liquid to carry the egg (e.g. 2 µL)
3. Using a sterile pipette tip, crush the egg against the side of the tube to release the embryo
4. Incubate for 30 min at 95˚C, and then cool on ice for 3-4 min
5. Add 50 µL neutralising solution (Tris-HCl 40mM, pH 5.0).
6. Vortex the tubes to mix and spin
7. Use 2 µL for PCR or freeze at -20˚C for long-term storage

amplified using the primers LCO1490 and HCO2198 (Folmer et al. 1994) or specific primers for the same mitochondrial region modified for *Artemia* (J. Muñoz, unpublished manuscript). PCR reactions were performed in a total volume of 20 µL using 2 µL of DNA extract, 1x PCR buffer, 0.5 µM of each primer, 1.5-2.0 mM $MgCl_2$, 0.2 mM dNTPs and 0.4-0.6 U *Taq* DNA polymerase (Bioline). The thermal profile consisted of a 3 min initial cycle at 94°C, followed by 35 cycles of 94°C for 45 s, 45-55°C for 1 min and 72°C for 1 min, with a final extension of 72°C for 5 min. Five µL of the PCR product were separated by electrophoresis in 0.5x SB buffer (Brody & Kern 2004) in a 1.5% agarose gel. Gels were stained with ethidium bromide and visualized under UV in a transilluminator.

Assessment

To test the performance of the HotSHOT protocol in diapausing stages we assessed the technique in different groups of zooplankton and compared the results with the Chelex technique. We also tested the success of DNA extraction on old ethanol preserved samples and in diapausing stages of different ages. In addition, we carried out HotSHOT DNA extractions using a range of different total volumes (20-140 µL) and quantified the DNA in the extracts. For this, we used diapausing eggs from rotifers and anostracans (the smallest and the largest diapausing eggs used in this study, respectively). Soluble DNA concentration in the HotSHOT extractions was quantified by measuring the absorbance at 260 nm using a GeneQuant II spectrophotometer (Pharmacia Biotech Co.). No comparison could be done with the Chelex extractions due to unreliable quantification of these extractions with spectrophotometers (JM, JM-P, personal observations).

DNA concentrations obtained with the HotSHOT method in *Artemia* cysts ranged between 25 and 5 ng/µL for the smallest (20 µL) and largest (140 µL) total extract volume, respectively, while lower concentrations were obtained for the smaller *Brachionus* diapausing eggs (maximum 5 ng/µL). Although in *Brachionus* the amount of DNA was not high, it was sufficient to perform PCR amplifications with the same success than for *Artemia* (Fig. 3.1). Both HotSHOT and Chelex methods resulted in reliable amplifications of diapausing stages for

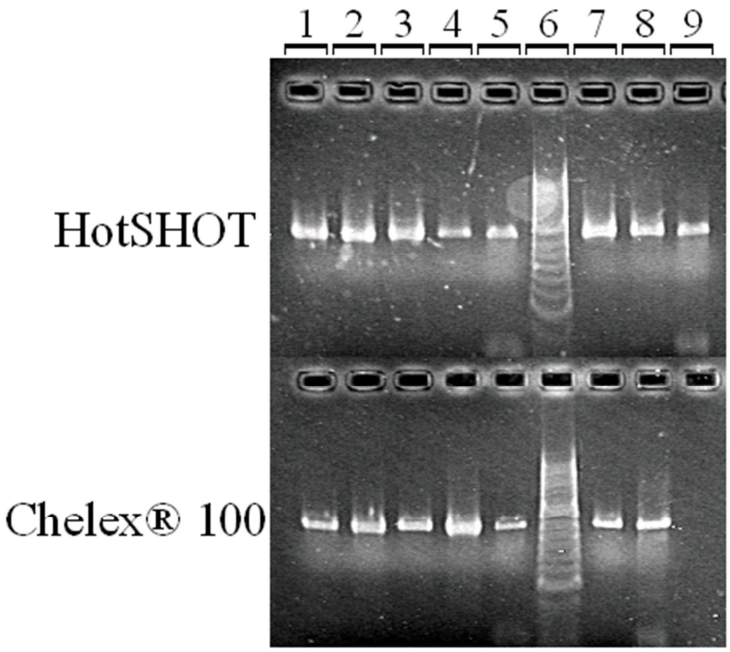

Figure 3.1. PCR products of diapausing eggs DNA extractions using HotSHOT and Chelex methods. Samples: 1 = *Brachionus* (rotifer); 2 = unidentified copepod; 3 = *Daphnia* (cladoceran); 4 = *Triops* (notostracan); 5 = *Artemia* (anostracan); 6 = 100 bp ladder; 7 = *Daphnia* from 2000; 8 = *Artemia* from 1985. More details of sample sources and their preservation history are described in Table 3.1.

freshly prepared DNA extractions in fresh and old preserved material, including dry (live) and ethanol-preserved samples, with the HotSHOT method providing slightly stronger bands (see Fig. 3.1; Table 3.1).

The success rate for individual cysts DNA extractions with the HotSHOT method (measured indirectly as successful PCR amplifications) was on the order of 97% for all taxonomic groups, and in tests in rotifers it increased when only healthy looking eggs were used in the extraction (JM-P, personal observation).

In addition, we successfully tested the HotSHOT technique for various adult zooplanktonic samples (abdomen *Artemia*, adult *Brachionus*, whole/half copepod, whole/antenna *Daphnia*).

In contrast to Chelex preparations, HotSHOT produced long-lasting DNA extractions, as 3 year-old DNA rotifer preparations using this method and stored at -20°C gave clear amplification products for COI and even for a larger fragment of mtDNA genome (1300 bp). These old extracts were also used to reliably score seven microsatellite loci in a large number of samples with no allelic dropout effect (JM-P, unpublished results), indicating that the quality and quantity of the DNA obtained with this method is adequate for PCR-based methods.

Discussion

We have shown that HotSHOT DNA extractions provide consistent and reliable PCR amplification in diapausing eggs in a range of invertebrate taxa. HotSHOT yielded as good or better results as the widely used Chelex technique in extracting DNA from single diapausing eggs, both for fresh and preserved material (Fig. 3.1). Although the rate of successful amplifications from both techniques is comparable, HotSHOT presents superior features such as being a single tube method with increases the efficiency and eases the implementation of high-throughput processing of diapausing egg samples, and at the same time minimises the risk of cross-sample contamination, and success of long-term preservation of DNA samples. The low failure rate in PCR amplifications found in our work is likely to be due to the deteriorated stage of the embryos rather than the age or mode of preservation of the diapausing eggs.

HotSHOT extraction is a highly suitable technique for PCR-based methods such as microsatellite genotyping, restriction fragment length polymorphism (RFLP) analysis, single stranded conformation polymorphism (SSCP) analysis, and sequencing. Its application to other methods requiring high DNA concentrations or high molecular weight DNA could be restricted (Truett et al. 2000).

In summary, the HotSHOT method is a rapid, inexpensive, high performance technique for PCR-quality DNA extractions from diapausing eggs, which avoids cross-contamination and, as larger volumes are used, it allows for more PCR reactions per sample. In addition, HotSHOT DNA extractions are stable for at least several years. All these advantages make HotSHOT a valuable method to use in diapausing eggs and a superior technique to the widely used Chelex method.

We have provided a protocol to perform high-throughput DNA extraction widely applicable for high-throughput PCR-based genetic screening analyses of virtually all zooplanktonic organisms with diapausing stages in their life cycle. This technique will facilitate the application of large-scale screening molecular techniques in several areas of molecular ecology, from population genetics to barcoding studies.

Acknowledgements

We thank D. Lunt for suggesting the use of HotSHOT, for technical help and for providing old *Daphnia* samples. We are grateful to T. Zierold for providing *Triops* samples and F. Amat for providing *Artemia* cysts. Rob Hammond is kindly thanked for his constructive comments on an earlier version of this manuscript. We also thank the valuable comments and

suggestions by two anonymous reviewers. This study was partly funded by a NERC Advanced Fellowship to AG. JM was supported in his stay in Hull by the Spanish Ministerio de Educación y Ciencia project (project CGL2006-05085/BOS) to A.J. Green, C. Rico, F. Hontoria and J. Figuerola. The Spanish Ministerio de Educación y Ciencia supported JMP during his stay in the University of Hull.

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4

Long-term coexistence of rotifer cryptic species

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Published in PLoS One 2011 6:e21530

Abstract

Despite their high morphological similarity, cryptic species often coexist in aquatic habitats presenting a challenge in the framework of niche differentiation theory and coexistence mechanisms. Here we use a rotifer species complex inhabiting highly unpredictable and fluctuating salt lakes to gain insights into the mechanisms involved in stable coexistence in cryptic species. We combined molecular barcoding surveys of planktonic populations and paleogenetic analysis of diapausing eggs to reconstruct the current and historical coexistence dynamics of two highly morphologically similar rotifer species, *B. plicatilis* and *B. manjavacas*. In addition, we carried out laboratory experiments using clones isolated from eight lakes where both species coexist to explore their clonal growth responses to salinity, a challenging, highly variable and unpredictable condition in Mediterranean salt lakes. We show that both species have co-occurred in a stable way in one lake, with population fluctuations in which no species was permanently excluded. The seasonal occurrence patterns of the plankton in two lakes agree with laboratory experiments showing that both species differ in their optimal salinity. These results suggest that stable species coexistence is mediated by differential responses to salinity and its fluctuating regime. We discuss the role of fluctuating salinity and a persistent diapausing egg banks as a mechanism for species coexistence in accordance with the 'storage effect'.

Introduction

The last two decades have witnessed an increasing awareness of the widespread phenomenon of cryptic species (Bickford et al. 2007). These morphologically undistinguishable taxa have been described in almost all phyla, although they appear to be especially abundant among aquatic organisms (Knowlton 1993; Gómez et al. 2002; Pfenninger & Schwenk 2007). The taxonomic uncertainty introduced by cryptic species has had a profound impact on various ecological fields: delaying the detection of biological invasions (Geller 1999; Mergeay et al. 2005), slowing down apparent evolutionary rates (Alizon et al. 2008), and confounding the ecological niche of biological species (Molbo et al. 2003; Blair et al. 2005; Smith et al. 2006). Additionally, cryptic species pose a major challenge to other aspect of ecological theory. As these species are often sympatric (e.g., Molbo et al. 2003; Leibold & McPeek 2006), they are so similar in their morphology and physiology, a high degree of ecological similarity is expected (Braune et al. 2008). Therefore, their existence in sympatry poses a challenge regarding niche differentiation theory and the mechanisms that facilitate species coexistence.

Many studies have addressed the mechanisms responsible for coexistence in terrestrial (e.g., Smith et al. 2006; Nicholls & Racey 2006) and marine sympatric cryptic species (e.g., Knowlton & Jackson 1994). In contrast, awareness of continental aquatic cryptic species is more recent and few studies deal with the ecological mechanisms mediating their coexistence (Ortells et al. 2003; Ciros-Pérez et al. 2004; Wellborn & Cothran 2007). The salt lake rotifer species *Brachionus plicatilis* and *B. manjavacas* share a virtually identical morphology. They belong to the *Brachionus plicatilis* species complex (Gómez et al. 2002; Suatoni et al.

2006) and, their species status has been supported by genetic and reproductive isolation analysis (Gómez & Snell 1996; Suatoni et al. 2006). Given their morphological similarity, the most reliable way to discriminate both species is molecular barcoding (Campillo et al. 2005; Fontaneto et al. 2007). *B. plicatilis* and *B. manjavacas* often co-occur in salt lakes in the Iberian Peninsula, where they are thought to have been present for at least several Pleistocene glaciations (Gómez et al. 2000; Gómez et al. 2007). Since *Brachionus* species reach very high population densities in short times in the field (for example, densities of thousands of individuals/L are commonly recorded for *B. plicatilis* in the Iberian Peninsula short after hatching (Carmona et al. 1995; Carmona et al. 2009), these species are likely to experience resource limitation in nature (Cordova et al. 2001). Laboratory experiments have shown competition between *B. plicatilis*, *B. rotundiformis* and *B. ibericus*, which belong to the same cryptic species complex but, are morphologically different (Ciros-Pérez et al. 2001).

Six species of the *Brachionus plicatilis* cryptic species complex have been found living sympatrically in the Iberian Peninsula in inland salt lakes and coastal lagoons (Gómez et al. 2000; Ortells et al. 2000; Lapesa et al. 2004). Until now, salinity and temperature (Gómez et al. 1997), resource partitioning and differential vulnerability to predators (Ciros-Pérez et al. 2002; Ciros-Pérez et al. 2004) have explained competitive outcomes for three of the species of the complex with the greater morphological differentiation (*B. plicatilis, B. ibericus* and *B. rotundiformis*). However, and given their morphological similarity, such factors appear unlikely to mediate *B. plicatilis* and *B. manjavacas* coexistence. As *B. plicatilis* is an osmoregulator, with its population

growth rate negatively affected by increasing salinity (Lowe et al. 2005), and the lakes where these species co-occur in the Iberian Peninsula have a highly variable salinity regime (Comín et al. 1992; Rodriguez-Puebla et al. 1998), it could be expected that salinity fluctuations affect the competition of both species.

Here, we explore the role of salinity fluctuations on the niche differentiation of *B. plicatilis* and *B. manjavacas*. We present data on (1) annual plankton population dynamics in two lakes, (2) historical population dynamics screening diapausing eggs from the sediment cores of a salt lake using a paleogenetic approach, and (3) laboratory growth rates at six salinities representative of the range experienced by both species in the wild. We provide evidence that both species co-occur in the water column and that salinity is a factor for niche differentiation. We propose that the fluctuating salinity regime can act as a stabilizing niche difference mediating their coexistence.

Methods

Annual population dynamics

The seasonal dynamics of *B. plicatilis* and *B. manjavacas* was studied in two Iberian salt lakes, Salobrejo and Pétrola (see Table 4.1), from October 2004 to April 2006. The lakes were visited every 2-3 weeks, and zooplankton samples taken whenever water was present. In each sampling event, salinity and conductivity were recorded using a WTW LF320 conductivity meter (Wissenschaftlich – Technische Werkstätten GmbH, Willheim, Germany). Quantitative samples were obtained to estimate rotifer density by collecting 6-30 L of lake water using a Van Dorn horizontal sampling bottle (6 L) and filtering them through a 30 µm

Nytal mesh. When the lakes were too shallow to allow the sampling bottle to be used, samples were obtained by filtering lake water collected by repeatedly sweeping a 1 L plastic container until the sampling volume was reached. Zooplankton samples were fixed in situ with formaldehyde. Rotifer density in the quantitative samples was determined using a CK2 Olympus inverted microscope at 40x-100x magnification. All rotifers identified morphologically as belonging to the *B. plicatilis/B. manjavaca*s morphotype were counted.

Samples to estimate the relative abundances of *B. plicatilis* and *B. manjavacas* using molecular barcoding were collected using a 30 µm mesh plankton net, preserved in 70% ethanol and stored at 4°C in the dark until used. From each sample, up to 50 individuals with *B. plicatilis/B. manjavacas* morphology were randomly picked for species identification using PCR-RFLP of a mitochondrial gene fragment (cytochrome *c* oxidase, cox1, COI) following Campillo et al. (2005). In those samples with few individuals, all individuals were barcoded. This way, the relative abundance of each species in the lake was estimated.

Long-term population dynamics

Four sediment cores of 57 mm diameter and 50 cm length were collected from Pétrola lake during December 2005-October 2006 (Fig. 1) using a piston core sampler (Eijkelkamp, Agrisearch Equipment). Cores were sliced into 0.5 cm width sections until a maximum depth of 10 cm, where diapausing egg bank had previously been shown to be extinct in this lake (García-Roger et al. 2006). Each section was weighted and then stored in a Petri dish at 4°C in the dark until it was processed.

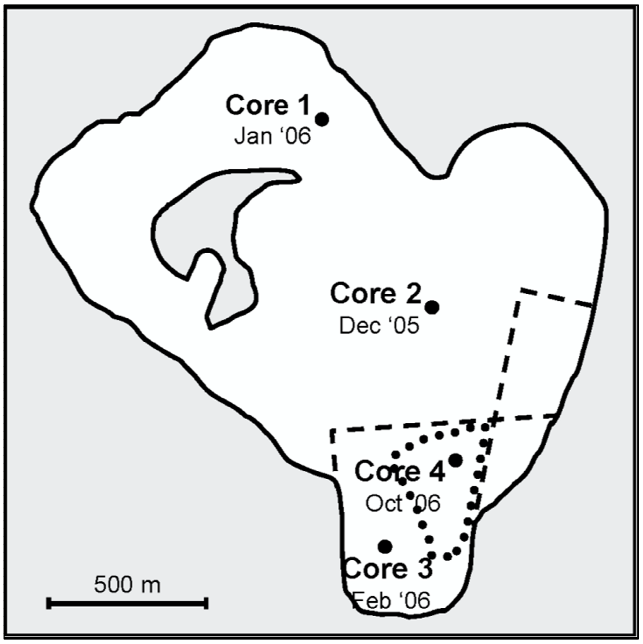

Figure 4.1. Location and date of sediment core sampling in Pétrola lake. Dashed lines show dikes of a disused salt evaporation plant that divide the lake into compartments. The more southern compartment receives the inflow of freshwater from a nearby sewage treatment plant. Dotted lines show the area with remaining water during a summer drought in August 2007 (from an aerial photograph in Google Earth 4.7). Note that Core 4 was obtained in the deeper point of the lake.

Diapausing eggs were extracted from the sediment samples following García-Roger et al. (2006), and, to establish whether cores were age-structured, each egg was assigned a degree of deterioration (i.e., on the proportion of egg occupied by the embryo) (García-Roger et al. 2005). Eggs with 50% or more of volume filled by the embryo were considered suitable for genetic analyses and were isolated and kept at 60 g/L, 4°C in the dark to prevent hatching until DNA extractions were performed.

Diapausing egg isolation was carried out in weighed subsamples of each sediment section until 30 eggs were isolated, until 2.5 g of sediment was processed without finding any eggs, or until 4 g of sediment was processed without finding any eggs suitable for genetic analyses. In Core 3 and 4, from depths 6.0-cm downwards we used a sucrose flotation technique (Gómez & Carvalho 2000) that allows to process larger volumes of samples, so all the sediment on each section could be processed. Due to the lower efficiency of this technique, extractions were repeated three times to ensure that all eggs were isolated. Diapausing egg densities were computed for each section.

For species identification in sediment cores (barcoding), we used PCR-Single Stranded Conformation Polymorphism (SSCP) analysis (Orita et al. 1989) of a 378 bp fragment of the mitochondrial gene 16S rRNA. SSCP allows processing a large number of samples in a more cost-effective way than PCR-RFLP. An average of 23 diapausing eggs was analyzed per section. DNA extractions were performed using a modified alkaline lysis protocol (Montero-Pau et al. 2008) in a final volume of 40 µL, and a fragment of the 16S rRNA gene was amplified using rotifer specific primers (Papakostas et al. 2005). PCR reactions were performed in a final volume of 10 µL with 2 µL DNA, 1x $(NH_4)_2SO_4$ buffer, 0.2 mmol/L of each deoxy nucleotide, 2.5 pmol of each primer and 0.15 U of *Taq* polymerase (Biotools), using the following PCR cycling conditions: 2 min at 94°C, 40 cycles of 30 s at 94°C, 30 s at 60°C and 40 s at 72°C and final extension of 3 min at 72°C. SSCP analysis was performed by mixing 2.5 µL of PCR product with 7.5 µL of denaturing buffer (95% formamide, 10 mmol/L sodium hydroxide, 0.25% bromophenol blue and 0.25%

xylene cyanol) and incubating this mixture for 5 min at 95°C and transferring it immediately to a 4°C bath. Thirty-two denatured samples were loaded in a gel 0.5x MDE® (Cambrex Bio Science Rockland), 0.6x TBE (Merck), 10% glycerol, 0.05% TEMED and 0.1% ammonium persulfate) and run at 40 V and 4°C in 0.6x TBE (Merck) for 16 h. Gels were stained with 1X SYBR® Gold (Molecular Probes, Invitrogen). Each sample was assigned to an electromorph pattern by eye and one sample from each electromorph per gel was sequenced. An additional 10% of the samples on each gel were sequenced as quality control.

PCR amplifications for the samples selected for sequencing were repeated under the same conditions described above but in a final volume of 50 µl. Products were purified using High Pure PCR Product Purification Kit (Roche) and sequenced using ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Biosystems) in both directions and run in an ABI 3700 sequencer (Perkin-Elmer Biosystems). Chromatograms were checked and edited using CodonCode Aligner v.1.6. (CodonCode Corporation).

We used a generalized linear model (McCullagh & Nelder 1989) with a binomial distribution and a logit link function to test the effect of sampling point (core) and depth on the relative frequency of *B. plicatilis* and *B. manjavacas*. Tests were performed with R v.2.7.1 (R Development Core Team 2006).

Effect of salinity on growth rate

Clonal growth rates of six *B. plicatilis* and six *B. manjavacas* clones were estimated in the laboratory at six salinities (5, 10, 20, 30, 40 and 45 g/L).

Clones were obtained by hatching diapausing eggs isolated from sediment samples of eight Iberian salt lakes covering a wide range of salinity conditions (see Table 4.1). Experimental salinities were chosen according to this range and data in the literature, suggesting dramatic growth rate decrease, even negative growth, in the range 40-50 g/L for different clones of these species (Miracle & Serra 1989). Clones of both species could be obtained for four lakes (Manjavacas, Pétrola, Salobrejo and Hondo Sur) (Table 4.1). Clones were identified at species level by PCR-RFLP of a COI fragment (Campillo et al. 2005). All clones except one from Charca Universidad de Cádiz and another from Capacete were obtained and identified as part of another study (Campillo et al. 2009).

Clonal growth rate was estimated in 216 cultures (2 species x 6 clones x 6 salinities x 3 replicates). Pre-experimental cultures were started by placing approximately 650 females of each rotifer clone in 2 L diluted artificial seawater (Instant Ocean®, Aquarium Systems) at 25 g/L salinity (i.e., the intermediate experimental salinity), and 25°C in the dark. Rotifers were fed inert cells of the microalgae *Tetraselmis suecica* (see below). The experimental cultures were set up by placing 12 egg-bearing females from each pre-experimental culture into a 6-cm diameter Petri dish with 50 ml diluted seawater at the experimental salinity and adding 100 µL of a suspension of 10^5 cells/mL inert cells of *T. suecica*. Cultures were kept in the dark at 25°C. Female rotifers in each experimental culture were counted at days 2 and 4, and exponential clonal growth rates (r) were estimated as $r = (\ln N_4 - \ln N_2) / 2$, where N_2 and N_4 are the number of female rotifers in the second and fourth day, respectively.

To produce the inert microalgal cells, two 5 L cultures of *T. suecica* were grown in diluted seawater fertilized with f/2 medium (Guillard & Ryther 1962), at 25 g/L salinity, 25°C and 35 µmol quanta m^{-2} s^{-1} (constant illumination). After 10-12 days, the cultures were concentrated by centrifugation (12.6 g for 5 min), and thoroughly mixed. 20 mL aliquots of 10 x 10^6 cells/mL for the pre-experimental cultures and 1.2 mL aliquots of 50 x 10^6 cells/mL for the experimental cultures were prepared and kept at -80°C until needed. This procedure allows us to control for food quality variation since a single algae stock is used throughout the experiment.

A three-way, fixed effect ANOVA was performed to test for the effect of salinity on clonal growth rates for each species. Kolmogorov-Smirnov test for normality and Brown-Forsythe (Brown & Forsythe 1974) test of homogeneity of variances were used to check the fit of data to ANOVA assumptions. Additionally, the response of each species to salinity was explored by fitting a quadratic function, where the independent variable was the *r* values averaged over replicates and clones, and the independent variable was salinity. Fitting was computed using least-squares regression. A quadratic function was selected because it is a simple one able to detect an intermediate maximum for the independent variable, accordingly with the expectation for the response to an environmental condition. All statistical tests were carried out using SSPS v 12.0.1 (SPSS Inc., Chicago, IL).

All planktonic and sediment samples used in this study were obtained under the permission of the corresponding environmental agencies (Junta de Andalucía, Junta de Castilla y la Mancha, Gobierno de Aragón and Generalitat Valenciana).

Table 4.1. Lakes where rotifer clones were isolated, with geographic location and their recorded salinity range. Salinity at which the maximum daily growth rate (r_{max}) is achieved in each lake for *B. plicatilis* and *B. manjavacas*, as estimated in the laboratory, is shown. Average optimal salinity, calculated as the average of the salinity at which each clone reaches their maximum growth rate, is also provided.

			B. plicatilis		**B. *manjavacas***	
Lake	**Geographic location**	**Salinity range (g/L)**	**Salinity (g/L)**	**r_{max}-value**	**Salinity (g/L)**	**r_{max}-value**
Hondo Sur	38° 10' 49'' N 0° 45' 19'' O	8 – 18 [1,2]	10	0.417	20	0.383
Manjavacas	39° 25' 00' N 2° 51' 49'' O	5 – 79 [2,3]	10	0.313	10	0.336
Salobrejo	38° 54' 50' N 1° 28' 11'' O	8 – 65 [1,4]	5	0.356	10	0.336

Pétrola	38° 50' 26' N 1° 33' 56'' O	10 – 280 [4,6]	10	0.267	30	0.313
Charca Universidad de Cádiz	36° 32' 02'' N 6° 12' 38'' O	39 [2]; 49 [4]	5	0.466	-	-
Capacete	37° 01' 22'' N 4° 49' 36'' O	3 – 6 [5]	-	-	5-30*	0.267
Camino de Villafranca	39° 21' 45''N 3° 15' 17'' O	6 – 108 [6,7]	-	-	10	0.363
Average optimal salinity			**7.5 g/L**		**17.5 g/L**	

*_B. manjavacas_ grew with the same r_{max} from 5 to 30 g/L.

1, Lapesa (2004); 2, Ortells et al. (2000); 3, García-Ferrer et al. (2003); 4, this study; 5, Rodríguez-Rodríguez & Moral Martos (2005); 6, Boronat et al. (2001); 7, Alonso (1990).

Results

Annual population dynamics

During the study period, Pétrola and Salobrejo lakes showed wide salinity fluctuation and both reached hypersaline conditions, although, salinity variation was more pronounced in Pétrola, which also reached much higher values than Salobrejo (Fig. 4.2). *B. plicatilis* and *B. manjavacas* showed very low prevalence and abundance in both lakes (Fig. 4.2) and populations appeared to grow opportunistically, with population peaks occurring during narrow time windows of lower salinity. *B. plicatilis* was detected in 14 and *B. manjavacas* in 10 out of 55 samples. Both species were found together in eight samples, although one of them was always much more abundant than the other. A contrasting pattern was found in the abundance of both species between the two lakes; in Salobrejo, *B. plicatilis* was the most abundant species, while *B. manjavacas* was the abundant species in Pétrola. In Salobrejo, *B. plicatilis* was detected when salinity decreased to around 25 g/L (from November 2004 to March 2005). In Pétrola, *B. manjavacas* was present in the water column only when salinity decreased to 45 g/L during a very short period of time (from September to October 2005). These results suggest that *B. plicatilis*, if compared to *B. manjavacas*, has a temporal distribution associated to low-range salinity periods in these lakes.

Other rotifer species (*Brachionus quadridentatus*, *Lecane* sp., *Notholca* sp. and *Keratella* sp.), a species of cladoceran and two species of copepods were also present in the water column in both lakes. Maximum densities of these species were lower than those of *B.*

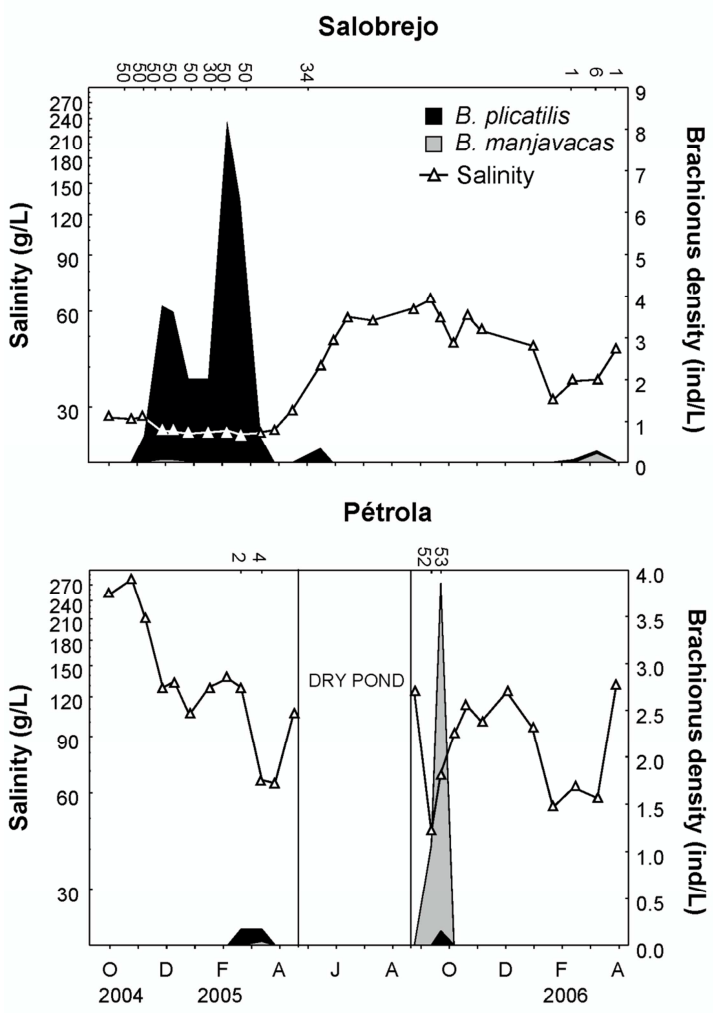

Figure 4.2. Salinity variation and population densities of *B. plicatilis* and *B. manjavacas* from October 2004 to April 2006 in Salobrejo and Pétrola lakes. Density estimates are based on the relative frequencies of the two species analyzed by RFLP. Numbers on top are the number of individuals analyzed. Note that population density scale is different in each graph.

plicatilis and *B. manjavacas*; they ranged from 0.08 to 2.63 individuals/L in Pétrola lake and from 0.03 to 5.18 individuals/L in Salobrejo lake. The only exception was one of the copepod species in Salobrejo lake that reached a maximum density of 198 individuals/L.

Long term population dynamics

A total of 9244 diapausing eggs belonging to the *B. plicatilis* species complex were isolated from the four sediment cores and classified according to their degree of deterioration. Total diapausing egg bank density (i.e., eggs in any deterioration state and empty egg shells) varied from 31.97 to 73.12 eggs cm^{-3} for the upper first cm and from 7.93 to 23.47 eggs cm^{-3} for the upper ten cm. Viable eggs – i.e., those eggs with more than 75 % of the space occupied by the embryo, which make up most of the diapausing eggs that contribute to the hatchings (García-Roger et al. 2005) – are 46.0-79.8% of the eggs in the upper 1-cm layer. Cores 1 and 2 were explored until a maximum depth of 7 cm where the egg bank was considered to be extinct, while densities in Cores 3 and 4 remained of about 5 eggs cm^{-3} in the deepest section analyzed (10.0-10.5 cm).

Both total diapausing egg and viable egg densities showed a clear negative relationship with depth in all four cores, although Cores 3 and 4 showed a subsurface peak of density at around 2.25 cm and 7.25 cm respectively (data not shown). In consequence, the diapausing egg bank showed a pattern of increased deterioration with depth. Both results suggest an age-structured egg bank. Although no data are available for Pétrola, sedimentation rates in other similar inland salt lakes of Eastern Iberian are about 0.05-0.35 cm/year (García-Roger et al. 2006). Using

this range of sedimentation rates, we estimated an approximate maximum age of 28.5 to 200 years for the first 10 cm. In addition, as the four cores were taken in different sites in the lake basin (see Fig. 4.1) they are likely to be affected by different hydrological and salinity regimes and thus, their sedimentation rates may differ. For example, Cores 3 and 4 were taken in the south side of the lake, separated from the north by a dike of a disused salt evaporation plant, and with a freshwater inflow from a nearby village sewage treatment plant; consequently, its salinity and hydrological regime differ from the north side.

A total of 1167 diapausing eggs were considered suitable for genetic analysis and were analyzed by SSCP. Of these, 255 were sequenced (GeneBank accesion numbers JN035646-JN035900). Six different SSCP electromorphs corresponding to six 16S rRNA haplotypes were found, three of them belonging to *B. plicatilis*, two to *B. manjavacas* and one to *B.* sp. 'Almenara' (a species of the *B. plicatilis* complex not formally described yet). All haplotypes found in the subsample of randomly sequenced diapausing eggs corresponded with those predicted by the SSCP analysis. There was a clear numerical dominance of *B. manjavacas* over *B. plicatilis* in the four sediment cores (Fig. 4.3), although this situation was reversed in some sediment depths. Despite *B. plicatilis* being rarer, it was detected consistently, being only absent in 5 samples out of 49, three of them with low sample size. There is no observable trend towards the exclusion of any of the species at any given depth. The generalized linear model comparing the relative frequency of both species showed significant differences among cores ($p < 0.001$), depths ($p < 0.001$), and an interaction effect depth x core ($p <$

0.001). Deviations accounting for the interaction effect show that *B. plicatilis* is more frequent than expected in the southern side of the lake (Cores 3 and 4), with lower salinity, while the opposite is found in the northern side (Cores 1 and 2). Diapausing eggs of *B.* sp 'Almenara', appeared twice in Core 3 at depths 1.25 and 2.25 cm, reaching an abundance of 5.6% and 11.1% respectively, and coinciding with an increase in the abundance in *B. plicatilis* in the same core.

Differences in the variance of the logarithm of the recruitment are informative on competition dynamics (Chesson 2003) (see below for further details). Therefore, we computed the variance of the ln of the total diapausing egg density for each core, assuming an exponential loss of eggs in the sediment for each slice, and we found that *B. plicatilis* showed a higher variance of the estimated recruitment than *B. manjavacas,* with an average difference for the four cores of 1.47 ± 0.45. Results for the different cores are: Core 1, 1.68 vs. 0.30 ($F_{3,3}$= 0.178, p-value = 0.190); Core 2, 1.03 vs. 0.17 ($F_{5,5}$= 0.112, p-value = 0.032); Core 3, 1.66 vs. 0.06 ($F_{18,18}$= 0.034, p-value < 0.001) and Core 4, 1.99 vs. 0.01 ($F_{19,19}$= 0.006, p-value < 0.001). Note that when density for any species was zero, it was replaced by the minimum density recorded in all the cores (0.064 eggs cm^{-3}), as a conservative approach. Similar qualitative results were obtained when no correction for egg loss in the sediment was used or different kinds of eggs were considered (i.e., with/without empty eggs; data not shown).

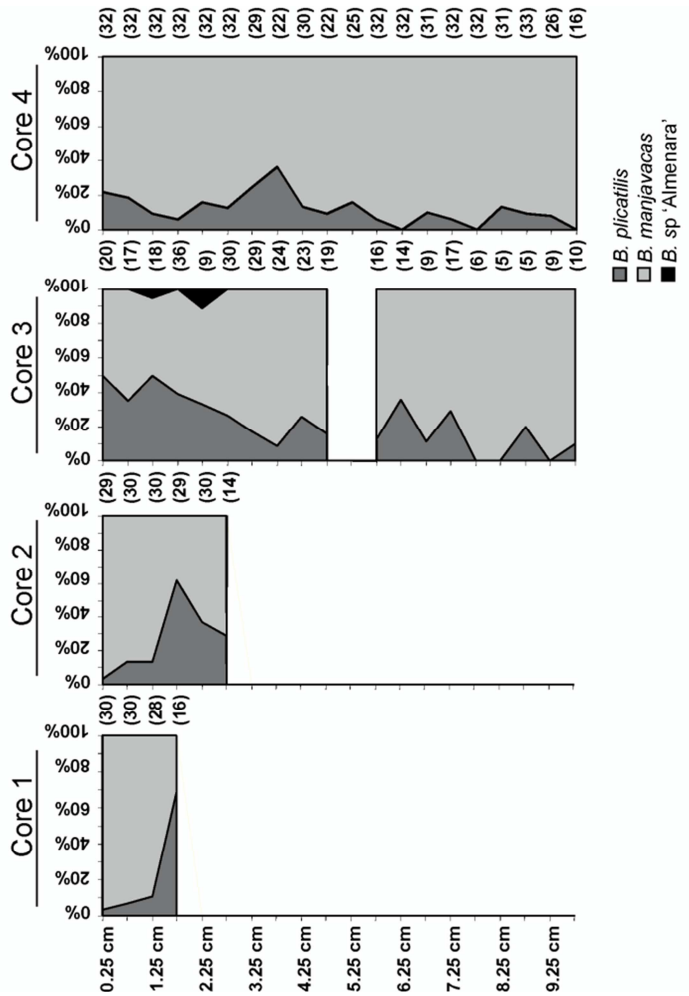

Figure 4.3. Relative abundance along the four cores of *B. plicatilis, B. manjavacas* and *B.* sp 'Almenara'. Number in brackets are sample sizes of diapusing eggs. Section 5.25 cm of Core 3 could not be analyzed due to failed DNA extractions. Dashed lines mark 50% of abundance.

Effect of salinity on growth rate

The analysis of variance revealed substantial variation in growth rates in response to salinity (Table 4.2). *B. manjavacas* tended to have higher growth rates than *B. plicatilis*, growth rates averaged over clones and salinities being 0.216 and 0.191 $days^{-1}$ respectively. This might reflect the effect of a pre-adaptation to the conditions in our experiments (e.g., temperature, food conditions, etc.). All 12 clones displayed positive mean growth rates at all but the highest salinity tested (45 g/L). The highly significant interaction between species and salinity (Table 4.2) indicates that both species differed in their response to salinity. Depending on the clone, *B. plicatilis* had their highest *r* at 5 or 10 g/L, while the ones for *B*. *manjavacas* were in the range 10-30 g/L. Within-

Table 4.2. ANOVA results of the effects of lake, species and salinity on the clonal growth rate (*r*).

Source of variation	**df**	***F***	**p-level**
Lake	7	9.121	<0.001
Species	1	38.933	<0.001
Salinity	5	142.448	<0.001
Lake x Species	3	1.926	0.128
Lake x Salinity	35	11.540	<0.001
Species x Salinity	5	4.440	0.001
Lake x Species x Salinity	15	4.293	<0.001
Error	144		

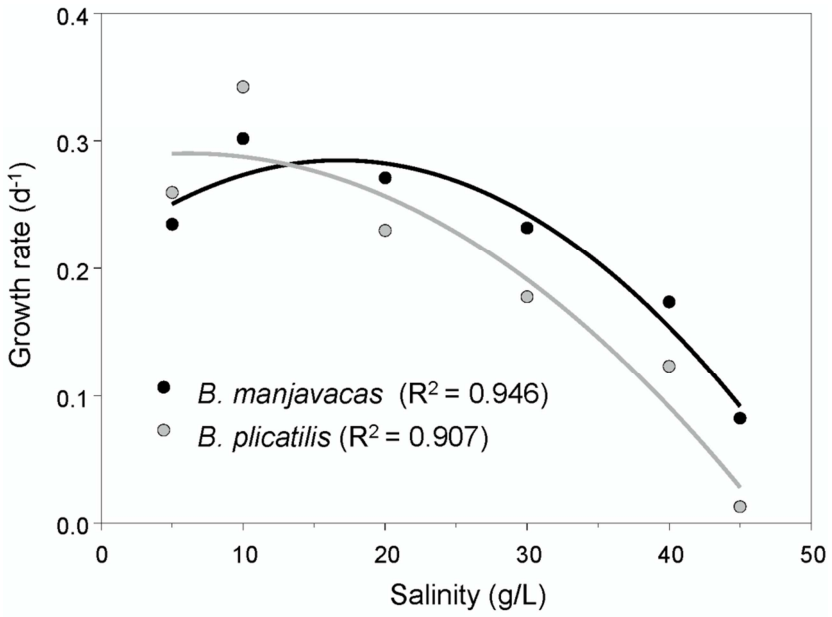

Figure 4.4. Relationship between mean *r* value, after averaging over clones and replicates, of *B. plicatilis* and *B. manjavacas*, and salinity. Curves are least-squares quadratic functions. Determination coefficients (R^2) are shown.

species variation in optimal salinity, as assessed by the maximum *r*, was wider in *B. manjavacas* than in *B. plicatilis* (Table 4.1). Values of *r* averaged over clones and replicates, and then fitted to quadratic functions point out the overall trend in the response of these species to salinity (Fig. 4.4). *B. manjavacas* had higher mean *r* than *B. plicatilis* at 20-45 g/L salinity, while the opposite was observed at 5-10 g/L. Moreover, at 45 g/L, three *B. plicatilis* clones (from Manjavacas, Pétrola and Universidad de Cádiz lakes) showed negative growth rates, but only one of *B*. *manjavacas* (from Camino de Villafranca lake) did it.

Discussion

We have shown that *B. plicatilis* and *B. manjavacas* have co-occurred in a stable way in a lake, with long-term population fluctuations in which no species was permanently excluded. Both species have differential growth rates in response to salinity, with *B, plicatilis* growing better at lower salinities than *B. manjavacas.* In addition, our field study reports for the first time quantitative evidence for the co-occurrence of *B. plicatilis* and *B. manjavacas* in the water column. Since *B. plicatilis* and *B. manjavacas* have co-occurred in the Iberian Peninsula from the Pleistocene, altogether these data suggest that the coexistence of these two morphologically identical species in the area is persistent, and not due to a lasting, but transient random walk towards the extinction of one of the species.

Populations of both species were found in the periods of lower salinity in both lakes, being *B. manjavacas* more abundant in Pétrola, the lake with a higher salinity, while the opposite pattern was found in Salobrejo, which had lower salinity. The sporadic occurrence and low population densities achieved were probably due to the extremely high salinity reached as a consequence of the drought during the sampling period. Rainfall during 2005 in the area is amongst the lowest since 1940 (data from Spanish Agencia Estatal de Meteorología). In fact, *B*. *plicatilis* and *B. manjavacas* were the most abundant species in the zooplankton during the studied period, only exceeded by a species of copepod in Salobrejo. In wetter years, individuals with *B. plicatilis/B. manjavacas* morphotype have been found in Salobrejo and Pétrola at much higher population densities (187 and 68 individuals/L) (Lapesa 2004), which makes competition a feasible scenario. Nevertheless, our data illustrates

that even a short time window of low salinity may offer environmental conditions for opportunistic growth of these species. We do not find evidence for seasonal succession, although we cannot rule it out.

Our results suggest that subtle differential responses to salinity play a role in niche differentiation of these two species. In other species of the *B. plicatilis* species complex, salinity is associated to their differential spatial and temporal distribution (Gómez et al. 1995; Ortells et al. 2003). In particular, *B. manjavacas* tends to occur in hypersaline lakes (Gómez et al. 2007). In agreement with this, our results show that there is a consistent trend for *B. manjavacas* to occur at higher salinities than *B. plicatilis* in the field. It supports our laboratory results showing an average higher salinity optimum for *B. manjavacas* than for *B. plicatilis* clones. As salinity tolerance ranges overlap, this factor will not be directly limiting species occurrence, but affecting their relative fitness, which has implications for competition. The role of salinity as a challenging factor for these species is also supported by physiological data. *B. plicatilis*, and most likely *B. manjavacas,* is an osmoregulator (Lowe et al. 2005); i.e. the higher the salinity, the more resources need to be allocated to maintain the internal osmotic pressure.

In absence of long-term limnological studies, paleolimnological analysis can provide a proxy for the long-term coexistence dynamics of species in a lake. Our combination of paleolimnology and paleogenetic analysis of Pétrola lake sediment cores, which have signatures of being age-structured, supports the absence of a replacement trend between *B. plicatilis* and *B. manjavacas* for at least several decades and possibly centuries. Correlations between species densities in the sediment and past densities in the water column, however, are not straightforward, as

they depend on taxon-specific recruitment to and from the egg bank and differences on the degree of preservation of eggs (Jankowski & Straile 2003; Nykänen et al. 2009). Although no published data exist on egg production or preservation of the eggs for both species in natural conditions, our long experience culturing both species in the lab does not indicate differences in diapausing egg production per female. In addition, differences in deterioration rates in sediment cores of diapausing eggs of species of the *Brachionus plicatilis* complex in different lakes were mainly due to lake sediment conditions regardless the species composition (García-Roger et al. 2006). All this, together with the highly similar morphology of *B. plicatilis* and *B. manjavacas* diapausing eggs, supports that the relative frequencies of their diapausing eggs in the sedimentary record are likely to give information on past species fluctuations in recruitment. Accordingly, *B. manjavacas* appears as the most abundant species in this lake, although *B. plicatilis* was able to reverse this situation several times along the recent history of the lake. The increases of *B. plicatilis* diapausing egg recruitment could have been associated with reductions of the lake salinity during past periods of higher rainfall, which is supported by the association of an increase of *B. plicatilis* in Core 3 with the presence diapausing stages of *B.* sp 'Almenara', a species of the same cryptic species complex associated with low salinities (Ortells et al. 2003). Core 3 was taken in a shallow part of the southern, lower salinity area of the lake, and given the correlation of salinity with depth in salt lakes (Comín et al. 1992) this core is likely to reflect recruitment during periods of lower salinity (i.e., when the shallower areas were covered with water at periods of lake filling). In a similar way, the differential response to salinity of both

species is also supported by the much higher frequency of *B. manjacavas* in both cores from the more saline north side of the lake.

We conclude that *B. plicatilis* and *B. manjavacas* have coexisted and coexist in a stable regime in the Iberian Peninsula. If, as expected, resource competition between them occurs, one or several stable coexistence mechanisms should be regulating their population dynamics. Mechanisms such as resource partitioning through food particle size or food quality, or differential predation vulnerability appear unlikely, due to the extreme resemblance in size and shape of the external morphology and the grazing and trophic structures (Fontaneto et al. 2007) of these two suspension-feeding rotifers. In addition, the salt lakes where these species coexist in the Iberian Peninsula are shallow and offer few opportunities for microhabitat differentiation. However, salt lakes are paradigmatic examples of fluctuating environments. Salinity fluctuates highly and unpredictably at a large range of temporal scales in these lakes (from seasonal to interannual) as a consequence of meteorological and climatic changes (Comín et al. 1992). This is specially marked in the Iberian Peninsula, on one hand, due to its Mediterranean climate, with remarkably large and largely unpredictable inter-annual fluctuations in rainfall (Comín et al. 1992;Rodriguez-Puebla et al. 1998;Domínguez-Castro et al. 2008), and on the other hand, due to the effect of global climatic phenomena such as El Niño-Southern Oscillation events, which have particularly strong effects in the SE region of the Iberian Peninsula, where the studied lakes are located (Rodó et al. 1997). Environmental fluctuations are in the base of several stable coexistence mechanisms (Hutchinson 1961; Chesson 2000). One of such mechanisms is the 'storage effect', based on

recruitment fluctuation. This has been suggested to be a coexistence mediating mechanism in many systems (Chesson 2003). The storage effect is also likely to mediate species coexistence in continental zooplankton – e.g., copepods, cladocerans and rotifers – (e.g., Hairston 1996; Brendonck & De Meester 2003). However, until now, just one study has demonstrated the storage effect in these organisms (Cáceres 1997). Our results allow a preliminary exploration of this mechanism for coexistence of *B. plicatilis* and *B. manjavacas*

Three components are needed to create the necessary population feedback that allows a competing species to recover from low densities in a fluctuating environment (Chesson 2000): (1) a life-cycle stage buffered from competition; (2) a differential response of the competing species to a fluctuating environment; and (3) covariance between environment and competition. Of these three components, the first and the second are met by *B. plicatilis* and *B. manjavacas*. Both species produce diapausing eggs – which do not compete for resources –, and we have shown here that they have a differential response to salinity, a highly fluctuating condition in the lakes where they coexist; with *B. manjavacas* outperforming *B. plicatilis* in the higher salinity range. We cannot rule out that instead of salinity, other physical environmental factors correlated to it might also contribute to this differential response. The third component measures the population response to the physical environment and acts as a stabilizing mechanism; the environment, by altering differentially the population density, modifies the competition, i.e. the species favored by the environmental conditions suffers from a greater competition. The greater the magnitude of the environment-competition covariance, the lower the

benefits are for the species when the environment is favorable and the greater the opportunity for a competitor species. Showing that this component actually happens has been proposed as a rigorous field test of the storage effect (Sears & Chesson 2007). However, to date, no experimental data exists assessing this component in temporal fluctuating environments. An indirect approach based on the comparison of the variance of ln recruitment ('recruitment variation') of the species has been developed to test this component and, thus, the storage effect (Chesson 2003). The test is based on the fact that a high environment-competition covariance results in a low variation in recruitment. Therefore, a difference in the recruitment variation between competitors is expected if a difference in environment-competition covariances occurs, as required by the storage effect. As explained in Chesson (Chesson 2003), the difference in recruitment variance between competing species is proportional to the community storage effect; and this comparison reflects the full magnitude of the storage effect in an invader-resident scenario, where the invader species will have a higher variance. That scenario can be expected when comparing a situation of high versus low-density species (Chesson 2003). This is a sound assumption for our system (Fig. 4.3), with *B. manjavacas*, the most abundant species in the paleolimnological record, being the resident species, and *B. plicatilis* the invader. Additionally, Chesson (2003) also recognizes that this scenario is expected when studying small fast growing organisms, which is the case of our species. Our data for the four cores show that *B. plicatilis* always has a higher estimated recruitment variance than *B. manjavacas*, suggesting an environment-competition covariance. Therefore, our data, even being preliminary, are

what we should expect if the storage effect is a factor mediating the coexistence of *B. plicatilis* and *B. manjavacas.*

Notwithstanding the storage effect associated to salinity fluctuation, other coexistence mechanisms could be interacting with it in stabilizing coexistence. Little explored ecological factors (e.g., oxygen concentration, pH or ionic composition) could also be involved in niche differentiation mediating the coexistence of both species. In addition mechanisms not based in niche differentiation such as density-dependent life-cycle switching (Montero-Pau & Serra 2011) are likely.

Despite the increasing use of paleolimnology in ecological and evolutionary studies (e.g., Cousyn et al. 2001; Mergeay et al. 2005; Decaestecker et al. 2007), this approach had not been used, to our knowledge, to document long-term coexistence of cryptic species before. Our results conclusively show long-term coexistence in a location of *Brachionus plicatilis* and *B. manjavacas,* two highly similar rotifer species, although with fluctuations in their relative densities. We have also shown that these species have different but overlapping responses to salinity. We propose that stable species coexistence is mediated by such differential responses to salinity in the context of habitat fluctuations. As King (1980) stated almost three decades ago, "the 'population' investigated in many [limnological] studies may be an artifact with closer affinities to griffins, unicorns and mermaids than to the population as a biological unit". Our work contributes to the increasing awareness that molecular techniques, by unveiling hidden species richness, can reveal concealed past and present ecological and evolutionary patterns.

Acknowledgements

We thank Sergi Campillo for allowing us to use the characterized clones used in this study. We are grateful to Robert L. Hammond, Maria José Carmona Navarro and two anonymous reviewers for constructive comments on previous versions of the manuscript. This work was funded by a grant from the Spanish Ministerio de Ciencia y Tecnología (BOS2003-00755) to M. S., A. G. was supported by a National Environment Research Council (NERC) Advanced Fellowship (NE/B501298/1), E. R-R was supported by a fellowship by the Secretaría General de Universidades e Investigación (Consejería de Educación y Ciencia, Junta de Andalucía) and J. M-P by a fellowship by the Spanish Ministerio de Ciencia y Tecnología (BES2004-5248).

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5

Measuring the potential for growth in populations investing in diapause

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Abstract

The intrinsic growth rate of population increase (*r*) is a common performance measure in many ecological and evolutionary studies. However, in life cycles with diapause investment, resources are split into a short-term (current population growth) and a long-term (population survival through periods of unsuitable habitat conditions) component, which questions the use of *r* as a single performance measure. Here we propose a new meassure which integrates both performance components into a single measure, the potential intrinsic growth rate, r_{pot}. This is the rate of increase that a population/genotype would be predicted to have if no investment in diapausing stages would occur. We show that r_{pot} can be computed using standard demographic data from temporal series or life table experiments and demonstrate the use of the r_{pot} for two common life cycles among zooplanktonic organisms: (1) a cyclically parthenogenetic life cycle where investment in diapause happens only during the sexual phase, and (2) an obligate sexual life cycle with a switch from non-investing females to investing females along the lifespan. Using case studies we show that the use of r_{pot} or the standard *r* affects comparisons between genotypes/populations or environmental factors. We suggest that r_{pot} can be estimated in life cycles not considered here if appropriate assumptions are made.

Introduction

Many aquatic organisms rely on resistant diapausing stages to cope with the variability in their environments through dispersal in time and space (Hairston 1996; Brendonck & De Meester 2003). However, investment in diapause results in a direct reduction of the current population growth rate, because a fraction of resources is allocated to the production of diapausing stages, which do not reproduce immediately. Thus, it affects the per capita population growth rate, which is a central parameter in population and evolutionary biology. This tradeoff between production of diapausing stages and current population growth has been well studied by applying life-history theory to zooplankton life cycles (Ellner 1997; Spencer et al. 2001; Serra et al. 2005), and has major evolutionary and ecological implications. For example, it might promote coexistence of competitive species (Montero-Pau & Serra 2011).

Under the exponential growth model, the per capita population growth rate becomes the intrinsic rate of increase (r). Obviously, exponential growth cannot last forever, however many empirical and theoretical studies observe or assume density-independent growth during relevant periods of the population dynamics, and despite the exponential model cannot be assumed in all cases, the biological relevance of the model and the intrinsic rate of increase holds in many situations. First, the intrinsic rate of increase is a predictor for the population recovery from low densities, as stressed in the *r-K* theory (Pianka 1970; Roughgarden 1971), in the concept of fugitive species (Hutchinson 1951), or in invasion analysis (Chesson 2000). Even when studying exploitative competition, the response of the intrinsic rate of increase in relation to constant food levels plays an important role

(Tilman 1982; Rothhaupt 1990; Ciros-Pérez et al. 2001). Second, as pointed out by Caswell (2001, p. 29), the exponential growth model can be interpreted as a projection rather than as a forecasting – i.e., the model describes what would happen if all the conditions remained constant. On that sense, the exponential model has parallels to the role of Newton's law of inertia (Turchin 2003, p. 22). Consequently, the intrinsic rate of increase has been widely used as a performance measurement to compare environmental effects, or the differences between species, populations or genotypes under the same conditions (e.g., Girma et al. 1990; Kocourek et al. 1994; Roy et al. 2003; Deutsch et al. 2008). Not surprisingly, the intrinsic rate of increase has also been used to assess the chronic effects of toxicants (Biesinger & Christensen 1972; Mount & Norberg 1984; Snell & Moffat 1992; Snell & Carmona 1995; Forbes & Calow 1999).

The investment in diapause, however, entangles the interpretation of the biological meaning of the intrinsic rate of increase. This problem is especially important when the intrinsic rate of increase is used to compare the performance of populations or genotypes with different diapause investment, or to compare environmental conditions (e.g., experimental treatments) resulting in a differential diapause investment. It may happen that a population/genotype in any given conditions has a lower current population growth rate than another not because is performing worse, but because is investing more in diapause – i.e. future growth. Also, the interpretation of the intrinsic rate of increase is affected by how this parameter is estimated. Two common methods are normally used to estimate this rate: (1) from population density time series as the slope of the log-density with time in a culture

growing exponentially (Lampert & Sommer 2007), or (2) performing life-table experiments (Carey 1993). Both methods estimate the same rate of increase if in the life table experiment individuals investing in diapausing stages are included for calculations but no fecundity is assigned to them, since they do not contribute to the current population growth in the time-series experiment. By contrast, if in the life-table experiment the diapausing-stage producers are dropped for data analysis, then a different parameter is estimated; the intrinsic growth rate of increase when diapause is suppressed. This parameter although is not useful to predict population dynamics under exponential growth (forecasting), it is still useful to assess the population performance for the environmental conditions in the experiment (projection). The different meaning of the growth rates resulting from the different experimental and estimation approaches points out the importance of reporting what approach was used.

The aim of this chapter is to clarify the biological meaning of the intrinsic rate of increase in organisms investing in diapause, and how this meaning is affected by the assumptions and methods used to estimate this parameter. We focus on zooplanktonic organism investing in diapause, and propose a new measure to estimate the intrinsic growth rate when the interest is to assess performance. Our approach is to propose a demographic measure of the resources captured by organisms and made available to invest either in current growth or in diapause. We propose that potential intrinsic rate of population increase (r_{pot}) is a suitable comparative measure of performance when an effect of diapause investment on population growth is suspected. We define

r_{pot} as the rate of increase that a population/genotype would have if no investment in diapausing stages would occur. That means that r_{pot} cannot be measured directly in a population dynamics, but inferred. Here, we show a methodology to integrate the diapause investment into the intrinsic rate of population increase to obtain r_{pot}.

Procedure

Integration of diapause investment into the intrinsic rate of population increase is not trivial as both measures have different metrics. Moreover, the estimation of r_{pot} is strongly dependent on details of the life cycle and on the type of demographic data available, and might require simplifying assumptions. The intrinsic rate of population increase is commonly estimated from data obtained either by following the population dynamics or by a life table experiment. When using data from time series, parameters (i.e., birth and death rate) are required to be density-independent. Normally, that requirement is (1) accomplished by using data from populations growing at low densities or (2) selecting the data showing a linear relationship between log-density and time. In the case of using data from life table experiments density-independence is guaranteed by an appropriate experimental set-up, particularly by approaching a constant environment through frequent medium renovation and offspring removal.

Life cycle features also play a decisive role when estimating r_{pot}. Important features are which stages contribute to the diapausing investment or when this investment occurs along the lifespan. For

example, in obligate sexuals and obligate asexuals, the investing individuals are usually females producing diapausing eggs, whereas, in cyclical parthenogens, diapause-investing individuals are females producing either males –which allocate their gametes only into diapausing eggs – or diapausing eggs. Also, diapause investment may or may not vary along an individual lifespan. In some organisms females are born determined to contribute uniquely either to current population growth or to diapausing stages (e.g., asexual vs. sexual females in rotifers), while in other organisms the individuals can switch along their lifespan from non-investing to investing in diapause (e.g., cladocerans, anostracans, and copepods). As the diversity of life cycles among zooplaktonic organisms is enormous, we demonstrate the computation of the r_{pot} for two common life cycles among zooplanktonic organisms: (1) a cyclically parthenogenetic life cycle where investment in diapause occurs only during the sexual phase, and (2) an obligate sexual life cycle with a switch from non-investing females to investing females along the lifespan. We will show how to obtain r_{pot} using data from time series and life table experiments. Before dealing with these case studies, we develop the equations describing the exponential growth for these two life cycles.

Exponential growth

Obligate sexuals with diapause investment switching. This life cycle is common for many zooplanktonic organisms like anostracans (e.g. *Artemia*) (Dodson & Frey 2001) or calanoid copepods (Santer 1998). We will illustrate this life cycle with the calanoid copepod *Onychodiaptomus* (formerly *Diaptomus*) *sanguineus* (Hairston et al. 1995). Active

individuals reappear in the water column when diapausing eggs begin to hatch. Females produce subitaneous eggs resulting on both females and males during several generations. Associated to habitat deterioration, some environmental cues – variation of photoperiod and temperature – induce females to switch from producing subitaneous eggs to producing diapausing eggs that do not hatch immediately. Interestingly, variation in the timing of diapause has been described in this species (Hairston & Olds 1984). Consequently, a differential investment in diapause among genotypes exists, which can lead to the problems stated above for comparing performance.

The current dynamic of the copepod females in the water column can be described as

$$\frac{dF}{dt} = b(1-\delta)F - dF$$

where F is female density, δ is the proportion of eggs going into diapause, and b and d are the instantaneous birth and death rates estimated for females (i.e., discounting male production from the offspring). This model assumes that the contribution of diapausing-egg hatching to the current population growth is negligible after the growing season has started. Assuming that b, d and δ are density-independent, which may be true for an observation period, this model reduces to the standard exponential growth model ($dF/dt = r\,F$), with intrinsic growth rate of population increase $r = b\,(1-\delta) - d$. Therefore, the potential intrinsic rate of population increase (i.e., if no investment in diapause is assumed, $\delta = 0$) will be $r_{pot} = b - d$, assuming that b and d are not dependent on δ (e.g., a higher proportion of diapausing eggs has no cost

for female survival, or the production of a diapausing egg is as costly as the production of a subitaneous egg).

Cyclical parthenogens with investment during the sexual phase. This life cycle is characteristic of many cladocerans and monogonont rotifers (De Meester et al. 2004). We will use the life cycle of monogonont rotifers of the genus *Brachionus* as a model here (Wallace & Snell 1991). The growing season of the population begins with asexual female hatching from diapausing eggs. These females produce genetically identical daughters by ameiotic parthenogenesis during several generations. When sexual reproduction is induced by environmental factors such as population density, asexual females start producing both asexual and sexual daughters. Sexual females produce haploid eggs that develop into haploid males or, if fertilized, diploid diapausing eggs. Diapausing eggs usually hatch after a dormant period of variable length. As in the case of the copepod, variation in the diapause investment has been observed among clones and populations (Aparici et al. 2001; Carmona et al. 2009; Campillo et al. 2011).

The population dynamics in the water column can be described by the number of asexual females (F_σ, for females producing daughters subitaneously) and sexual females (F_δ, for females investing in diapause, either directly or mediated by male production). A model after Serra and King (1999) is

$$\frac{dF_\sigma}{dt} = b(1-\delta)F_\sigma - dF_\sigma$$

$$\frac{dF_\delta}{dt} = b\delta F_\sigma - dF_\delta$$

where b and d are the intrinsic birth and death rates for females, and δ is now the proportion of eggs from non-investing females that develop into females investing in diapause. As in the copepod model, contribution to population from diapausing egg hatching after the growing season starts is assumed to be negligible. Following the same reasoning as above, the first equation in the model tells us that F_σ grows exponentially with $r = b(1-\delta) - d$. By contrast, this model could raise concern about whether the whole female population ($F = F_\sigma + F_\delta$) grows exponentially. Nevertheless, it can be proved (see Appendix B) that F converges to be

$$\frac{F_\sigma}{1-\delta}$$

Therefore, F converges to exponential growth as F_σ, with $r = b(1-\delta) - d$. This convergence is analogous to the convergence to a stable age distribution and exponential growth in age-structured populations with density-independent age-specific fecundity and age-specific mortality (Caswell 2001).

Keeping the assumption that b and d are independent on δ, $r_{pot} = b - d$. Notice that, although the cost of producing diapausing egg is higher than the cost of a subitaneous egg (e.g., Serra et al. 2005), here δ is the proportion of eggs developing into sexual females, so the assumption that b is not dependent on δ is likely.

Calculating r_{pot} from time series

The use of time series is a common and simple procedure to estimate the intrinsic rate of population increase (r). Two main kinds of experiments can be performed. The first one is starting a culture from a

known number of individuals and after a given lapse of time, count the number of individuals again. Then, r is estimated as $[ln\ F(t) - ln\ F(0)]/t$. The second one is monitoring population abundance along time, and then r is estimated by adjusting the time series to the function $ln\ F(t) = ln\ F(0) + rt$. In order to estimate r properly, the population in the initial conditions has to be growing exponentially, which means that it must have reached the stable age-distribution (Caswell 2001). In some long-lived temporary populations this convergence might not occur. Nevertheless, experimental procedures where the convergence does occur are possible.

As shown above, the potential intrinsic growth rate ($r_{pot} = b - d$) cannot be inferred directly from r and δ, but an estimation of b and d is needed. Different approaches can be used to obtain b (Gabriel et al. 1987). Here we will use Edmonson-Paloheimo's method (EP) (Paloheimo 1974), which is simple and has been proved to be accurate (Lynch 1982; Gabriel et al. 1987). EP method estimates instantaneous birth rate as

$$b_{EP} = \frac{ln\left(\frac{E_{EP}}{F_{EP}} + 1\right)}{D_{EP}}$$

where E_{EP} is the number of eggs, F_{EP} is the number of females and D_{EP} is the egg development time. Estimates for D_{EP} for a range of zooplanktonic organisms are available in the literature (e.g., Herzig 1983; Galkovskaja 1987).

In the copepod case, EP method can be applied by ignoring diapausing eggs (i.e., E_{EP} and D_{EP} being respectively the number of observed subitaneous eggs and their development time, and F_{EP} being the total number of females, F). In this way, since the subitaneous eggs

are divided by both diapausing investing and non-investing females, the estimation of b_{EP} equals b (1-δ) (i.e., the actual birth rate with diapausing investment) and d is estimated as $d = b_{EP} - r \cdot \delta$ is obtained from identification and counting of eggs (Lohner et al. 1990) as number of diapausing eggs/total number of eggs, and b can be estimated from b_{EP} and δ. Finally, r_{pot} is obtained as $r_{pot} = b - d$.

In the rotifer case, the numbers of sexual F_δ and asexual females F_σ have to be estimated. This can be performed by (1) counting separately the non-egg-bearing females and the egg-bearing females, (2) classifying the latter as sexual and asexual accordingly to the type of eggs carried (e.g., Carmona et al. 1995), and (3) applying the proportion found in the egg-bearing females to the total number of females. Notice that the proportion found in (2) allows estimating δ, as $F_\delta/(F_\delta + F_\sigma)$. Besides counting females, the number of ameiotic subitaneous eggs (i.e., those hatching into asexual females) needs to be recorded. It is important to keep in mind that the rotifer population as a whole grows exponentially with $r = b(1- \delta) - d$. Hence, as in the copepod case, if E_{EP} is the number of ameiotic subitaneous eggs, F_{EP} is $F_\delta + F_\sigma$, and D_{EP} is the development time of subitaneous eggs, then EP equation gives $b_{EP} = b(1-\delta)$, again, the actual birth rate. Estimation of d can be carried out as above, and, analogously to the copepod case, b can be obtained from b_{EP} and δ.

Calculating r_{pot} from life table data

Dynamic life tables are another common technique to calculate r in laboratory studies of zooplanktonic organisms. Typically, a cohort of new born females is followed until all have died and their survival and

offspring recorded. Intrinsic growth rate of population increase is obtained from life table data by solving Euler-Lotka equation (e.g., Stearns 1992). This equation provides the *r* that a population has with the same fecundity, mortality and investment in diapause as the cohort.

In dynamic life table experiments for rotifer cohorts, the type of reproduction of the females is ignored a priori. However, offspring inspection allows knowing if a female in the cohort is either asexual (producing daughters) or sexual (producing sons as non-fertilized sexual females only produce males). If the whole cohort (i.e., including both sexual and asexual mothers) and the produced daughters are used to estimate net age-specific fecundity – l(x)m(x) –, then Euler-Lotka equation gives *r*. Alternatively, if age-specific fecundity is computed using only the sub-cohort of asexual females and the produced daughters are all regarded as asexual, then, Euler equation gives r_{pot} .

Similarly, in the copepod case, when the whole cohort and the daughters produced subitaneously are taken into account to calculate net age-specific fecundity, Euler-Lotka equation gives *r*. However, a strategy to infer r_{pot} is based on the cost of producing diapausing eggs relative to the cost of producing a subitaneous egg. As a simplifying assumption, it can be considered that production of a diapausing egg is not more costly for the females than the production of subitaneous eggs, or alternatively, an approximate cost can be estimated (for instance, see Serra et al. 2005 for an example of this estimate in rotifers). Then, the development time, the egg survival proportion from laying to hatching, and the sex ratio observed in the subitaneous eggs could be applied to the number of diapausing eggs produced by the cohort, as if these eggs were subitaneous ones. This allows estimating

the number of daughters that would emerge from the diapausing eggs under that assumption, and therefore the net age-specific fecundity under the assumption that all the eggs are subitaneous. Using this net age-specific fecundity the Euler equation gives r_{pot}.

Assessment

Calculating r_{pot} from time series

Gabaldón et al. (in preparation) experimentally studied the combined effect of salinity (seven levels) and temperature (three levels) on the growth of two rotifer cryptic species (*Brachionus plicatilis* and *B. manjavacas*). Three replicates were performed for each combination; from acclimated cultures at the same experimental salinity and temperature 20 individuals were transferred to flasks containing an excess of food and allowed to grow. Cultures were fixed after four days and the number of females and eggs counted. Proportion of sexual females (δ), E_{EP}/F_{EP} and b_{EP} were computed. E_{EP}/F_{EP} was calculated as the total number of ameiotic eggs (i.e., parthenogenetic eggs contributing to the current growth) per female (either sexual or asexual). Table 5.1 summarizes the results for a temperature (25^{o}C). *B. manjavacas* showed higher *r* than *B. plicatilis* at any salinity. However, *B. plicatilis* had higher r_{pot} than *B. manjavacas* in the lower range of the salinities tested, showing in this way its higher performance in such conditions. Gabaldón and her coworkers interpreted this result as a strategy of *B. plicatilis* to use its higher performance to produce diapausing stages, even with the cost of a lower current growth rate. This observation support previous results that suggest that *B. plicatilis* is adapted to relatively low salinities if compared to *B. manjavacas* (Montero-Pau et al. 2011).

Table 5.1. Demographic response to salinity of populations of the rotifers *B. plicatilis* (Bp) and *B. manjavacas* (Bm), growing at 25°C. The highest per capita growth rate at each salinity is in bold type. Values are averages over 3 replicates, with two exceptions[a]. Time for egg development was assumed to be 0.462 days (Galkovskaja 1987) when applied to estimate birth rate using Edmonson-Paloheimo method (b_{EP}).

Salinity (g/L)	Species	δ (%)	E_{EP}/F_{EP}	b_{EP} (d^{-1})	d (d^{-1})	r_{pot} (d^{-1})	r (d^{-1})
5	Bp	10.4	0.406	0.736	0.227	**0.593**	0.509
	Bm	7.1	0.496	0.871	0.258	0.613	**0.613**
10	Bp	25.3	0.535	0.925	0.202	**1.043**	0.723
	Bm	5.3	0.716	1.166	0.430	0.799	**0.736**
20	Bp	30.5	0.615	1.036	0.426	**1.064**	0.610
	Bm	6.2	0.763	1.225	0.568	0.738	**0.658**
30	Bp	1.8	0.816	1.289	0.726	0.587	0.563
	Bm	0.4	0.754	1.215	0.632	**0.588**	**0.583**
40	Bp	0.0	0.511	0.892	0.497	0.395	0.395
	Bm	3.9	0.537	0.925	0.448	**0.512**	**0.477**
50	Bp	0.0	0.591	0.997	0.800	0.198	0.198
	Bm	0.0	0.447	0.798	0.423	**0.375**	**0.375**
60	Bp	0.0	0.484	0.852	0.910	-0.058	-0.058
	Bm	0.0	0.361	0.663	0.605	**0.058**	**0.058**

a. b_{EP} estimation gave slightly negative mortality rates in one (Bp) and two (Bm) replicates at 5 g/L salinity, and then average mortality in the rest replicates was used.

Calculating r_{pot} from life table data

Serra (1987) conducted life-table experiments to study the combined effect of salinity (3 levels) and temperature (three levels) on three genotypes of rotifers belonging to the *Brachionus plicatilis* species complex. For each combination a cohort of 50 females was monitored every 12 or 24 h and survival and fecundity schedules recorded. The

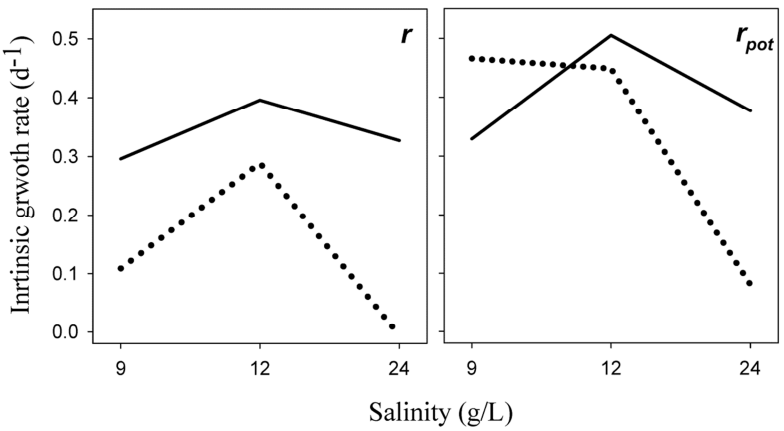

Figure 5.1. Comparison between intrinsic rate of population increase (*r*) and potential intrinsic rate of population increase (r_{pot}) for two rotifer species of the genus *Brachionus* at three different salinities. This example shows how the inclusion of the diapause investment can lead to a different interpretation of the results. Data obtained from Serra (1987). Intrinsic growth rates were computed using a life table approach.

cohort females were identified as sexual or asexual. From this data set, both *r* and r_{pot} were computed. Fig. 5.1 shows the results for two of these genotypes. Genotype A is predicted to have faster growth than genotype B in all three salinity treatments, since the former has higher intrinsic rate of increase. However, r_{pot} shows that the situation is reversed for the lower salinity treatment. This means that, if diapausing-stage production was inhibited, genotype B would growth faster than genotype A, implying that the former has higher capability to convert resources in growth (i.e., higher performance).

Discussion

Intrinsic growth rate of population increase is a common measure of performance in many ecological and evolutionary studies. However, investment in diapause entangles this use of the intrinsic growth rate, because the reproductive potential splits into investment in effective, current population growth and investment in future offspring, i.e., in diapausing stages with delayed hatching needed for survival through periods of unsuitable habitat conditions. Population biologists are often interested in comparing the performance of different genotypes of the same or different species in a given environment, or testing how different environments affect the performance of a given genotype. If performance is evaluated demographically (e.g., as the proliferation potential), diapause investment needs to be considered besides the current growth. Here we have developed a simple method to integrate both growth components (current and future) into a single measure, the potential intrinsic growth rate (r_{pot}). Computation of this parameter does not require any additional experimental set-up or technique to those commonly used in the lab to estimate the intrinsic growth rate. As illustrated in the case studies shown above, comparisons of the performance of species or strains based on the effective and the potential growth rate may differ, and the discrepancies need to be analyzed after knowing what the different measurements mean. Failure to consider the potential for growing can lead to misleading conclusions on relative performance when genotypes or environments are compared.

Although we have exemplified the computation of r_{pot} for two common life cycles, this method can be extended to other life cycles if

appropriate assumptions are made. To do so, first, it is necessary to parameterize the population dynamics for exponential growth and to find the intrinsic growth rate of population increase (*r*) for the model. In cases where systems of equations arise, convergence of the whole population to exponential growth has to be demonstrated. Notice that some steps to estimate r_{pot} require simplifying assumptions (e.g., cost of a diapausing egg compared to the cost of subitaneous one) that can be inaccurate and thus the estimation of r_{pot} should be refined. Despite this, we propose that a rough estimation of r_{pot} is a better measurement of the demographic performance than intrinsic growth rate with no correction. Although a statistical analysis of this methodology to obtain r_{pot} is needed, such analysis is beyond the scope of this paper.

Besides a performance measure, the intrinsic population growth rate is used as a fitness measure in some ecological scenarios. Using different approaches, Charlesworth (1980) and Lande (1982) showed that, if selection is density-, frequency-independent (plus additional, relatively minor assumptions), the intrinsic rate of increase is the fitness measure. The relevance of this result for natural populations depends on how much the exponential growth phase lasts. In non-equilibrium populations, selection of high intrinsic rates of increase is expected to occur during long periods (Caswell 2001), shaping life histories in this way. However, this is not the case of temporary zooplankton populations, where only the total number of diapausing eggs hatching at the beginning of the next growing season is considered a between-year fitness measure (Serra & King 1999; Campillo et al. 2011). Unfortunately, measuring the total production of diapausing eggs and their viability involves difficult and time-consuming experiments or field observations.

Thus, fitness estimations based on short-term measurements like intrinsic growth rate of population increase or diapausing investment can be useful. However, if short-term intrinsic growth rate is used, then investment in the ultimate fitness component (i.e., diapausing stage production) is completely neglected. In contrast, if short-term production of diapausing stages is considered, then future return in diapausing stage production from current population growth is neglected. Therefore, investments in both diapause and current growth need to be combined, and in order to make comparisons, they should be expressed in a single metric. r_{pot} can work as such a integrated measure. An important assumption to use r_{pot} as a measure of fitness is that the resource allocation in diapausing stages is optimal; in order words, this allocation maximizes the total production of diapausing stages for a given growing potential. Optimization of investment in diapause is a sound assumption because it only implies a mechanism to split the acquired resources, rather than a mechanism to acquire resources, which is likely more constrained. In fact, empirical evidence on rotifers suggests that this optimization evolves easily (Carmona et al. 2009; Campillo et al. 2011).

Our concern on using the effective intrinsic growth rate of population increase (r) to estimate population/genotype performance emerges from the fact that diapause investment inflicts a cost on current population growth. However, this same problem will arise when considering other trade-offs over population growth. For instance, it is well known that investment in sex leads to a cost for population growth (Maynard Smith 1978). Thus, our results can be generalized to species of

(1) obligate sexuals with a variable sex ratio or (2) cyclical parthenogens and facultative sexuals with a variable proportion of sexual offspring.

Acknowledgments

This article has greatly benefited from discussions with Sergi Campillo and Eduardo M. García-Roger about the evolutionary and ecological role of investment in diapause, and two anonymous reviewers This work was funded by a grant from the Spanish Ministerio de Ciencia e Innovación (CGL2009-07364) to MS. JM-P was supported a fellowship by the Spanish Ministerio de Ciencia y Tecnología (BES2004-5248) and CG by a fellowship by the Spanish Ministerio de Ciencia e Innovación (BES2010-036384).

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6

Life-cycle switching and coexistence of species with no niche differentiation

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Published in PLoS One 2011 6:e20314

Abstract

The increasing evidence of coexistence of cryptic species with no recognized niche differentiation has called attention to mechanisms reducing competition that are not based on niche-differentiation. Only sex-based mechanisms have been shown to create the negative feedback needed for stable coexistence of competitors with completely overlapping niches. Here we show that density-dependent sexual and diapause investment can mediate coexistence of facultative sexual species having identical niches. We modelled the dynamics of two competing cyclical parthenogens with species-specific density-dependent sexual and diapause investment and either equal or different competitive abilities. We show that investment in sexual reproduction creates an opportunity for other species to invade and become established. This may happen even if the invading species is an inferior competitor. Our results suggests a previously unnoticed mechanism for species coexistence and can be extended to other facultative sexual species and species investing in diapause where similar density-dependent life-history switches could act to promote coexistence.

Introduction

Maintenance of species diversity is a central topic in ecology, a critical issue being the limiting similarity of competing species allowing coexistence (MacArthur & Levins 1967). Most of the mechanisms that have been proposed to allow stable coexistence rely on niche differentiation (i.e. resource partitioning, differential vulnerability to predation, or differential response to temporal fluctuation or spatial variation). However, the number of cryptic species reported has dramatically increased since the introduction of the molecular techniques (Bickford et al. 2007), and these species are frequently found in sympatry (Molbo et al. 2003; Wellborn & Cothran 2004; Braune et al. 2008). In many cases cryptic species do not show any clear niche differentiation, thus long-term co-occurrence of cryptic species may indicate that stable persistence of ecologically equivalent species is possible (Leibold & McPeek 2006). Therefore, explanations other than neutrality (i.e., lasting unstable coexistence) will be needed, and mechanisms able to explain stable coexistence not based on niche differentiation may be required.

A necessary condition for stable coexistence is population growth from low densities (i.e., invasibility criterion) (Chesson 2000). For this to happen, the species with the highest density should affect their own growth more negatively than that of the rare species. Zhang and Hanski (1998) showed that negative feedback could arise through features of sexual reproduction and recognized three mechanisms that could promote stable coexistence of identical competitors: density-dependent adjustment of sex ratio, sexual conflict, and sexually transmitted diseases. We propose another mechanism for stable coexistence of

ecologically equivalent species based on density-dependent investment in sexual reproduction and/or dormancy. This mechanism may be especially important in facultatively sexual species (e.g., cyclical parthenogens), and species with diapause stages.

Sexual reproduction and diapause both impart a cost on population growth. Sexual reproduction typically incurs the "two-fold cost of males", while diapausing stages usually exhibit a delay in hatching/germinating, so that part of the resources allocated to their production is lost to current population growth and competition efficiency (Serra & King 1999). This cost of sex and diapause could provide an opportunity for ecologically equivalent species making a lower investment in sexual reproduction or diapause to invade an assemblage. Hence, if sexual reproduction or diapause investment is density-dependent and controlled by species-specific signals a separate density-dependence occurs. It might create the negative feedback necessary for coexistence, even between species with otherwise completely overlapping niches. By investing in sex or diapause, a high-density competitor would decrease its own population growth rate more than that of its low-density competitor, allowing the rare competitor to grow faster. This could be the case for obligate sexuals, like the copepod species of the cryptic complex *Eurytemora affinis,* where crowding is the signal to produce dormant stages (Ban & Minoda 1994); or for obligate asexuals, like *Bacillus* species where sporulation is triggered, among other cues, by quorum sensing (Lazazzera 2000). Also, such a mechanism might be relevant for the coexistence of facultative sexuals, such as plants with a density-dependent switching between vegetative and sexual reproduction (Takada & Nakajima 1996; Coelho et al. 2005) and

cyclical parthenogens, where the costs of sex and dormancy are common. Cyclical parthenogenesis is a reproductive mode shared by approximately 15000 species (De Meester et al. 2004) and is characteristic of aphids and two common zooplanktonic taxa – cladocerans and monogonont rotifers. This life cycle combines an extended phase of exclusive asexual (parthenogenetic) reproduction alternating with a phase of combined asexual reproduction and sexual reproduction, the latter leading to the production of diapausing stages. Sexual reproduction is known to be induced, among other clues, by population density in several groups of cyclical parthenogens (Gyllström & Hansson 2004; Zadereev & Lopatina 2007), and in at least three rotifer genera (*Epiphanes*, *Rhinoglena* and *Brachionus*) it is exclusively induced by population density (Schröder 2005). One of the best-known mechanisms of sex induction is that of the rotifer *Brachionus plicatilis* species complex, where sexual reproduction is induced by a protein released into the environment by the rotifers (Snell et al. 2006). As population density increases, this protein accumulates, and at a threshold concentration it triggers sexual reproduction, in a process akin to quorum sensing in bacteria (Kubanek & Snell 2008). Recently, it has been shown that some degree of specificity exists among these species regarding the induction of sex (García-Roger et al. 2009).

Here, we address the hypothesis that a density-dependent life cycle switch like the asexual to sexual transition can promote coexistence of otherwise ecologically equivalent species. Using the cryptic species complex *Brachions plicatilis* as a model, we develop and analyze a simple Lotka-Volterra competition model describing the dynamics of two competing species with a density-dependent

investment in sex. We explore whether coexistence is possible in the extreme case of complete niche overlap between the competing species, first by assuming that density-dependent investment is exclusively dependent on conspecific density, and later by including the heterospecific density. We also explore the consequences of equal and unequal competitive ability between species.

Methods

Model

A modification of the model proposed by Serra and King (1999) was used to describe the dynamics of the asexual (A_i) and sexual (S_i) individual densities for two competing species (i = 1, 2) having identical niches:

$$\frac{dA_i}{dt} = b_i(N_T) \cdot \left[1 - m_i(N_i)\right] \cdot A_i - q_i A_i \qquad \text{[Eq. 6.1a]},$$

$$\frac{dS_i}{dt} = b_i(N_T) \cdot m_i(N_i) \cdot A_i - q_i S_i \qquad \text{[Eq. 6.1b]},$$

where $b_i(N_T)$ is the birth rate of species i at total density N_T ($N_T = \Sigma_i [A_i+S_i]$), q_i is the mortality rate, assumed to be density-independent, and $m_i(N_i)$ (where $N_i = A_i + S_i$) is the proportion of sexual individuals in the offspring of an asexual individual and is a measure of sexual investment. This proportion is assumed to be dependent on the species-specific density following a non-decreasing function. A species-specific dependence is a critical assumption which will discussed below. The exact definition of a sexual individual depends on details of the life cycle. For instance, in monogonont rotifers density of sexual individuals (S_i) refers exclusively to sexual females since males are short-lived and do

not feed, while in cladocerans and aphids it refers to sexual females (i.e., those producing haploid eggs) and males. Notice that S does not contribute births to the dA/dt or dS/dt of the current population because sexual reproduction is assumed to produce diapausing stages, and the model focus on the dynamics of the active stages. However, diapausing eggs matter for the long-term coexistence, and these implications will be discussed below. We assume a functional equivalence of sexual and asexual individuals except for their reproductive mode. Thus, birth rate and mortality rate are assumed to be equal for both types of individuals. Notice that, contrasting with the output of sexual reproduction – i.e., diapausing eggs –, sexual individuals are active, consume recourses and account for competition.

Density effects on the birth rate are modeled according to the Lotka-Volterra assumption of a linear relationship between birth rate and total population density:

$$b_i(N_T) = b_{\max,i} - \frac{b_{\max,i} - q_i}{K_i} N_T \quad \text{[Eq. 6.2]},$$

in which $b_{max,\,i}$ is the birth rate of the i^{th} species without density effects (i.e. the intrinsic birth rate), and K_i is the carrying capacity. Note that no competition coefficients are included, so the effect of a competitor on the birth rate is the same as that of a conspecific. In other words, the two species have completely overlapping niches. Eq. 6.2 gives $b_i(K_i) = q_i$, so that growth rate of the i-th species in absence of sexual reproduction is zero when $N_T = K_i$. Moreover, the parameters in the model are time-independent, so that, if found, coexistence is not an effect of environmental fluctuations.

As a conservative approach for species similarity, the parameters of the model $b_{max,i}$ and q_i, are considered to be equal for both species (hereafter, the species index in these parameters is dropped). By contrast, carrying capacity of Species 2, K_2, is assumed to be a proportion of the carrying capacity of Species 1, K_1 (i.e., $K_2 = \beta K_1$, with $0 < \beta \leq 1$). This allows us to introduce an asymmetry in the competitive abilities. As convention, if asymmetry exists, Species 1 is always the best competitor (i.e., $\beta < 1$).

Results

Model analysis

Density-dependent sexual/diapause investment can be described by a sharp sigmoid function accounting in a continuous fashion for the occurrence of a population growth phase with negligible sexual or diapause investment and a population growth phase with both sexual and asexual reproduction (Fig. 6.1). An simple instance of such a function for density-dependent sexual investment, $m_i(N_i)$ is:

$$m_i(N_i) = \frac{m_{\max,i}}{1 + e^{z_i (T_i - N_i)}} \quad \text{[Eq. 6.3]},$$

where $m_{max,i}$ is the maximum asymptotic investment in sexual reproduction, T_i is the population density threshold for sex induction, defined as the density at which $m_i(N_i) = m_{max,i} / 2$, and z_i is a parameter related to the slope of the response. However, the model resulting from combining Eq. 6.1 and 6.3 cannot be analyzed algebraically. Equilibrium analysis for a single species yields transcendental equations, and a Taylor expansion of Eq. 6.3 truncated to second order is a poor

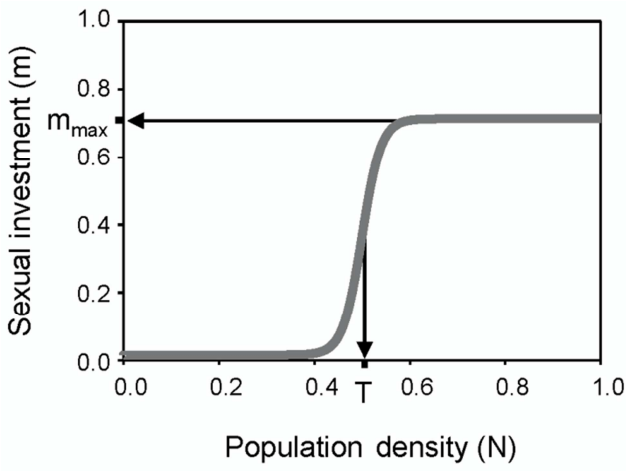

Figure 6.1. Relationship between sexual investment (m) and population density (asexual +sexual), as modelled by Eq. 6.3 (T = 0.5, m_{max} = 0.7 and z = 50).

approximation and gives extremely complex equations. This makes it unfeasible to determine the equilibrium values for a two-species system as well as to perform an invasibility analysis, in which the equilibrium density for a system with a single species, the resident, needs to be found.

Alternatively, details of the functional relationship between sexual investment and density can be ignored, while the well-known features (i.e., sex investment determined by density, and sex induced at a density threshold) are taken into account. It can be assumed that, if only one species (the resident) occurs, an equilibrium population density that is greater than zero is achieved, and at that density sexual investment is m_i*, while sexual investment is m_0 for the low-density invader species. Therefore, if $dA_i/dt = dS_i/dt = 0$, $A_i > 0$, and $N_{j\neq i} = 0$, then $m_i(N_i) = m_i$*.

Alternatively, if $N_i \rightarrow 0$, then $m_i(N_i) \rightarrow m_0$. Using these assumptions, an invasibility analysis is possible.

Two different scenarios are possible: (a) Species 1 (i.e., the superior competitor if an asymmetry exists) as a resident at its single-species equilibrium and Species 2 as invader; and (b) the opposite situation. Densities at equilibrium (A_i^* and S_i^*) for both scenarios were obtained for the resident species. Non trivial solutions for each scenario (Sol. 6.1a is for the scenario a) are:

$$A_1^* = \frac{b_{\max} K_1 - b_{\max} K_1 m_1^* - K_1 q}{b_{\max} - q}$$
$$S_1^* = \frac{b_{\max} K_1 (m_1^*)^2 + K_1 m_1^* q - b_{\max} K_1 m_1^*}{(m_1^* - 1)(b_{\max} - q)} \qquad \text{[Sol. 6.1a],}$$

$$A_2^* = \frac{b_{\max} \beta K_1 - b_{\max} \beta K_1 m_2^* - \beta K_1 q}{b_{\max} - q}$$
$$S_2^* = \frac{b_{\max} \beta K_1 (m_2^*)^2 + \beta K_1 m_2^* q - b_{\max} \beta K_2 m_2^*}{(m_2^* - 1)(b_{\max} - q)} \qquad \text{[Sol. 6.1b],}$$

Notice that $A_i^* > 0$ needs $m_i^* < (b_{max} - q)/b_{max}$. If not, birth rate is overcompensated by the combined effect of investment in sex and mortality.

The per capita rate of increase for the invader species was obtained by equaling the resident species densities to their densities at equilibrium (A_i^*, S_i^*), assuming that the invader was composed exclusively by asexual individuals, and the sex investment of the invader

is m_0. The per capita growth rates corresponding to scenario a and b are respectively (see Appendix C):

$$\frac{dA_2}{dt}\frac{1}{A_2} = \frac{-b_{\max}(m_o - 1)(m_1^* - 1)(\beta - 1) + q(m_0 + \beta - \beta\, m_1^* - 1)}{\beta\,(m_1^* - 1)} \quad \text{[Sol. 6.2a]}$$

$$\frac{dA_1}{dt}\frac{1}{A_1} = \frac{b_{\max}(m_o - 1)(m_2^* - 1)(\beta - 1) - q(m_2^* + \beta - \beta\, m_0 - 1)}{(m_2^* - 1)} \quad \text{[Sol. 6.2b]}$$

As the invasion analysis assumes a low density of invader, no investment in sexual reproduction is likely to happen ($m_0 = 0$), consistent with the observation in the wild of a completely asexual phase in cyclical parthenogens. However, at equilibrium density sexual reproduction is expected to occur. From these assumptions ($m_i^* > 0$, $m_0 = 0$), the following relationships are found for an invasion to occur (i.e., for $(dA_i/dt)(1/A_i) > 0$)

Species 2 is able to invade if

$$\frac{1}{\beta}\left(1 - \frac{b_{\max}}{r} m_1^*\right) < (1 - m_1^*) \quad \text{[Sol. 6.3a]}$$

Species 1 is able to invade if

$$\beta\left(1 - \frac{b_{\max}}{r} m_2^*\right) < (1 - m_2^*) \quad \text{[Sol. 6.3b]}$$

Here, $r = b_{max} - q$, $0 < \beta \leq 1$ and $0 < m_i^* \leq r/b_{max}$ and these parameters belong to $\mathbb{R}$ [0, + ∞[. According to Sol. 6.3b, Species 1 (the superior competitor) is always able to invade. Note that b_{max}/r is larger than 1. Sol. 6.3a shows that the invasion capability of Species 2 depends on the amount of investment in sex of the resident species and the level of competitive asymmetry (Fig. 6.2). Therefore, Sol. 6.3a is the condition

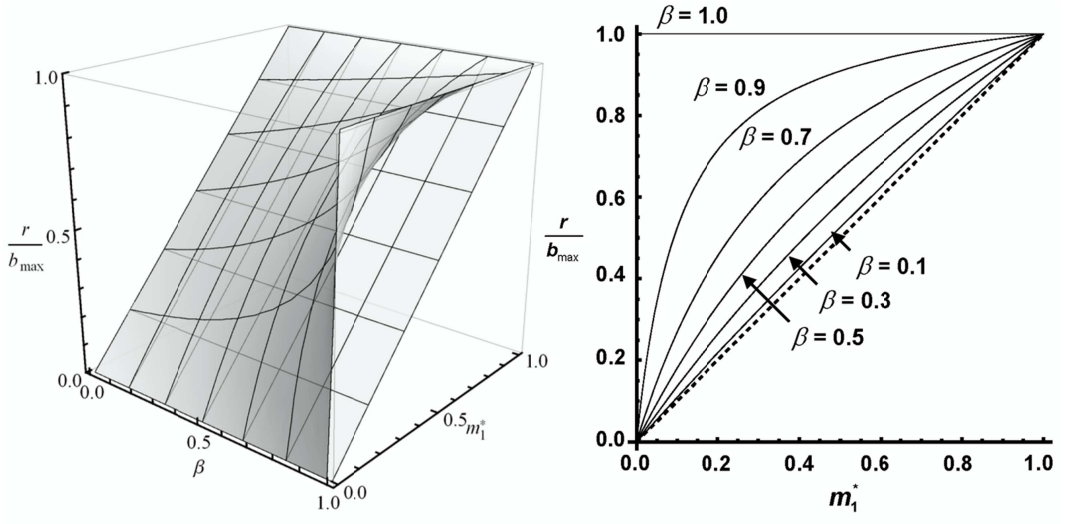

Figure 6.2. Invasion capability of an inferior competitor when it is not investing in sex ($m_0 = 0$). *Left panel*. Parametric space defined by r/b_{max}, the density-dependent sexual investment at equilibrium of the resident species (m_1^*) and relative competitive ability of the invader (β). The linear surface is defined by the maximum possible sexual investment at equilibrium ($m_i^* = r/b_{max}$), so that the values below that surface do not allow permanence of the resident species. The non-linear surface defines an edge for a positive growth rate of an invader being competitively inferior or equivalent to the resident, so that all the values below that surface imply successful invasion. Hence, all the values between both surfaces allow stable coexistence. Right panel. Slides for different values of β of the parametric space shown on the left panel. Dotted line shows the maximum possible investment in sex. Values between solid lines and the dotted line are the parametric values allowing stable coexistence.

for reciprocal invasibility and hence for stable coexistence. Accordingly, species with identical competitive abilities (β = 1) are able to coexist if some investment in sexual reproduction is made by the resident. Interestingly, as sexual investment increases, higher degrees of asymmetry in competitive abilities are still compatible with stable coexistence.

According to Sol. 6.3, if no species is investing in sex ($m_i^* = m_0 = 0$), invasion capability $(dA_i/dt)(1/A_i)>0$ results in $\beta > 1$ and $1/\beta > 1$ for scenario a and b respectively, which never can be accomplished. Thus, Species 2 (i.e. the inferior competitor) is not able to invade in any case. The second condition is always accomplished except for $\beta = 1$. That is, with no sex, Species 1 will be able to invade the resident population except if it is competitively equivalent. These results are the expected ones under a conventional Lotka-Volterra model for interspecific competition.

As stated in Eq. 6.1a, our model assumes that the sexual investment of a species is dependent only on the conspecific population density; that is, signal for sex is species-specific. Additionally, we modified the model to allow partial cross induction between species. In this modification, we used Eq. 6.3 but with the variable N_i substituted by $N_i+\delta N_j$, where δ accounts for the similarity between species in their sex-inducing signals (i.e. δ =1 and δ =0 imply respectively complete cross-induction of sex and total specifity of the signal). We explored this scenario by numerical integration. The model was parameterized using a cyclical parthenogenetic rotifer as biological model. K_1 and b_{max} were rescaled to 1, and q was assumed to be 0.2, which, if $b_{max} = 1\ d^{-1}$, gives a

maximum population growth of 0.8 d^{-1}, a realistic value for rotifers (Serra et al. 2005). Several values for two of the three parameters controlling investment in sex, the threshold density (T) and the maximum theoretical investment in sexual reproduction (m_{max}), were explored. The other parameter, z, was fixed at a value (50) high enough to cause an almost "on-off" response, in agreement with empirical observations (Snell & Boyer 1988). δ values in the range 0.0-1.0 were tested and we found that stable coexistence, is still possible, although it becomes more unlikely as value of δ increases (Fig. 6.3).

Discussion

In this paper we identify a heretofore unidentified mechanism that could explain the coexistence of ecologically equivalent species by means of density-dependent sex and/or diapausing investment. In our model, the ultimate reasons for the loss of competitive ability with increasing density are the negative effects of male production and diapause on current population growth. Sexual reproduction and diapause allocates resources that do not translate into current population growth or competition efficiency. Thus, depending on the amount of sex and/or diapause investment, this creates an opportunity for another species to invade a sex and/or diapause-investing resident population, even if the invader is an inferior competitor. This is a novel extension of the conclusions of Zhang and Hanski (1998) of how mechanisms based on sexual reproduction could allow the coexistence of ecologically equivalent species without niche differentiation.

It is unlikely that any pair of species can be identical in all of their ecological traits, yet our results for identical species demonstrate that

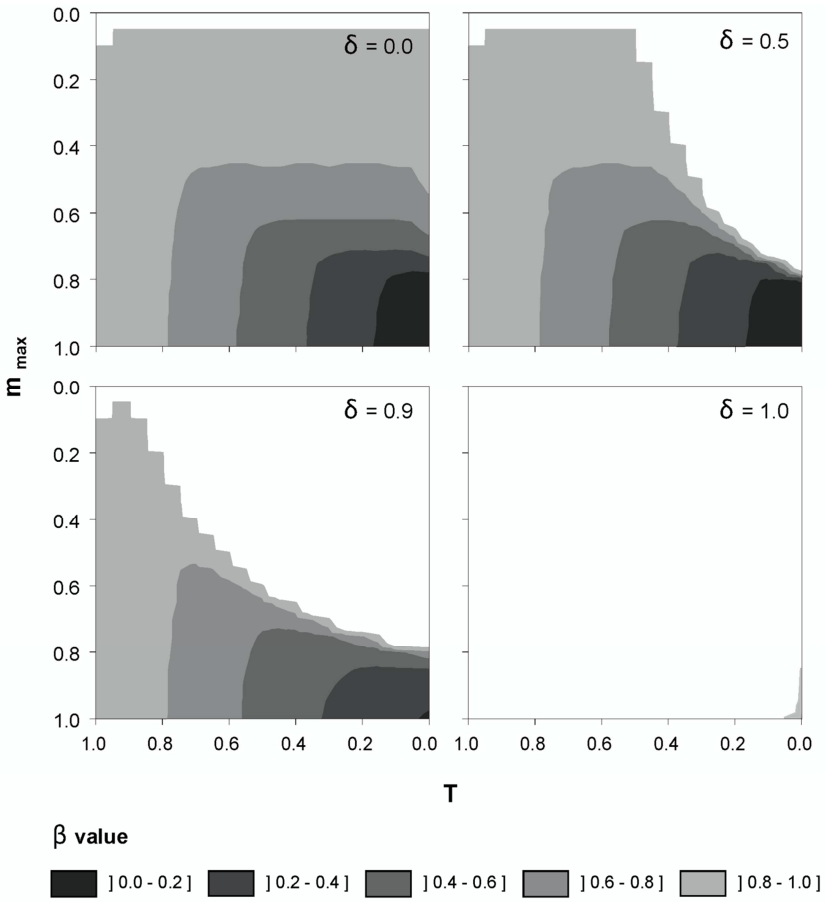

Figure 6.3. Stable coexistence of two species with density-dependent investment in sex under different degrees of heterospecific induction of sexual investment (δ). Sex investment is defined in terms of T and m_{max}. The range of asymmetry in the competition (β) that allows coexistence is also shown. Note that no stable coexistence was observed for $m_{max} = 0$.

coexistence is possible even in this most stringent case. To date, no study has focused on the effect of density-dependent sex or diapause investment on coexistence. However, Ciros et al. (2002) studying the competitive success of three sympatric cryptic cyclical parthenogenetic species from the *Brachionus plicatilis* species complex found a negative

relationship between their sexual reproduction investment and competitive success. Our results based on modelling provide guidelines for future empirical studies on competition. For instance, the coexistence of species with no differential predation vulnerability, exploiting a single resource in a constant environment has been shown here to be theoretically possible, and can be tested with simple experiments. We further advocate that studies attributing species coexistence to niche differentiation, also need to consider the possibility of differential sex and diapause investment.

A potential concern about our study is that no species differentiation in the timing of sex would be expected if species niches completely overlap. This concern is based on the assumption that the switch to sexual reproduction occurs when conditions are adverse, e.g., when the density of both the resident and invading species is high, so that birth rate decreases due to competition. This is called the habitat deterioration hypothesis of sex initiation and is only one of several plausible scenarios (Serra et al. 2004). For example, species could have evolved different population density signals in allopatry, as a response to physical conditions in their environments. It is known that cryptic rotifer species, which currently coexist, had separate refugia during Pleistocene glaciations (Gómez et al. 2002). Perhaps of most importance, the timing of sex in facultative sexuals is expected to be shaped not only as a response to anticipated environmental adversity, but by mate encounter probability, which is strictly species-specific. That is, signals for initiating sex are part of quorum sensing mechanism (Kubanek & Snell 2008). Because our model deals with real –i.e., reproductively isolated—species, sex induction at low density is not

expected to evolve. Otherwise, male-female encounter is unlikely to occur. Moreover, our model suggests this specificity will be evolutionarily stabilized by competition, since that the low density species has invading opportunities by delaying sex (see below).

A complete differentiation in the signals for sex and diapause seems unlikely in the case of closely related species and some level of cross-induction due to heterospecific population density could be expected. For example, in rotifers of the genus *Brachionus* some degree of cross-induction has been reported (Stelzer & Snell 2006; García-Roger et al. 2009). Our simulation results also have shown that partial cross-induction still allows coexistence between cyclical parthenogenetic species sharing identical niches. However, an open question is how other density-independent sex or diapause inducing signals would interact with density-dependent sex induction. For example, sexual reproduction in the cladoceran *Daphnia magna* is induced by a suite of factors including crowding, temperature, food level and photoperiod (Gyllström & Hansson 2004). Some of these factors may exert their effects by altering patterns of temporal niche differentiation.

A second concern about our model is that sex is assumed to make no immediate contribution to current population growth, whereas the diapausing eggs produced sexually could be relevant to coexistence through evolutionary time. However, since sex typically is associated with diapause, sex involves a short-term cost additional to the two-fold cost. This cost results from longer generation times and lowered survival (Serra & Snell 2009). As a result, sexual offspring are expected to make a negligible contribution to population growth, which is primarily the result of asexual reproduction with short generation times. Of course,

these costs should be compensated if, as expected, the life cycle is adaptive. During adverse periods, which are recurrent in many habitats, the active populations disappear, and recolonization relies on the diapausing stages. Then, a longer timescale becomes relevant. However, as long as both species are able to produce viable diapausing eggs, our conclusion on coexistence stands. This is because the invasion process analyzed by our model is merely suspended when habitats are unsuitable, and resumes when conditions favorable for growth return. If invasion is successful in one of the favorable periods, it will be successful the following one as well.

An additional question is how stochasticity interacts with the deterministic dynamics of our models. The coexistence showed by our model is not neutral, and the recovery of rare species found in our analysis is expected to provide some protection against extinction due to random walks. However, the abundance reached by the inferior competitor is relevant to evaluate the effects of demographic stochasticity on random extinction. Nevertheless, at least for some groups where our model is applicable, even low population densities imply large population sizes, making unlikely a strong effect of demographic stochasticity. Moreover, the formation of diapausing banks could buffer against stochasticity, protecting the inferior competitor (Chesson 2000). Interestingly, weak stochastic effects might suggest that coexistence of ecologically equivalent species might be neutral. Even accepting this hypothesis as plausible, it needs to be contrasted with non-neutral models incorporating relevant lifecycle features of the species involved, and accounting for stable coexistence, as the model developed here.

A question arising from our results is whether the evolution of sexual and/or diapause investment patterns might be evolutionarily shaped by interspecific competition. For instance, a superior competitor would not be invaded by an inferior one if the former is not investing in sex or diapause. However, this investment is necessary to survive through adverse environments or to generate genetic variability. A species that invested less in sexual reproduction and became a better competitor might be compromising its own long-term persistence. Therefore, a trade-off is likely to exist, and an optimal level of sexual and/or diapause investment is expected to evolve. Our results suggest that this optimal level would still mediate coexistence, since coexistence was found with low sex investment. As another example, a highly species-specific response to the sex-inducing signal could be costly (e.g. it could require the maintenance of complex enzymatic machinery to produce the signalling molecule) and be selected against due to this cost. However, it could confer a competitive advantage to an inferior competitor, particularly if the superior competitor has not evolved a species-specific signal. It is an open question if the rates of competitive exclusion would provide time enough for the evolution of differentiation in sexual signals.

The coexistence mechanism identified here could be extended to other species with life cycles where cost of males or cost of diapause are density-dependent (i.e., obligate sexual and obligate asexuals investing in diapause, and facultative sexuals that are not cyclical parthenogens). More generally, density-dependent life-cycle switches – such density-dependent sex or diapause investment –, density-dependent sex ratio mediated by local mate competition (Zhang & Hanski 1998; Zhang et al.

2004), and perhaps other density-dependent switches – such as investment in dispersal in aphids (Braendle et al. 2006) – show that plasticity in life-history traits could cause a decrease in growth rates with density, so that coexistence of competitors would be promoted. As life history theory has demonstrated, these traits are evolutionarily shaped by a suite of selective factors including intraspecific relationships, interspecific competition, predation, parasitism and abiotic conditions (Roff 1992). Thus, where optimal life-history trait values are not determined uniquely by interspecific competition, competitor coexistence might be possible.

Acknowledgements

We are grateful to María José Carmona, Africa Gómez, Charles E. King and Terry W. Snell for helpful suggestions and comments, and to Paula Tuzón for mathematical advice. We also want to thank to several anonymous referees that worked on previous versions of this paper.

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7

Final remarks and conclusions

Diapause is a fascinating phenomenon; a “time travelling” stage to endure adverse periods and a “prophetic” life history trait that foresees the deterioration of the habitat. Diapause is involved in a wide variety of ecological and evolutionary processes ranging from the population to the ecosystem level: from population demography to community structure, from current population growth to long-term dynamics, from trade-offs on the life history traits of the individuals to genetic differentiation and divergence of populations. Evolutionary and ecological processes act at overlapping time scales, interplaying and resulting in a complex eco-evo dynamics. The awareness and understanding of such dynamics has increased enormously in the last decade. This thesis inserts in that conceptual framework. It explores the ecological and evolutionary implications of diapause at a population

level and explores its impact at a community level, an aspect that had attracted little attention until now.

In temperate latitudes, continental water bodies typically suffer changes that make continuity of life along the year unfeasible due to the adverse physical environmental conditions. In many cases, this variation is highly unpredictable; a prototypical example is the salt lakes in the Mediterranean regions (e.g., Comín et al. 1992). To cope with such discontinuity and variability, long term diapause has evolved among many hololimnetic invertebrates – i.e., those not having a terrestrial phase in their life cycle – (De Stasio 1989). Diapause allows these species to survive in their habitats during extended periods of time when conditions are adverse. Diapausing eggs accumulate in the sediment waiting to hatch and forming extensive banks (billions of eggs can be found in moderately sized ponds; De Meester et al. 2004). Such huge dormant reservoirs affect many ecological and evolutionary characteristics. The number of individuals hatching from the bank every season, even when they represent a small fraction of the total number of eggs, is large enough to almost reach the whole carrying capacity immediately after the habitat becomes suitable again. This makes the establishment of migrants very difficult ("high-density blocking"; Hewitt 1993), which reduces the effective gene flow. Thus, diapausing egg banks favor increased among population genetic differentiation (Boileau et al. 1992), and in a longer run, the establishment of strong phylogeographic structures (Waters 2011). Besides this blocking and given the high population growth rates of these organisms, after a new habitat is founded by a few individuals (even by a single individual in the case of parthenogenetic species) high population densities can be

achieved promptly, as well as large diapausing eggs banks, which creates a persistent founder effect that avoids the attainment of the migration-drift equilibrium (Boileau et al. 1992).

Persistent founder effects have been proposed as the main responsible factor involved in the observed pattern in zooplanktonic organisms of high between-population differentiation in neutral genetic markers, despite the high dispersal capability of zooplankton (also called "Dispersal-Gene Flow Paradox"; De Meester et al. 2002). De Meester and coworkers (2002) expanded this explanation to include also the likely effect of local adaptation into neutral genetic differentiation. In this thesis we have studied the relative role of such factors resulting in this Dispersal-Gene Flow Paradox (Chapter 2), and we have shown that persistent founder effects are, indeed, rapidly built and strongly resilent. In contrast, we have found that local adaptation does not seem to play an important role, once founder effects have been established. Nevertheless, local adaptation could be important when the numerical effects are less intense (i.e., in small ponds holding reduced diapausing egg banks or lower population sizes). Our results have opened the path to associate the ecological framework to the genetic differentiation levels, as the most important factors driving genetic differentiation of neutral markers are driven by population size and diapausing egg banks, which are quite dependent on the ecological conditions.

Diapausing egg banks are not only useful to cope with abiotic adversity but also to avoid harsh biotic environments, like for example predators (Hairston & Olds 1984) or, as we show in Chapter 4, intraspecific competition. Thus, diapause interacts at a community level by modifying the trophic and competitive structure. Coexistence of

species can be mediated by diapausing eggs through the storage effect (Chesson 1983; Cáceres 1997), where exclusion is avoided by investing in a competition-insensitive phase. As we have shown (Chapter 4), this mechanism is able to mediate coexistence even when ecological differences between species are subtle, like those expected between cryptic species.

However, investment in diapause can also result in stable coexistence of competing species not by protecting the inferior competitor from extinction (as shown in Chapter 4) but by affecting the competitive ability of the superior competitor. Diapause investment reduces current population growth (see Chapter 5), so an inferior competing species with a low investment could outcompete a superior one with high investment. If this investment is density-dependent, then a stable mechanism arises (Chapter 6), which able to explain coexistence of ecologically equivalent species. Although this "life-cycle switching" has been proposed to be a coexistence mechanism for cyclical parthenogens with a density-dependent diapause and sexual investment, it is a general mechanism which can be extended to other species and to any other different density-dependent investment that results on a reduction of current population growth. Life-cycle switching belongs to a group of coexistence mechanisms not based on niche differentiation, which are scarce (Zhang & Hanski 1998), and could be of great importance to explain, for example, the coexistence of cryptic species or phylogenetically close species, which can present strongly overlapping niches.

In this thesis, experimental, observational, and theoretical approaches have been used to gain insights on the implications of

diapause. We would like to stress the importance of using theoretical models and modeling approaches for the study of evolution and ecology (Levins 1966; Hastings 1997; Lynch & Walsh 1997). Such approaches allow dissecting complex and multifactorial hypothesis, as we have shown in Chapter 2 regarding the Monopolization Hypothesis, or to understand and predict the ecological and evolutionary implications of diapause for species coexistence (Chapters 5 and 6). Besides the theoretical and empirical contributions to the understanding of diapause, we have developed two different methodologies with a high potential for aquatic organisms investing in diapausing eggs. We have described a simple and inexpensive DNA extraction method for diapausing eggs of a wide range of aquatic organisms (Chapter 3). This method is based in an alkaline cell lysis protocol, which outperforms the widely used Chelex method as it improves long-term storage and avoids the problems with iminodiacetic acid groups, which can inhibit PCR by chelating Mg^{+2} cations.

The other method developed here allows integrating diapause investment into current population growth (Chapter 5). In life cycles with diapause investment, resources are split into a short-term (current population growth) and a long-term (population survival through periods of unsuitable habitat conditions) component. This division hinders the use of the intrinsic growth rate of population increase (*r*) as performance measure, which is commonly used in many ecological and evolutionary studies, as a lower *r* could result from investing more in diapause and not due to worse performance. This is of special interest when comparing genotypes, populations or species with variable diapause investment or treatments that can lead to a variable

investment. In this thesis, we have derived a related measure to *r*, the potential intrinsic growth rate (r_{pot}), which is the rate of increase that a population/genotype would have if no investment in diapausing stages occured. Despite the computation of r_{pot} requires some simplifying assumptions, we propose that this new performance measure is superior to *r*, and should be used when a variable diapause investment is expected.

Globally, in this thesis an effort to show the implications of diapause at population and community level in zooplankters has been performed. This effort focused in the interplay between ecology and evolution. We anticipate that, in the future, ecological and evolutionary research would increase their integration by incorporating relevant life cycle complexities, and this research will address to bridge the gap between evolution and community ecology. This task requires using modeling, field observations, and field and or laboratory experiments, three arms moving from being loosely connected to becoming a siege to assault scientific questions.

Conclusions

The main conclusions derived from this thesis are enumerated below:

1. Persistent founder effects drive the genetic differentiation of passively dispersed aquatic organisms. These founder effects mainly arise from the formation of extensive diapausing egg banks, high population densities and fast population growth rates (i.e., demographic effects).

2. Demographic effects are able to counteract the impact of migrantion and local adaptation, and only when population sizes are small or no diapausing egg banks are present, do migration, local adaptation, or genetic hitchhiking have a role on shaping the genetic structure of populations.

3. The results of this thesis show the importance of the ecological framework in order to dissect the relative weights of the forces acting on the population genetic structure of passively dispersed aquatic organisms. Aspects such habitat temporal continuity, available resources or body size, can be key to understand the importance of the demographical effects, and thus, its relationship with neutral and selective forces.

4. A new simple, reliable, and inexpensive DNA extraction method has been developed for a wide range of aquatic diapausing eggs. This method outperforms the widely used Chelex method as it improves long term storage and it is a suitable technique for DNA extraction for genetic barcoding or genetic population studies.

5. We have provided for the first time quantitative evidence of the long-term historical co-occurrence of *B. plicatilis* and *B. manjavacas*, two highly similar cryptic rotifer species with potentially wide overlapping niches.

6. *B.* plicatilis and *B. manjavacas* present a differential response to salinity, with *B. plicatilis* growing better at lower salinities than *B. manjavacas.* However, as salinity tolerance ranges widely

overlap, this factor does not limit species occurrence directly, but rather affects their relative fitness.

7. Salinity fluctuations are likely to mediate the coexistence of *B. plicatilis* and *B. manjavacas*, which results in the observed long-term population fluctuations. This suggests the hypothesis that the storage effect is the underlying mechanism explaining the coexistence of these two species.

8. Diapause investment inflicts a quantifiable cost on current population growth, and thus in the competitive abilities of species or genotypes. In cyclical parthenogens, where sex is linked to the production of diapausing eggs, diapause cost includes the cost of sexual reproduction.

9. A new performance measure (r_{pot}) is introduced to integrate diapause investment into current population growth evaluated as the intrinsic population growth rate of increase. r_{pot} is suggested be useful when comparing genotypes or species where differential diapause investment can be expected.

10. The cost of diapause and sex investment creates opportunities for competitively inferior species to outcompete superior ones. We identify a heretofore unidentified coexistence mechanism (i.e. life-cycle switching) that could explain the coexistence of ecologically equivalent species by means of density-dependent sex and/or diapausing investment. Coexistence through this mechanism is possible even when the density-dependent signals

that trigger the cost are partially shared between competing species.

11. Life-cycle switching is a general coexistence mechanism not based on niche differentiation. This mechanism could be extended to species where costs arise from density-dependent life-cycle switches different from diapause and sex investment.

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“But then ...” I venture to remark, “you are still far from the solution...”
“I am very close to one,” William said, “but I don’t know which.”
“Therefore you don’t have a single answer to your questions?”
“Adso, if I did I would teach theology in Paris.”
“In Paris do they always have the true answer?”
“Never,” William said, “but they are very sure of their errors.”

U. Eco, “The name of the rose”

-Pero entonces -me atreví a comentar-, aún estáis lejos de la solución…
-Estoy muy cerca, pero no sé de cuál.
-¿O sea que no tenéis una única respuesta para vuestras preguntas?
-Si la tuviera, Adso, enseñaría teología en París.
-¿En París siempre tienen la respuesta verdadera?
-Nunca, pero están muy seguros de sus errores.

U. Eco, “El nombre de la rosa”

Appendix A

Genetic differentiation in neutral and locally-selected loci when diapausing egg bank is absent (Chapter 2)

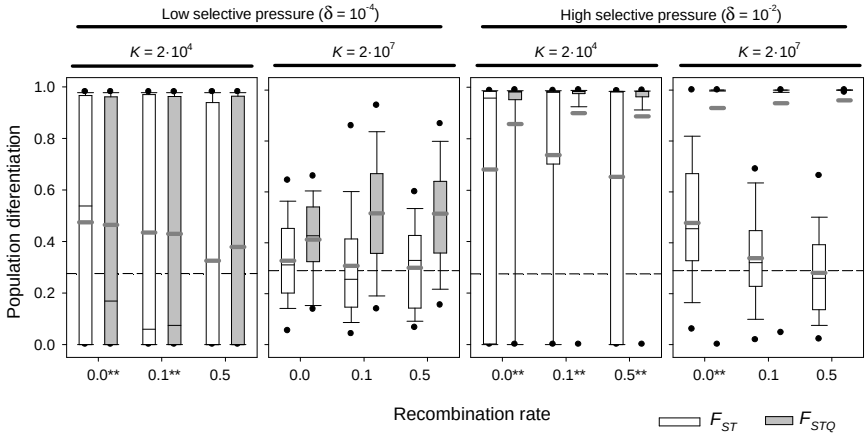

Figure A.1. Box plot graph of F_{ST} and F_{STQ} values after 1000 sexual generations with different recombination rates for two different values of fitness components ($\delta = 10^{-4}$ and 10^{-2} d^{-1}) and without a diapausing eggs bank. For each of the fitness scenarios, the left panel refers to $K = 2 \cdot 10^4$ and the right panel to $K = 2 \cdot 10^7$. The rest of parameters were $r = 0.3$ d^{-1}, $n = 5$, $s = 5$, $F = 1$ and $M = 2$. Data are based on 100 replicates. Boxes represent 25^{th} /75^{th} percentile and black dots 5^{th}/95^{th} percentile. Thin black lines and thick gray lines in each bar represent the median and the mean respectively. Dashed lines show the initial value of F_{ST} after foundation. Asterisks indicate F_{ST} statistically different from those without selection ($\delta = 0$) (**, $\alpha = 0.05$).

Appendix B

Asymptotic convergence to exponential growth in a rotifer population with constant investment in sex (Chapter 5)

The model describing the population dynamic of a parthenogenetic monogonot rotifer (after Serra & King 1999) is:

$$\frac{dF_\sigma}{dt} = b(1-\delta)F_\sigma - dF_\sigma$$

$$\frac{dF_\delta}{dt} = b\delta F_\sigma - dF_\delta$$

where F_σ are the females producing daughters subitaneously and F_δ are the females investing in diapause, either directly or mediated by male production. Dependence on t of both variables is understood. b and d are the intrinsic birth and death rates, and δ is the proportion of eggs from non-investing females that develop into females investing in diapause.

We want to prove that the whole population grows exponentially. First, we obtain the integrated equations for the model

$$F_\sigma = \int_0^t dF_\sigma = F_{\sigma,0}\, \mathrm{e}^{[\mathrm{b}\,(1-\delta)-d]t}$$

$$F_\delta = \int_0^t dF_\delta = \left[F_{\delta,0} + \frac{\delta(\mathrm{e}^{\mathrm{b}\,(1-\delta)\mathrm{t}} - 1)F_{\sigma,0}}{(1-\delta)} \right] e^{-dt}$$

where $F_{\sigma,0}$ and $F_{\delta,0}$ are the values for the functions at $t = 0$.

The ratio between both types of females is

$$\frac{F_\delta}{F_\sigma} = \frac{\left[F_{\delta,0}(1-\delta) + \delta\left(e^{b(1-\delta)t} - 1\right)F_{\sigma,0}\right]}{F_{\sigma,0}\,(1-\delta)} e^{-b(1-\delta)t}$$

Asymptotic convergence of the ratio is given by

$$\lim_{t\to\infty} \frac{F_\delta}{F_\sigma} = \frac{\delta}{1-\delta}$$

Thus, the whole population $F = F_\sigma + F_\delta$ converges to $F_\sigma / (1 - \delta)$, and since F_σ grows exponentially, F converges to exponential growth.

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Appendix C

Model for two identical niche species with diapause/sex investment: solving for coexistence (Chapter 6)

Scenario a: Species 1, resident; Species 2, invader

First, we consider Species 1 to have a sexual investment at equilibrium m_1^* and to be the unique species in the system ($A_2 = S_2 = 0$).

$$\frac{dA_1}{dt} = \left[b_{\max} - \frac{b_{\max} - q}{K_1}(A_1 + S_1) \right] \left(1 - m_1^*\right) A_1 - q\, A_1 = 0$$

$$\frac{dS_1}{dt} = \left[b_{\max} - \frac{b_{\max} - q}{K_1}(A_1 + S_1) \right] m_1^* A_1 - q\, S_1 = 0$$

By setting these equations equal to zero and solving for A_1^* and S_1^*, Sol. 6.1a was obtained as a non-trivial solution. Then, the equilibrium values given by Sol. 6.1a were used to compute the per capita growth rates of asexual and sexual individuals of Species 2, assuming it is composed by asexual individuals having m_0 investment in sex:

$$\frac{dA_2}{dt}\frac{1}{A_2} = \left[b_{\max} - \frac{b_{\max} - q}{\beta\, K_1}(A_1^* + S_1^* + A_2) \right] (1 - m_0) - q$$

In order to obtain the growth rate of Species 2 when $A_2 \to 0$ (i.e., as an invader), we computed

$$\lim_{A_s \to 0} \frac{dA_2}{dt}\frac{1}{A_2} = \frac{-b_{\max}(m_0-1)(m_1^*-1)(\beta-1)+q(-\beta m_1^*+\beta+m_0-1)}{\beta(m_1^*-1)}$$

which needs to be larger than zero for the invasion to occur. Hence, in case the invader is not investing in sexual reproduction ($m_0 = 0$), the condition for the invasion to occur is

$$\frac{dA_2}{dt}\frac{1}{A_2} = \frac{b_{\max}(m_1^*-1)(\beta-1)+q(-\beta m_1^*+\beta-1)}{\beta(m_1^*-1)} > 0$$

which, as $m_1^* < 1$, gives

$$b_{\max}(m_1^*-1)\ (\beta-1)+q\ (-\beta(m_1^*-1)-1) < 0$$

$$(b_{\max}-q)\beta(m_1^*-1)\ -b_{\max}(m_1^*-1)-q) < 0$$

and terming $r = b_{max} - q$ gives

$$\beta r(m_1^*-1)\ -b_{\max}m_1^*+(b-q) < 0$$

$$\frac{1}{\beta}(1-\frac{b_{\max}}{r}m_1^*) < (1-m_1^*)$$

which is Sol. 3a.

Scenario b: Species 2 at equilibrium, Species 1 invades

First, we consider Species 2 to have a sexual investment at equilibrium m_2^* and to be the unique species in the system ($A_1 = S_1 = 0$).

$$\frac{dA_2}{dt} = \left[b_{\max} - \frac{b_{\max}-q}{\beta K_1}(A_2+S_2)\right]\left(1-m_2^*\right)A_2 - q\,A_2 = 0$$

$$\frac{dS_2}{dt} = \left[b_{\max} - \frac{b_{\max}-q}{\beta K_1}(A_2+S_2)\right]m_2^*A_2 - q\,S_2 = 0$$

By setting these equations equal to zero and solving for A_2 and S_2, Sol. 1b was obtained as a non-trivial solution. Then, the equilibrium values given by Sol. 6.2a were used to compute the per capita growth rates of asexual and sexual individuals of Species 1, assuming it is composed by asexual individuals with m_0 investment in sex:

$$\frac{dA_1}{dt}\frac{1}{A_1} = \left[b_{\max} - \frac{b_{\max} - q}{K_1}(A_2^* + S_2^* + A_1) \right](1 - m_0) - q$$

In order to obtain the growth rate of Species 1 when $A_2 \to 0$ (i.e., as an invader), we computed

$$\lim_{A_s \to 0} \frac{dA_1}{dt}\frac{1}{A_1} = \frac{b_{\max}(m_0 - 1)(m_2^* - 1)(\beta - 1) - q(-\beta m_0 + \beta + m_2^* - 1)}{m_2^* - 1}$$

which needs to be larger than zero for the invasion to occur. Hence, in case the invader is not investing in sexual reproduction ($m_0 = 0$), the condition for the invasion to occur is

$$\frac{dA_1}{dt}\frac{1}{A_1} = \frac{b_{\max}(m_0 - 1)(m_2^* - 1)(\beta - 1) - q(-\beta m_0 + \beta + m_2^* - 1)}{m_2^* - 1} > 0$$

which, as $m_2^* < 1$, gives

$$-b_{\max}\ (m_2^* - 1)\ (\beta - 1) - q\ (m_2^* - 1 + \beta) < 0$$

$$-\beta\, b_{\max}(m_2^* - 1)\ + (b_{\max} - q)(m_2^* - 1) - \beta\, q\ < 0$$

and terming $r = b_{\max} - q$ gives

$$r(m_2^* - 1) - \beta\, b_{\max} m_2^* + \beta\,(b_{\max} + q\ ) < 0$$

$$r(m_2^* - 1) - \beta\, b_{\max} m_2^* + \beta\, r < 0$$

$$\beta\,(1 - \frac{b_{\max}}{r} m_2^*) < (1 - m_2^*)$$

which is Sol. 6.3b.