Heterocypris bosniaca
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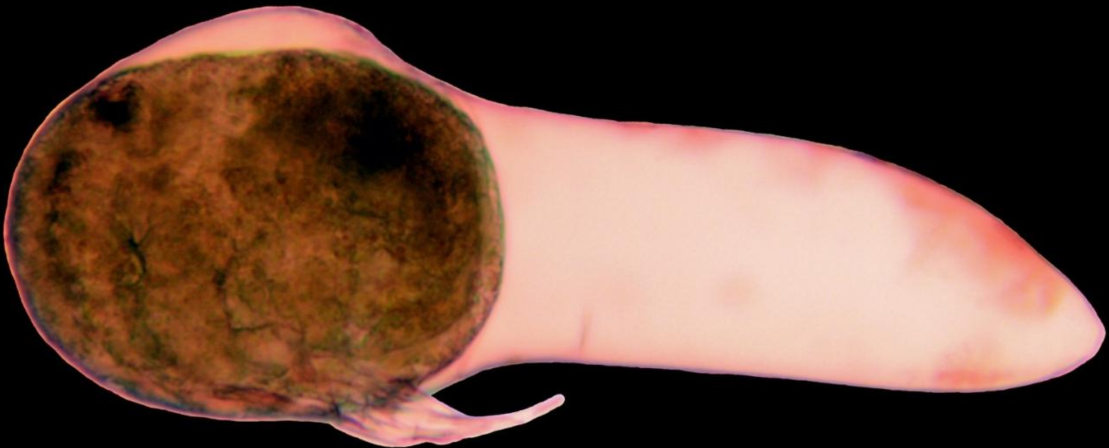

TESIS DOCTORAL

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Heterocypris bosniaca (Petkowski *et al.*, 2000):

Ecología y ontogenia de un ostrácodo (Crustacea: Ostracoda) de pozas temporales.

Tesis doctoral presentada por

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Imagen de cubierta: Vista lateral de la fase eclosionadora de *Heterocypris bosniaca*.
Más detalles en el capítulo V.

Tesis titulada "*Heterocypris bosniaca (Petkowski et al., 2000): Ecología y ontogenia de un ostrácodo (Crustacea: Ostracoda) de pozas temporales*" presentada por JOSEP ANTONI AGUILAR ALBEROLA para optar al grado de Doctor en Ciencias Biológicas por la Universitat de València.

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A Laura, Paco,

i la meua família

Resumen

Los ostrácodos son un grupo de pequeños crustáceos con amplia distribución mundial, cuyo cuerpo está protegido por dos valvas laterales que suelen preservarse con facilidad en el sedimento. En el presente trabajo se muestra la primera cita del ostrácodo *Heterocypris bosniaca* Petkowski, Scharf y Keyser, 2000 para la Península Ibérica. Se trata de una especie de cipridoideo muy poco conocida que habita pozas de aguas temporales. Se descubrió el año 2000 en Bosnia y desde entonces solo se ha reportado su presencia en Israel (2004) y en Valencia (presente trabajo).

En primer lugar se lleva a cabo una descripción del área de estudio. Tras conocer varias pozas con la presencia de la especie se realizó un seguimiento semanal para conocer el ciclo de vida y la dinámica poblacional. A continuación se estudia la supervivencia y otros aspectos ecológicos en respuesta a diferentes tratamientos (desecación, temperatura, contaminantes) controlados en el laboratorio para poder conocer mejor la ecología de las poblaciones naturales. Finalmente se presentan en detalle los cambios en la morfología externa a lo largo de la ontogenia y en los procesos de eclosión.

Los datos obtenidos indican que *H. bosniaca* tiene una estrategia de vida adaptada a tolerar el elevado estrés de ambientes acuáticos temporales de ciclo irregular, donde se observa que puede completar varias generaciones en un año. Su ciclo vital está fuertemente influenciado por la temperatura: la velocidad de crecimiento aumenta con la temperatura, aunque no siempre cumplen la regla de alcanzar mayor tamaño corporal a temperaturas más bajas. La especie muestra una gran resistencia a los contaminantes comparado con otros crustáceos, lo cual puede ofrecerle ventajas en la colonización de nuevos ambientes. Se ha conseguido describir la morfología de todos los estadíos postembrionarios, incluyendo el poco conocido prenauplio, esencial en el proceso de eclosión.

Abstract

The ostracods are a group of small crustaceans with worldwide distribution, whose body is protected by two lateral valves that are easily preserved in the sediment. In this paper we present the first record of the ostracod *Heterocypris bosniaca* Petkowski, Scharf and Keyser, 2000 for the Iberian Peninsula. It is a poorly known cipridoidean species inhabiting temporary pools. It was discovered in 2000 in Bosnia and since then its presence has been reported only in Israel (2004) and Valencia (this work).

Firstly, we describe the study area; after discovering several pools with the presence of the species, these were monitored weekly to investigate its life cycle and population dynamics. Then, we tested the survival and other ecological aspects of the species in response to different treatments (desiccation, temperature, pollutants) under laboratory controlled conditions in order to better understand the ecology of natural populations. Finally, the changes suffered in individual external morphology through the ontogenetic and hatching processes are presented in detail.

The data obtained indicate that the life strategy of *H. bosniaca* is adapted to tolerate the high stress of temporary aquatic environments with irregular hydrological cycles, and it is able to complete several generations in one year. Its life cycle is strongly influenced by temperature: the growth rate increases with temperature, but not always follows the rule of attaining larger body size at lower temperatures. The species shows a great resistance to pollutants compared to other crustaceans, which may offer advantages in the colonization of new environments. In addition, we were able to fully describe the morphology of all postembryonic stages, including the neglected prenauplius, essential in the hatching process.

Índice

Introducción general

Listado de artículos

Esta Tesis Doctoral ha sido escrita en la modalidad de compendio de trabajos previamente publicados o aceptados para su publicación. Consta de las siguientes publicaciones:

- **I.** Aguilar-Alberola, J.A. & Mezquita, F. 2008. On the presence of parthenogenetic populations of *Heterocypris bosniaca* Petkowski *et al*., 2000 (Crustacea: Ostracoda) in temporary rock pools of the eastern Iberian Peninsula, with insights into the ecology and life history of the species. *Bulletin de la Société des naturalistes luxembourgeois* 109: 149-158.

- **II.** Aguilar-Alberola, J.A. & Mesquita-Joanes, F. 2011. Population dynamics and tolerance to desiccation in a crustacean ostracod adapted to life in small ephemeral water bodies. *Limnologica* 41: 348-355.

- **III.** Aguilar-Alberola, J.A. & Mesquita-Joanes, F. (en revisión, 2013). Breaking the temperature-size rule: Thermal effects on growth, development and fecundity of a crustacean from temporary waters. *Journal of Thermal Biology.*

- **IV.** Aguilar-Alberola, J.A. & Mesquita-Joanes, F. 2012. Acute toxicity tests with cadmium, lead, sodium dodecyl sulfate, and *Bacillus thuringiensis* on a temporary pond ostracod. *International Review of Hydrobiology* 97: 375-388.

- **V.** Aguilar-Alberola, J.A. & Mesquita-Joanes, F. 2013. Ontogeny of *Heterocypris bosniaca* (Ostracoda: Cyprididae): Description of postembryonic instars and rediscovery of the neglected A-9 stage. *Journal of Crustacean Biology* 33: 348-371.

Los artículos no se presentan en un orden cronológico de publicación sino en un orden lógico para una mejor comprensión del trabajo global. Los artículos forman los principales capítulos de la Tesis y por lo tanto se les ha adaptado el formato para que sea uniforme y así ofrecer una lectura más cómoda. También se proporciona una copia electrónica de los artículos con el formato original de cada revista.

Ecosistemas acuáticos temporales

Los ecosistemas acuáticos temporales pueden encontrase casi en cualquier parte del mundo. Proporcionan un hábitat muy distinto del que ofrecen los sistemas permanentes puesto que su ciclo hidrológico no es continuo en el tiempo, y por lo tanto la mayoría de especies que los habitan están adaptadas a hacer frente a la pérdida de agua. La presencia de estos tipos de hábitats tan cambiantes favorece la diversidad biológica (Rossetti *et al*., 2006; Jocqué *et al*., 2007), pero, a pesar de que pueden ser focos importantes de biodiversidad, los pequeños cuerpos de aguas temporales han recibido poco interés científico comparado con las aguas permanentes (Céréghino *et al*., 2008). Aparte del interés biológico, muchos de éstos hábitats han estado en conflicto con las actividades humanas puesto que suponen reservorios para vectores de enfermedades como son los mosquitos.

Clasificación de las aguas temporales

Las aguas temporales son increíblemente diversas en cuanto a los hábitats que presentan. Varían desde pequeñas acumulaciones de agua de lluvia que dura unos pocos días hasta grandes charcas que no suelen secarse debido a que están alimentadas por ríos o aguas subterráneas (Williams, 2006). A lo largo de la historia se han intentado clasificar según diferentes factores, pero todavía carecemos de una clasificación ampliamente aceptada.

Un primer intento de clasificación fue atendiendo a su tamaño (Decksback, 1929), en la que se dividen en tres categorías: micro-, meso- y macrohábitats; pero con este sistema se tiende a englobar hábitats que pueden contener comunidades muy dispares. Atendiendo a las adaptaciones de los organismos presentes en el agua, Schmitt (1971) hace distinción entre aguas temporales (si contiene especies adaptadas a la desecación, como por ejemplo que los huevos eclosionan únicamente tras una rehidratación) y permanentes. En otras ocasiones se han intentado clasificar en función de especies indicadoras, pero tienen aplicabilidad únicamente a pequeña escala.

La duración de la fase seca es probablemente el factor medioambiental dominante que afecta la composición de la comunidad de organismos. Por esta razón las principales clasificaciones de las aguas temporales se hacen en base al hidroperiodo. En muchas ocasiones se han utilizado términos como estacional, anual, efímera, temporal o intermitente de manera ambigua. Para evitar confusiones, Comín y Williams (1994) sugieren que el término aguas temporales debe aplicarse únicamente a aquellas con un periodo imprevisible mientras que intermitentes son las que experimentan un secado cíclico. La clasificación más extensa la proporciona Wissinger (1999) en la que toma en consideración componentes importantes para la supervivencia de los organismos como son la permanencia (permanente, semipermanente, temporal), previsibilidad (estacional, interanual, relativamente impredecible), fenología (estacionalidad) del llenado y secado, duración de la fase seca y húmeda, y la severidad del ambiente durante las dos fases. Mediante este sistema se diferencian gran cantidad de tipos de aguas temporales, aunque resulta en un argot muy extenso.

Una propuesta, en la que se intentan evitar las ambigüedades que pueden surgir en las anteriores, es la clasificación multivariante ofrecida por Williams (2006). Consiste en cuatro pasos en los que se van describiendo las características de las aguas temporales. En el primero se especifica el bioma global en el que se sitúa, en segundo lugar se asigna a un carácter hidrológico (siguiendo a Comín & Williams, 1994). A continuación se clasifican según el tamaño (siguiendo a Decksback, 1929) y para finalizar se indica si son de agua dulce o salada porque muchas aguas lénticas temporales son ricas en minerales disueltos (el límite se establece con una concentración límite de 3 g L^{-1}). Se trata de una clasificación con gran flexibilidad puesto que con ella se pueden clasificar desde las lagunas temporales más abundantes hasta las más atípicas. Según esta clasificación, las aguas temporales que encontramos en la zona de estudio se clasificarían como microhábitat dulceacuícola intermitente en matorral Mediterráneo, y según Wissinger (1999) serían pozas de verano seco.

Factores abióticos

En los ecosistemas acuáticos, el balance hídrico es el resultado entre las entradas (precipitación, escorrentía, aguas subterráneas...) y salidas (evaporación, transpiración de las plantas, percolación...) de agua (Hulsmans *et al.*, 2008). Los sistemas temporales se caracterizan por presentar grandes fluctuaciones en ambos parámetros. El clima es el factor que determina la precipitación y evaporación, así que es fundamental en la duración del hidroperiodo. La geomorfología es otro factor importante que define las aguas temporales. En general son poco profundas, así que la composición y tamaño de las partículas de substrato condiciona muchas variables físico-químicas del agua y por tanto puede llegar a determinar la biota presente porque les puede ofrecer protección durante la fase seca.

La temperatura es una variable ambiental muy importante para los organismos. En el caso de pequeñas acumulaciones de agua ésta no sólo varía a lo largo del año sino que también presenta fuertes fluctuaciones diarias. El viento, especialmente por la noche, es el que enfría el agua y también puede remover el substrato del fondo, resuspendiendo partículas y aumentando así la turbidez. La radiación solar es la que calienta el agua, aunque si hay turbidez la radiación se absorbe en la capa más superficial y la temperatura del fondo se puede mantener con menos fluctuaciones. A parte de ser un factor importante que regula la producción primaria, la luz puede afectar muchos aspectos vitales de los organismos como por ejemplo el ciclo vital gracias al fotoperiodo.

El oxígeno disuelto también presenta un patrón de variación circadiano como resultado de la fotosíntesis y la respiración porque el intercambio con el aire tiene relativamente poca importancia (Wetzel, 1983). En algunas charcas pequeñas, el dióxido de carbono puede consumirse completamente durante el día debido a la fotosíntesis mientras que por la noche la respiración produce grandes cantidades de dióxido de carbono (Chan *et al*., 2005). Esto puede hacer variar los valores de pH en el agua si no hay suficientes compuestos disueltos o en el sedimento que puedan ejercer un efecto amortiguador o tampón. La concentración de substancias disueltas varía mucho más que en aguas permanentes debido principalmente al llenado, secado y posibles congelaciones. Desde que se llenan hasta poco antes del secado completo, la conductividad eléctrica del agua se incrementa especialmente debido a la evaporación, aunque el congelado tiene un efecto similar. Estas fuertes variaciones químicas influyen en la composición de especies (Margalef, 1983).

Factores bióticos

A pesar de la gran distancia geográfica, diferencias climáticas y otras características locales, existen muchas similitudes entre la fauna que habita las aguas temporales de todo el mundo. Este tipo de ecosistemas exhiben grandes variaciones en sus características físico-químicas, así que los organismos habitantes deben estar adaptados a esas condiciones. De todos los parámetros ambientales, la perdida de agua es la que más afecta a las especies y especialmente a su dinámica poblacional (Eitam *et al*., 2004; Sanderson *et al*., 2005). Por tanto, la supervivencia depende ampliamente de una excepcional tolerancia fisiológica, modificaciones en el ciclo de vida que les permitan tolerar la desecación y/o habilidades migratorias efectivas.

Las adaptaciones de los organismos a un ambiente acuático intermitente se pueden dividir en tres categorías: latencia, de crecimiento y etológicas. La latencia es una adaptación que permite parar el desarrollo para hacer frente a cambios desfavorables del entorno. Se puede mostrar en forma de quiescencia, respuesta inmediata y directa a un factor limitante, o diapausa, interrupción profunda del desarrollo no directamente controlada por factores ambientales y que precede a la aparición de las condiciones adversas (Alekseev *et al*., 2007). El caso más habitual es la formación de huevos diapáusicos o de resistencia, los cuales pueden sobrevivir varios ciclos de secado, formando así un banco de huevos. Las adaptaciones de crecimiento son aquellas que conllevan modificaciones en el ciclo de vida y programación del momento de aparición. En general, los organismos adaptados a ambientes temporales muestran una curva de crecimiento asimétrica sesgada a la izquierda porque tienen un rápido crecimiento al principio del desarrollo seguido por un crecimiento más lento cuando se acercan a adultos. De esta manera habrán llegado al estado adulto, o cercano, antes de que se produzcan cambios significativos en el ambiente. Muchos insectos completan rápidamente su desarrollo y el estado adulto emerge antes de que el medio acuático desaparezca. Algunas especies depositan huevos de resistencia en el fondo de las charcas secas mientras que otros permanecen vivos hasta que se vuelven a llenar y depositan los huevos directamente en el agua (esta técnica de recolonización suele ser importante en ambientes con carácter cíclico, Tauber *et al*., 1986). Las adaptaciones etológicas son la evitación de la falta de agua a través de la conducta. Por ejemplo, los individuos voladores pueden evitar la fase seca migrando a aguas permanentes, mientras que otros organismos pueden resguardarse excavando túneles en el sedimento.

Los modos de dispersión de estos organismos se pueden agrupar en dos grandes categorías, los activos y los pasivos. La dispersión activa requiere mucha energía, tanto para la producción de las estructuras necesarias como para el desplazamiento en sí. Se conoce bastante la dispersión de insectos adultos alados, la cual incluye diferentes tipos de movimientos que conllevan importantes consecuencias para entender la estructura de las poblaciones como son la migración denso-dependiente de aguas permanentes a otros lugares, la colonización oportunista entre aguas temporales y la colonización cíclica entre aguas permanentes y temporales (Wissinger, 1999). Respecto a la dispersión pasiva, lo más común es que se produzca mediante cuerpos reproductivos en reposo (por ejemplo huevos de resistencia), aunque en algunos casos la etapa larvaria y adulta pueden participar (Vanschoenwinkel *et al*., 2008). Los agentes dispersivos pueden ser abióticos, tales como el viento cuando arrastra formas diapáusicas depositadas en el sedimento mientras las pozas están secas, o el agua, debido a que el nivel puede subir de tal manera que las pozas rebosen y se formen torrentes que esparcen individuos a través de la corriente que se forma. También pueden hacerse servir de vectores bióticos como son los organismos que acuden a las pozas a beber y/o alimentarse. La dispersión pasiva es un proceso más lento y que en general ocurre a saltos cortos mientras que la dispersión activa es más directa (Bilton *et al*., 2001).

El hecho de que exista dispersión no significa necesariamente que haya colonización puesto que existen muchos factores que le pueden afectar. Para poder establecerse en un nuevo sistema es necesario que tengan capacidad de vivir en los nuevos hábitats y sus características físico-químicas, deben ser competitivamente eficaces frente a otros organismos y mostrar una capacidad reproductiva elevada.

El caso concreto de las pozas de roca

Las pozas de roca son cualquier tipo de depresión que pueda haber en el substrato rocoso y que puede contener agua. Aunque existen algunas que se llenan con agua de ríos o subterránea, se va a prestar especial atención a las que se alimentan únicamente por agua de lluvia puesto que son las que se pueden encontrar en la zona de estudio.

Se pueden encontrar sobre granito, arenisca y caliza. La forma y tamaño depende de la climatología de la zona y la erosionabilidad de la roca. Como el granito es difícilmente erosionable, las pozas que se encuentran sobre esta roca suelen ser muy antiguas comparado con las de arenisca que es una roca con un grado de erosión medio. Debido a la rápida erosión que sufre la roca caliza, se pueden formar pozas rápidamente, aunque también pueden perder la capacidad de retención de agua con rapidez. Todas estas pozas son muy susceptibles de sufrir colmatación por acumulación de sedimentos o crecimiento de la vegetación, aunque en ocasiones el viento o algunos animales pueden remover el sedimento y restaurar el ecosistema acuático.

La profundidad de la columna de agua suele ser un buen indicador de la duración del hidroperiodo porque las pozas suelen tener paredes verticales y un fondo bastante plano (Vanschoenwinkel *et al*., 2009). Debido a que este tipo de ambientes depende completamente de la duración y frecuencia de las inundaciones, las comunidades de organismos que contienen reflejan las condiciones climáticas predominantes. A diferencia de otros sistemas mucho más complejos, los pequeños cuerpos de agua son sistemas que pueden usarse para poner a prueba de forma más simple algunas teorías ecológicas (De Meester *et al*., 2005; Scheffer *et al*., 2006).

En la zona de estudio podemos encontrar pozas formadas sobre la roca calcárea que se llenan con agua de lluvia, especialmente en otoño, y permanecen llenas durante el invierno hasta su desecación en primavera. En general contienen poco sedimento, compuesto principalmente por restos vegetales. Las larvas de dípteros y los ostrácodos son los organismos que podremos encontrar con mayor abundancia.

Un mayor detalle en la descripción del área de estudio y las características de las pozas estudiadas se ofrece en el primer capítulo y en el anexo 1.

Introducción a los ostrácodos

El término ostrácodo proviene del griego *óstrakon*: concha, y *eidés*: con aspecto de. Se trata de unos pequeños crustáceos que suelen medir de 0.1 a 5 mm de longitud y su característica más distintiva es la posesión de un caparazón bivalvo que en las especies dulceacuícolas siempre está calcificado (Keyser & Walter, 2004). Estas valvas tienen un elevado potencial de preservación en el ambiente, lo que les permite tener el registro fósil más completo de los artrópodos y por tanto les hace particularmente útiles en estudios paleoecológicos (Rodriguez-Lazaro & Ruiz-Muñoz, 2012). En este uso también les ayuda que hayan recorrido una larga historia geológica puesto que existen restos evidentes desde el periodo Ordovícico, hace unos 485 M.a. (Martens & Horne, 2009). Desde entonces se han dispersado ampliamente por todos los sistemas acuáticos, pudiéndose encontrar tanto en ambientes marinos como dulceacuícolas, e incluso en ambientes semi-terrestres. Gracias a su larga historia geológica los ostrácodos han llegado a ser el grupo con mayor diversidad entre los crustáceos, pues se conocen entre 10.000 y 15.000 especies actuales, llegando a más de 60.000 si también consideramos el registro fósil (Meisch, 2000).

Aunque se los ha llegado a clasificar como una subclase de los Maxillopoda, actualmente está más aceptado que los ostrácodos forman una clase separada dentro de los crustáceos. Se dividen en dos subclases (Martin & Davis, 2001). La subclase Myodocopa (que se subdivide en los órdenes Myodocopida y Halocyprida) únicamente tiene representantes marinos. La subclase Podocopa se subdivide en tres órdenes: Platycopida (con especies marinas y unas pocas de aguas salobres), Palaeocopida (conocida exclusivamente por representantes fósiles) y Podocopida (grupo más diverso de ostrácodos con representantes en aguas dulces, salobres y marinas) (Martens *et al*., 2008). En esta tesis se analizan aspectos vitales de la especie dulceacuícola *Heterocypris bosniaca*, la cual pertenece al orden Podocopida, superfamilia Cypridoidea (Tab. 1).

Tab. 1. Posición taxonómica de las familias de ostrácodos no marinos actuales en la que se indica en número total (N) de géneros (gen) y especies (sp) descritas según Martens y Savatenalinton (2011) y Mestre *et al*. (2012).

	N	
Clase Ostracoda Latreille, 1806		
Subclase Podocopa Müller, 1894		
Orden Podocopida Sars, 1866		
Suborden Cypridocopina Baird, 1845	gen.	sp.
Superfamilia Cypridoidea Baird, 1845		
Familia Candonidae Kaufmann, 1900	98	666
Familia Cyprididae Baird, 1845	51	1002
Familia Ilyocyprididae Kaufmann, 1900	1	34
Familia Notodromadidae Kaufmann, 1900	8	35
Superfamilia Darwinuloidea Brady & Robertson, 1885		
Familia Darwinulidae Brady & Robertson, 1885	5	31
Superfamilia Cytheroidea Baird, 1850		
Familia Cytheridae Baird, 1850	2	15
Familia Cytherideidae Sars, 1925	7	100
Familia Cytheruridae Müller, 1894	2	5
Familia Entocytheridae Hoff, 1942	34	220
Familia Hemicytheridae Puri, 1953	1	1
Familia Kliellidae Schäfer, 1945	2	2
Familia Leptocytheridae Sars, 1925	1	21
Familia Limnocytheridae Klie, 1938	22	151
Familia Loxoconchidae Sars, 1925	4	16
Familia Xestoleberidae Sars, 1866	1	5
Superfamilia Terrestricytheroidea Schornikov, 1969		
Familia Terrestricytheridae Schornikov, 1969	1	4

Morfología básica de los ostrácodos de agua dulce

El **caparazón** está formado por dos valvas laterales que cubren todo el cuerpo y están articuladas en la parte superior (Horne *et al*., 2002). Cada valva está formada por una excrecencia doble de la pared corporal, dando lugar a una lamela interna y otra externa. Mientras que la lamela externa está completamente calcificada, la calcificación en la interna está restringida a una banda periférica más o menos ancha y la parte proximal al cuerpo es membranosa (Fig. 1). El caparazón tiene una funcionalidad importante debido a que engloba las partes blandas y las protege de los depredadores y otros agentes externos. En general, pueden encerrar las extremidades entre las valvas, pero cuando las abren, el extremo de algunos apéndices puede sobresalir. También forma parte del exoesqueleto sirviendo de anclaje para algunos músculos de los apéndices como los mandibulares. En algunas especies ofrece protección contra la desecación del hábitat cuando lo cierran por completo.

La segmentación del cuerpo está muy reducida, con lo que resulta muy complicada la separación entre las típicas divisiones corporales de los artrópodos en céfalon, tórax y abdomen (Namiotko *et al*., 2011). Se considera que los apéndices de los ostrácodos derivan de un apéndice birrámeo excepto el primer par de apéndices que se considera, del mismo modo que el resto de los crustáceos, unirrámeo (Boxshall *et al*., 2010). Los apéndices varían considerablemente en cuanto a su tamaño, morfología y función, tanto entre especies como a lo largo de la ontogenia dentro de la misma especie, y están adaptados al hábitat y modo de vida (Karanovic, 2012). La mayoría tienen ocho pares de apéndices cuando son adultos (Fig. 1): cuatro cefálicos (anténula, antena, mandíbula y maxílula), tres torácicos (quinta pata, pata caminadora y pata limpiadora) y el cuerpo terminado en la rama caudal.

La **anténula** es el primer apéndice cefálico y tiene una función locomotora y sensorial. El segundo apéndice es la **antena** y aunque es claramente birrámea, el exopodio está muy reducido. Tiene función locomotora y sensorial, aunque también las pueden hacer servir para sujeción y manipulación de alimentos gracias a las garras que tienen en el extremo. La **mandíbula** es el siguiente apéndice e interviene principalmente en la alimentación. En los Cypridoidea está formada por una coxa robusta con dientes, un palpo con función sensorial que también sirve para la manipulación de los alimentos y una placa respiratoria que se corresponde con el exopodio. El último apéndice cefálico es la **maxílula**. Orientado hacia la abertura bucal presenta tres enditos y el palpo maxilular (endopodio), que intervienen en la alimentación. En sentido opuesto está el exopodio, el cual se corresponde con una gran placa branquial. Los apéndices cefálicos se unen al céfalon en una estructura que también alberga los llamados órganos rastrillo o **RLO** (rake-like organ) que ayudan a masticar los alimentos. El quinto par de apéndices es el **primer toracópodo**. En los Cypridoidea aparece con forma de extremidad caminadora y a lo largo del desarrollo se va modificando hasta alcanzar la morfología de un maxilípedo con dimorfismo sexual. La base de la extremidad (protopodio) tiene setas sensoriales y en la parte delantera termina con numerosas setas con función alimenticia. El exopodio es una placa branquial muy reducida mientras que el endopodio o palpo en los machos de algunas especies está transformado en ganchos (*claspers*), unos órganos que usan para sujetar a las hembras durante la cópula (Athersuch *et al*., 1989). El segundo toracópodo es una **extremidad caminadora** con una morfología muy similar en todos los Podocopida. El tercer toracópodo es una extremidad caminadora excepto para los Cypridocopina, en los que se da la vuelta hacia la parte dorsal y poseen una pinza con función de limpieza, y por ello se la suele conocer como **extremidad limpiadora**. El último par de apéndices es la **rama caudal**, la cual tiene una función locomotriz.

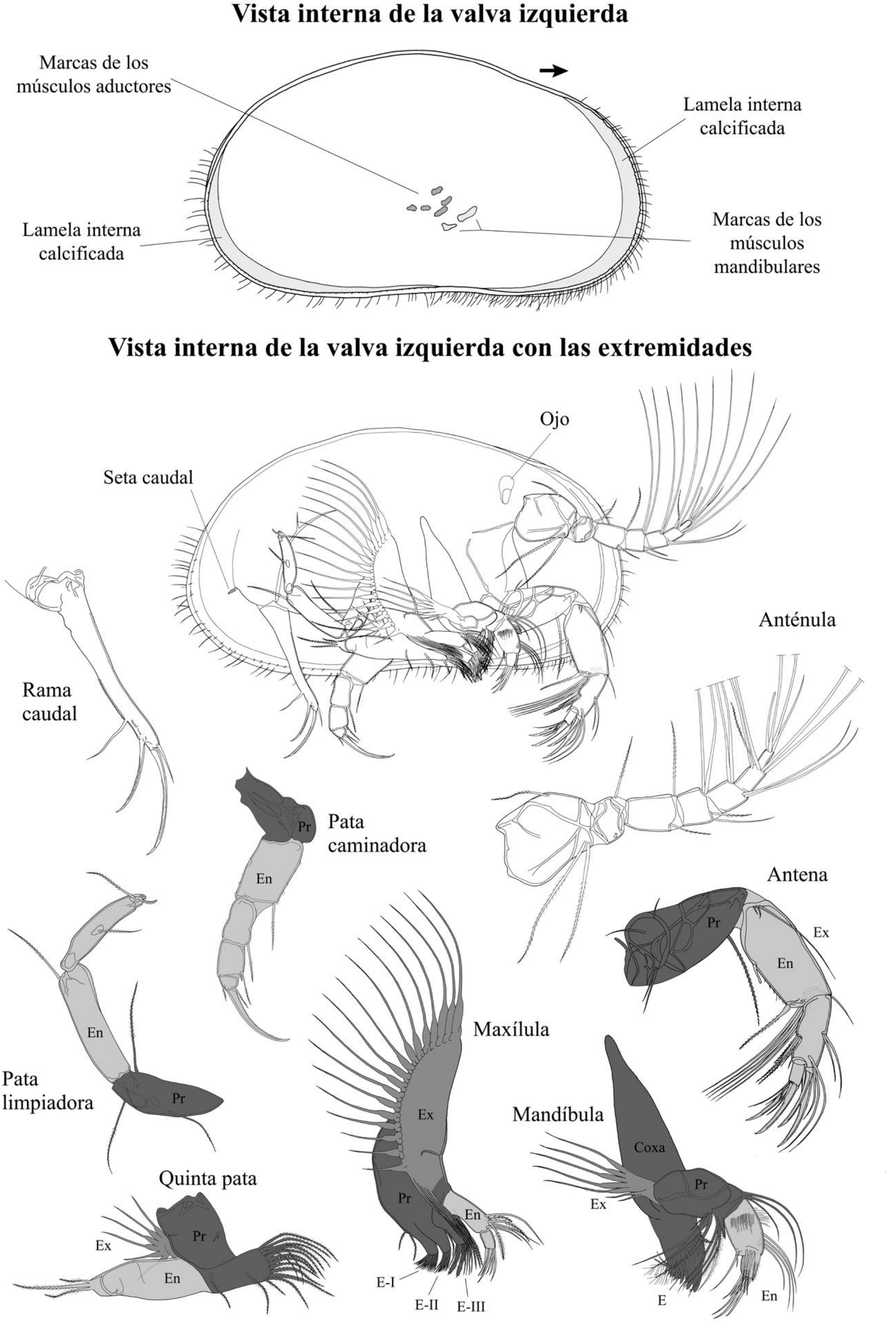

Fig. 1. Morfología de una hembra de *Heterocypris bosniaca* como representante de un ciprídido típico (Podocopida, Cypridoidea, Cyprididae). Se proporciona información más detallada en el capítulo V. Pr: protopodio; En: endopodio; Ex: exopodio; E: endito.

Reproducción

El dimorfismo sexual es común en los ostrácodos. A menudo pueden tener grandes diferencias en los rasgos externos de las conchas y en general el macho es más grande que la hembra (Cohen & Morin, 1990), aunque en el caso de la mayoría de los Cyprididae del género *Heterocypris*, al que corresponde la especie estudiada, los machos suelen tener menor tamaño (Meisch, 2000).

Los órganos reproductores son pares en ambos sexos. El sistema reproductor del macho es destacable por su complejidad y tamaño, llegando a ocupar hasta un tercio del volumen corporal (Danielopol *et al*., 1989). El par de órganos copuladores se conocen como hemipenes, aunque se cree que han evolucionado desde un par de apéndices abdominales, y están situados por detrás del tercer par de apéndices torácicos. Una particularidad, en especial de los Cyprididae, es la presencia de espermatozoides gigantes, los cuales pueden llegar a medir hasta más de 10 veces la longitud corporal. Así que en términos relativos, tienen el espermatozoide más largo del reino animal (Wingstrand, 1988; Matzke-Karasz, 2005). Las gónadas de las hembras están formadas por lóbulos genitales, en los cuales se aloja el ovario, el oviducto y la abertura vaginal. En los cipridoideos llegan a adentrarse en el espacio entre las lamelas de las valvas, dejando marcas en la parte calcificada. Los espermatozoides se reciben en los receptáculos seminales de las hembras alojados en los lóbulos genitales. Los huevos pueden ponerlos aislados o en grupos, aunque en algunas especies pueden guardarlos en unas cámaras de cría situadas en la parte posterior del caparazón de la hembra hasta su eclosión y en algunas ocasiones hasta que los juveniles alcanzan cierto tamaño (los Cypridoidea nunca muestran cuidado parental, Cohen & Morin, 1990).

Se han identificado cuatro modos de reproducción en ostrácodos no marinos (Meisch, 2000). La reproducción **bisexual** o completamente sexual es la que hace referencia a la amfimixis en la que siempre intervienen machos y hembras. En la reproducción **mixta** existen individuos con reproducción sexual y otros que se reproducen mediante partenogénesis ameiótica (en la que no existe meiosis y el huevo se forma por mitosis y por tanto es diploide). Cuando una especie muestra este tipo de reproducción podemos encontrar tres tipos de poblaciones, las bisexuales, las partenogenéticas, y las mixtas, en las que hay machos y hembras diploides sexuales y hembras partenogenéticas, que si se reprodujeran con los machos podrían dar lugar a hembras multiploides y así introducir variabilidad genética en las poblaciones clonales (Butlin *et al*, 1998). Las especies que muestran una reproducción **partenogenética en poblaciones recientes** son aquellas que parecen completamente partenogenéticas, pero en realidad se conocen unas pocas poblaciones en las que hay machos. Cuando la especie ha persistido largos periodos geológicos exclusivamente con reproducción partenogenética se considera que **tiene reproducción partenogenética ancestral**.

Cuando una especie muestra reproducción mixta, las poblaciones partenogenéticas suelen tener una distribución más extensa que las poblaciones sexuales (Simon *et al*., 2003). En el caso de los ostrácodos, las poblaciones asexuales son raras en ambientes marinos o en lagos antiguos, pero a menudo son dominantes en hábitats dulceacuícolas pequeños de reciente formación geológica (Bell, 1982). Esta predominancia de las poblaciones asexuales se debe probablemente a dos factores (Chaplin *et al*., 1994): (i) las transiciones a la asexualidad pueden ser más comunes en estas condiciones, y (ii) la mayor capacidad dispersiva de los partenogenéticos puede ayudar a ocupar esos hábitats debido a que un simple individuo en cualquier fase del desarrollo (desde el huevo hasta el adulto) puede formar una población completa.

Se ha postulado que la estabilidad climática durante la última parte del Holoceno ha podido tener un fuerte impacto en la expansión de las poblaciones asexuales hacia el norte de Europa debido a que son más exitosas estableciendo poblaciones en ambientes estables a corto plazo, mientras que las sexuales permanecen con una distribución circummediterránea (Horne & Martens, 1999). Aunque la mayoría de los modelos teóricos predicen que la reproducción sexual debe tener una ventaja selectiva en entornos inestables, los resultados con ostrácodos no resultan del todo esclarecedores (Chaplin & Ayre, 1989; Chaplin, 1992). Las especies que tienen reproducción partenogenética, especialmente las ancestrales, deben poseer mecanismos para evitar los posibles efectos negativos que tiene la ausencia de recombinación (acumulación de mutaciones deletéreas) como por ejemplo mecanismos de reparación de ADN muy eficientes (Schön & Martens, 2003).

Desarrollo

Los ostrácodos se desarrollan a partir de huevos, que tienen una envoltura formada por una pared doble de quitina impregnada de carbonato cálcico con un fluido que rellena el espacio entre las dos (Wohlgemuth, 1914). Esta cubierta hace que el huevo sea una estructura resistente capaz de soportar condiciones ambientales muy desfavorables (Vandekerkhove *et al*., 2013). Los Cypridoidea pueden producir huevos resistentes a la desecación que permanecen viables durante más de 50 años (Martens, 1994). Acerca del desarrollo embrionario en el interior del huevo y el proceso de eclosión se conoce muy poco (Weygoldt, 1960).

Los ostrácodos crecen mediante mudas del mismo modo que el resto de los crustáceos, pero a diferencia de ellos, no reabsorben el calcio del exoesqueleto antes de la muda ni lo almacenan en el cuerpo para construir la siguiente concha sino que lo absorben del agua (Turpen & Angell, 1971). Presentan un crecimiento determinado, lo que significa que una vez alcanzan la madurez sexual no vuelven a mudar. En general tienen nueve estadíos separados por ocho mudas (Kesling, 1951). Los estadíos se designan como: “A” para el adulto, y el resto de estadíos “A-1”, “A-2”... en orden decreciente de tamaño, siendo el “A-8” el primer estadío juvenil de vida libre tras la eclosión. La larva que eclosiona del huevo (estadío A-8) es un nauplio porque tiene tres pares de apéndices bien desarrollados, además tiene el caparazón completamente formado, pero poco o nada calcificado. En las siguientes mudas se van añadiendo extremidades que pueden ir variando de tamaño, forma y función, completando así la quetotaxia (Broodbaker & Danielopol, 1982; Smith & Martens, 2000). Únicamente el adulto está completamente formado y es sexualmente maduro, aunque los órganos reproductores pueden empezar a aparecer en el estadío A-1 o incluso el A-2. La información sobre la duración del desarrollo está limitado a relativamente pocos taxa. Se ha documentado que el ciclo de vida completo puede llevar desde pocos meses hasta varios años (Cohen & Morin, 1990).

Ecología y capacidad dispersiva

Los ostrácodos no marinos son organismos bénticos. Se pueden separar en nadadores y no nadadores según presenten o no setas plumosas largas en las antenas (Smith & Delorme, 2010). Aunque algunas especies pueden ser excelentes nadadoras, ningún ostrácodo dulceacuícola se puede considerar verdaderamente planctónico puesto que suelen nadar entre la vegetación sumergida y cerca del litoral. Los no nadadores usan las antenas para desplazarse o escarbar en el substrato. La mayoría de ostrácodos son predominantemente herbívoros y detritívoros, aunque algunas especies pueden mostrar un comportamiento carnívoro tanto depredador como carroñero o incluso caníbal (Rossi *et al*., 2011).

Su presencia está condicionada por las características físico-químicas del agua, siendo la salinidad y la temperatura los factores que más influyen en su distribución y abundancia. La salinidad y la composición iónica son importantes en la fisiología de los ostrácodos. La concentración de sales afecta la regulación osmótica y el equilibrio entre la calcificación y la regulación de iones (Mezquita *el al*., 1999). Existe una relación entre la distribución de las especies y la composición iónica del agua debido a las necesidades que tienen para calcificar su caparazón. La temperatura del agua afecta al desarrollo, fecundidad y supervivencia de manera específica. Debido a que los ostrácodos son organismos ectotermos, y por lo tanto no poseen mecanismos de termorregulación, la temperatura corporal varía con la del ambiente y en general se acelera el desarrollo al aumentar la temperatura, aunque se alcanzan tamaños menores que a menor temperatura (Atkinson & Sibly, 1997; Angilletta *et al.*, 2004). El oxígeno disuelto es crucial para la supervivencia de los organismos acuáticos. Las necesidades medias de oxígeno para los ostrácodos de agua dulce oscilan entre 7.3 y 9.5 mg L^{-1} (Delorme, 2000), aunque debido al tipo de vida bentónico, algunas especies están adaptadas a tolerar condiciones de hipoxia (Rossi *et al.*, 2002).

La dispersión dentro de un mismo sistema acuático puede ser activa, pero no se pueden dispersar entre diferentes sistemas a no ser que se conecten temporalmente, por ejemplo por una subida del nivel freático. La dispersión a larga distancia ocurre por transporte pasivo gracias a vectores como los invertebrados, vertebrados, humanos y el viento. Los huevos de muchas especies son resistentes a la desecación, así que pueden ser transportados largas distancias durante mucho tiempo. Algunos individuos de ciertas especies, y también los huevos, pueden atravesar vivos el conducto digestivo de peces y aves (Proctor, 1964). Los ostrácodos también se pueden agarrar al pelo o plumas mediante el cierre de las valvas y pueden ser transportados largas distancias de una manera más efectiva que con la dispersión por el viento que es más azarosa. Gracias a los humanos se han podido dispersar por el comercio de peces, plantas acuáticas, semillas, etc. Por eso suele ser habitual encontrar especies foráneas en invernaderos o campos de arroz (Escrivà *et al.*, 2012).

Los ostrácodos resultan muy útiles en estudios ecológicos por su amplia distribución, reducido tamaño y relativamente elevada velocidad de desarrollo, que junto con la facilidad para criarlos en acuarios les convierte en buenos organismos para su estudio en el laboratorio (Mesquita-Joanes *et al.*, 2012).

La característica que mejor define a los ostrácodos habitantes de pozas temporales es que la fase de resistencia a la falta de agua (tanto por desecación como por congelación) ocurre solo en forma de huevos, los cuales son depositados en las zonas más profundas de las charcas (Ganning, 1971). La densidad de individuos en las pozas es usualmente baja (pocos individuos por litro), aunque se han llegado a registrar densidades de más de 10 individuos mL^{-1}, al parecer relacionado con varios factores, especialmente la cantidad y calidad de alimento disponible (Juárez-Franco *et al.*, 2009). Según la revisión de Jocque *et al.*, (2010), de las 35 especies de ostrácodos que podemos encontrar en estos ambientes, la mayoría pertenecen a las familias Cyprididae y Limnocytheridae, siendo la subfamilia Cyprinotinae y dentro de ella los géneros *Hemicypris* y *Heterocypris* los mejor representados. En particular, *Heterocypris incongruens* es una de las pocas especies consideradas cosmopolitas dentro del grupo, con una elevada capacidad dispersiva y tolerancia ambiental (Fryer, 1997).

Justificación de la temática y objetivos

El hallazgo inicial de *Heterocypris bosniaca* en Valencia fue sorprendente puesto que se trata de la primera cita de la especie para la Península Ibérica y una de las pocas localidades en las que se conoce su presencia. Debido a que existe muy poca información disponible acerca de la especie, en la presente Tesis se pretenden aumentar los conocimientos generales sobre ella. Los objetivos particulares para cada capítulo se explican a continuación.

Capítulo I: Inicialmente se conoció la presencia de *H. bosniaca* a través de un cultivo disponible en el laboratorio, así que se decidió investigar la distribución de la especie en el área dónde se recogió la muestra original y saber si había establecido poblaciones estables mediante el monitoreo de dos pozas. También se quiso saber si la ausencia de machos inicialmente detectada era anecdótico o una característica constante en las poblaciones de la zona. Debido a la falta de información disponible acerca de la especie, se realizaron cultivos con individuos aislados con la finalidad de poder discriminar entre los diferentes estadíos encontrados en el campo y conocer mejor el ciclo de vida de la especie.

Capítulo II: Tras los resultados obtenidos acerca de la dinámica poblacional anterior y observaciones personales acerca de la capacidad de algunos individuos de *H. bosniaca* para enterrarse en el substrato y soportar durante cierto tiempo eventos de desecación, parecía indispensable seguir indagando en la respuesta de la especie a variaciones ambientales. Para ello se inició un programa de monitoreo de alta resolución (semanal) que empezó después del verano de 2005, cuando las pozas habían estado secas durante un largo periodo, y abarcó el hidroperiodo completo. Con ello se pretendía entender cómo la dinámica poblacional de *H. bosniaca* responde a corto plazo a los cambios ambientales que tienen un gran impacto en las pequeñas pozas como por ejemplo la lluvia, la variabilidad de la temperatura y la desecación.

Además de la información de campo, otro objetivo era poner a prueba la tolerancia a la reducción de contenido de agua en el substrato y el efecto de la velocidad de desecación en la tasa de supervivencia de ostrácodos en condiciones controladas de laboratorio, lo que potencialmente podría permitir descifrar como algunos invertebrados pueden resistir repetidos eventos de desecación de corta duración en pequeñas lagunas temporales.

Capítulo III: Los ectotermos carecen de mecanismos termorreguladores y por lo tanto su crecimiento y desarrollo tienen una fuerte dependencia térmica. En este trabajo se analizan los efectos de la temperatura sobre la velocidad de crecimiento, el tamaño de cada estadío de desarrollo y la fecundidad de las hembras adultas partenogenéticas en *H. bosniaca*. Esto permite la comparación con los resultados de campo obtenidos en los capítulos I y II, así como comprobar la variabilidad de la regla del tamaño-temperatura (en inglés *temperature-size rule:* TSR) a través de la ontogenia en un artrópodo acuático con crecimiento determinado.

Capítulo IV: Teniendo en cuenta los efectos negativos que ciertas sustancias químicas y productos biológicos de origen antrópico pueden tener sobre el medio acuático, y dada la limitada información disponible sobre su impacto en los ostrácodos, se propuso evaluar la respuesta de la especie de estudio al cadmio, plomo, SDS y las esporas de *B. thuringiensis*. De esta manera, se espera ampliar el conocimiento sobre el uso y aplicabilidad de los ostrácodos en las evaluaciones ecotoxicológicas, como ya se ha demostrado para otros miembros de este género. Además, con este trabajo también se propone analizar los posibles efectos de los contaminantes en la distribución y ecología de este ostrácodo poco conocido.

Capítulo V: Mediante el análisis de la morfología de las valvas, la completa quetotaxia de las extremidades, y el cuerpo completo de cada etapa postembrionaria desde el huevo hasta el adulto, el propósito de este trabajo es proporcionar una descripción detallada de la ontogenia de la especie y examinar las condiciones potencialmente pedomórficas del adulto. Otro objetivo es comparar la información detallada con otras especies, identificando de esta manera las diferencias morfológicas entre las mismas, además de las características conservadas en el desarrollo, lo cual puede resultar útil para el análisis de tendencias morfológicas en la evolución de los Cypridoidea. También se investiga especialmente la morfología de la poco conocida fase de eclosión debido a las implicaciones que puede tener en la filogenia de los ostrácodos.

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Capítulo I

On the presence of parthenogenetic populations of *Heterocypris bosniaca* Petkowski *et al*., 2000 (Crustacea: Ostracoda) in temporary rock pools of the eastern Iberian Peninsula, with insights into the ecology and life history of the species

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Abstract

Heterocypris bosniaca Petkowski *et al*., 2000 was abundantly collected from eight temporary rock pools on carbonate substrate near Rafelguaraf (València, Spain). As only females were found, reproduction is inferred to be parthenogenetical. *H. bosniaca* was the only ostracod occurring in the pools investigated. The populations at two sites were weekly monitored from early December 2004 to late January 2005 to investigate the environmental conditions, the population dynamics, larval growth and life history. In parallel, a number of juveniles were reared in the laboratory for controlled instar growth and life-span measurements. The variation in shape throughout the larval development is shown with the help of scanning electron micrographs. The length measurements taken from animals reared separately in the laboratory permitted to better establish size boundaries between the instars in general and in particular in those collected from the field. In the laboratory, the youngest instars stretched over about 50 hours, the last one (A-1) over about 100 hours. Three of the females each laid between 7 and 12 eggs. The species was found to be resistant to desiccation and tolerant to hypoxic conditions.

Keywords: Temporary rock pools, population ecology, geographical parthenogenesis, nonmarine Ostracoda, València, Iberian Peninsula.

Introduction

Heterocypris bosniaca was first described by Petkowski *et al*., (2000) from a well in Bosnia (Balkan peninsula). Both males and females were present. More recently, Martens *et al*., (2002) reported the finding of three populations, two of them with males, in temporary rock pools in Israel. In 2004, we first identified the ostracods kept since 1998 in a small aquarium in the laboratory of our institute as belonging to *H. bosniaca*. The aquarium contained substratum and water taken from a pool located in the mountains of Racó de Rius in Rafelguaraf (València, Spain).

The finding of *H. bosniaca* was the first for the Iberian Peninsula (Aguilar-Alberola & Mezquita, 2005), and therefore we decided to investigate the distribution of the species in the area. We also wanted to find out if the absence of males was anecdotic or a constant feature of these populations. We wanted to find out if the species has built up stable populations in the area or if the populations undergo wide changes that could bring them to local extinction. We weekly monitored the populations at two sites from the beginning of the rainy period to the complete drying-up of the pools. Also, a number of individuals were reared in the laboratory in order to study the life-cycle and the morphology of the carapace during larval development.

Material and methods

Sampling area

The area of study is situated in the mountains of Racó de Rius in Rafelguaraf, province of València, Spain. It is covered by typical Thermo-Mediterranean vegetation, composed of pine trees and shrubs. The geological substrate consists of limestone, which through weathering by rainwater forms a high number of small, rounded stony holes that fill up with water during rainy periods. The bottom of the pools is covered mainly with pine needles. The water evaporates relatively quickly so that either in spring, if the season is dry, or otherwise in early summer the habitats have completely dried out.

Exploratory sampling campaigns

Shortly after the rainy period, we started four sampling campaigns between the 27th of November 2004 and the 18th of December 2004. During these surveys, we searched through flat and deep zones of the area looking for sites with a high probability of holding rain water for several weeks, such as wide stony flat surfaces. We avoided areas with sandy substrate or with a cover of high shrubby vegetation.

At each sampling site, we filled a standardised field form with general information of the pool and surrounding area. This protocol included taking a picture of the pool, and recording GPS coordinates. Size (maximum length and width) and depth at three points were recorded in order to get an approximate idea of the surface (considering they are approximately circular in shape) and volume (most pools have vertical walls and hence a cylindrical structure). *In situ* measurements of water temperature, electric conductivity, pH (HANNA instruments), and oxygen concentration (WTW F/set3 instrument) with field probes were taken. Water samples were collected using plastic jars and transported to the lab in order to analyse chloride concentration and alkalinity using standard volumetric methods (APHA-AWWA-WEF, 1992). Ostracods were collected with a small hand net (mesh size 100 μm, diameter 15 cm) swept through the substrate, and fixed in the field with 30% ethanol. In some pools, where densities were very high, the ostracods were collected with a small plastic jar (5 cm in diameter), which was swept smoothly through the muddy substrate.

After several ponds with dense populations of *H. bosniaca* had been found, two of them were selected for the weekly monitoring of the population dynamics and the environmental factors associated to them (see below).

Weekly monitoring

The two study pools were visited once in the week from the 27th of November 2004 to the 22nd of January 2005, when eventually they both dried up. The two pools, designated as X1 and X6, were selected because of their easy access, high ostracod densities and differences in size and habitat conditions.

On each visit, we measured a number of environmental variables and collected water and ostracod samples. Ostracods were collected with a 100 ml, 5 cm diameter plastic jar, which was introduced into the substrate to a depth of about 1 cm and swept through it over about 3-5 cm. We chose this method because the high densities of *H. bosniaca* in the selected pools allowed collection of high numbers of individuals in this way, and also in order to minimise the impact on these small habitats. The samples collected were fixed with ethanol and later in the lab ostracods were handpicked under a binocular microscope and stored in 70% ethanol. The carapace length of all the individuals collected was measured in order to sort them into size groups that reflected the developmental stages. Adult and A-1 individuals were measured with a precision of 20 μm, A-2 and A-3 with an 10 μm error level, and the rest of instars were measured to the nearest 5 μm.

Ostracod cultures

In order to obtain precise information on the number, size and duration of the different instars, a few juvenile individuals collected from pool X1 were grown separately at room temperature (20-27ºC). The cultures were carried out using multi-well plates filled with filtered water from the pool and dried *Spirulina* sp. as food (Schmit *et al*., 2007). We monitored each animal individually in its well in order to check moulting events and collect information on the time elapsed between moults and total duration of the larval development.

Daily, sometimes twice per day, each individual was checked for size, valve remains and survival. In order to calculate life span for each instar, we used the between-moults time assessed, or the time to death since last moult in the case of adults. The time since the beginning of the experiment to the first moult is not considered here, so that we do not present information on A-8 life time, because we did not measure time at hatching events. Ostracod valves shed during moulting were collected and measured in order to have a more precise information about the size of each instar.

Results

The rock pools

During the survey *H. bosniaca* was found in eight rock-pools (Tab. 1). All of them are holes on rocky surfaces and result from the chemical weathering by rainwater. They are surrounded by Mediterranean-type shrubs rooted in neighbouring holes and fissures (Fig. 1). The rockpools being mostly situated on gently sloping land, the intensity of the incoming sunlight mainly depends on the surrounding vegetation. The only exception is pool X3, which is located on a steep slope, but is exposed to low sunlight intensity, this because the water level is about one meter below the rock surface.

The pools differ only slightly in their water chemistry. For instance, pH values ranged between 7.6 and 8.2, and electric conductivity (EC) varied between 150 and 580 μS cm^{-1}, this mainly in relation to pool size, rain events (decreasing EC) and evaporation periods (increase in EC).

Tab. 1. Physical and chemical variables recorded for the eight pools in which *H. bosniaca* was found.

Pool	UTM Coordinates	Date	Temp (ºC)	EC (µS cm^{-1})	pH	Cl$^-$ (mg L^{-1})	HCO$_3^-$ (mmol L^{-1})	pO$_2$ (%)	O$_2$ (mg L^{-1})	Length (cm)	Width (cm)	Depth (cm)	Area (dm^2)
X1	30S 0721767 4325537	11/12/2004	11	180	7.9	14	1.9	75	7.5	59	32	4.6	16
X2	30S 0721754 4325545	27/11/2004	16	420	8.2	30	4.1	93	9.5	38	25	7.3	8
X3	30S 0722134 4325146	27/11/2004	17	380	7.9	32	3.5	84	9.2	51	37	8.1	15
X4	30S 0722158 4325260	27/11/2004	14	350	7.9	30	4.5	88	10.0	60	42	13.2	20
X5	30S 0722158 4325259	27/11/2004	13	370	7.6	31	3.8	85	11.0	75	57	5.7	34
X6	30S 0721884 4325380	11/12/2004	14	160	8.0	8	1.9	93	8.8	120	92	28.1	88
X7	30S 0721887 4325367	11/12/2004	14	160	8.2	10	1.9	111	11.0	60	41	5.1	20
X8	30S 0721896 4325365	18/12/2004	13	150	7.7	n.a.	2.1	91	12.0	56	48	7.7	21

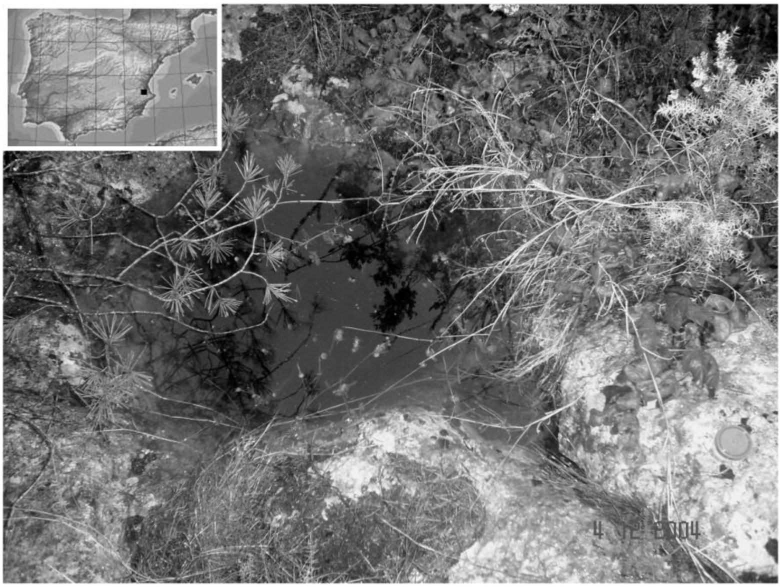

Fig. 1. Site X6, where its general shape can be seen, so as the type of rocky substrate and surrounding shrub vegetation rooting on surrounding holes and crevices.

Out of these eight sites, the pools X1 and X6 were selected for weekly monitoring. These two sites differ in size (32 x 59 and 92 x 120 cm, respectively), the intensity of direct sunlight, and the fact that X6, receives organic matter not only from pine needles but also from fig tree leaves (*Ficus carica*) in autumn. It also harbours a population of *Branchipus schaefferi*, an anostracan which in lab cultures we saw to be subjected to predation by *H. bosniaca*.

In general, pool X1 shows greater fluctuations of the environmental variables than X6 (Fig. 2). During the study, conductivity increased gradually in X6 whereas in X1 large fluctuations were recorded. The oxygen concentration was less variable in X1 (between 5.5 and 11 mg L^{-1}) than in X6, where a minimum of 2.4 mg L^{-1} was measured.

Sex, size and carapace shape of the instars

Heterocypris bosniaca was found to be the only ostracod occurring in the eight pools. All the individuals collected were females, so far no males have been found in the pools studied. From this finding we would like to infer that reproduction in the pools is exclusively parthenogenetical.

The A-8 instars have a rounded quadrate shape with an almost straight dorsal margin when seen laterally (Fig. 3). The oblong carapace shape of the adults is progressively reached throughout the larval stages.

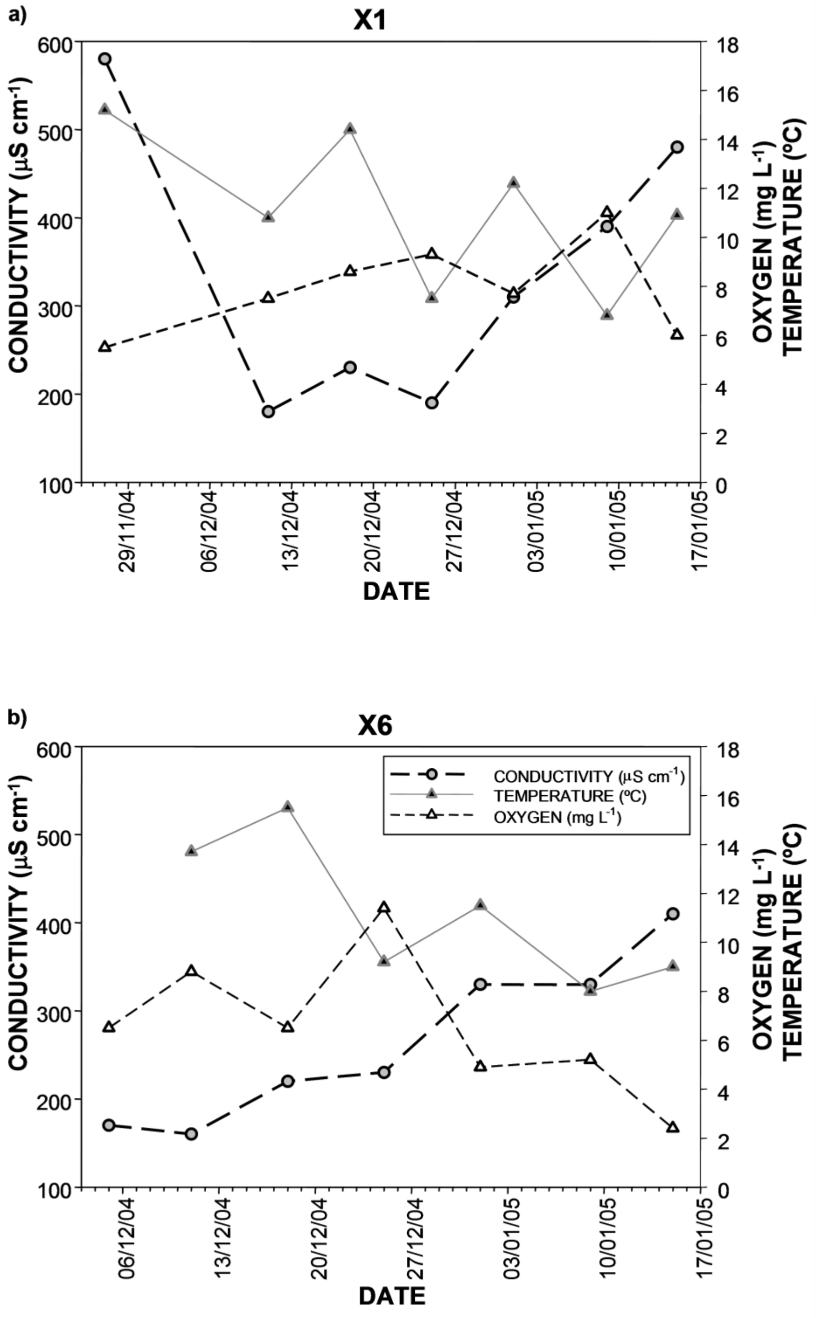

Fig. 2. Graphs showing temporal changes in water temperature, electrical conductivity and oxygen concentration in pools X1 (a) and X6 (b) during the study period.

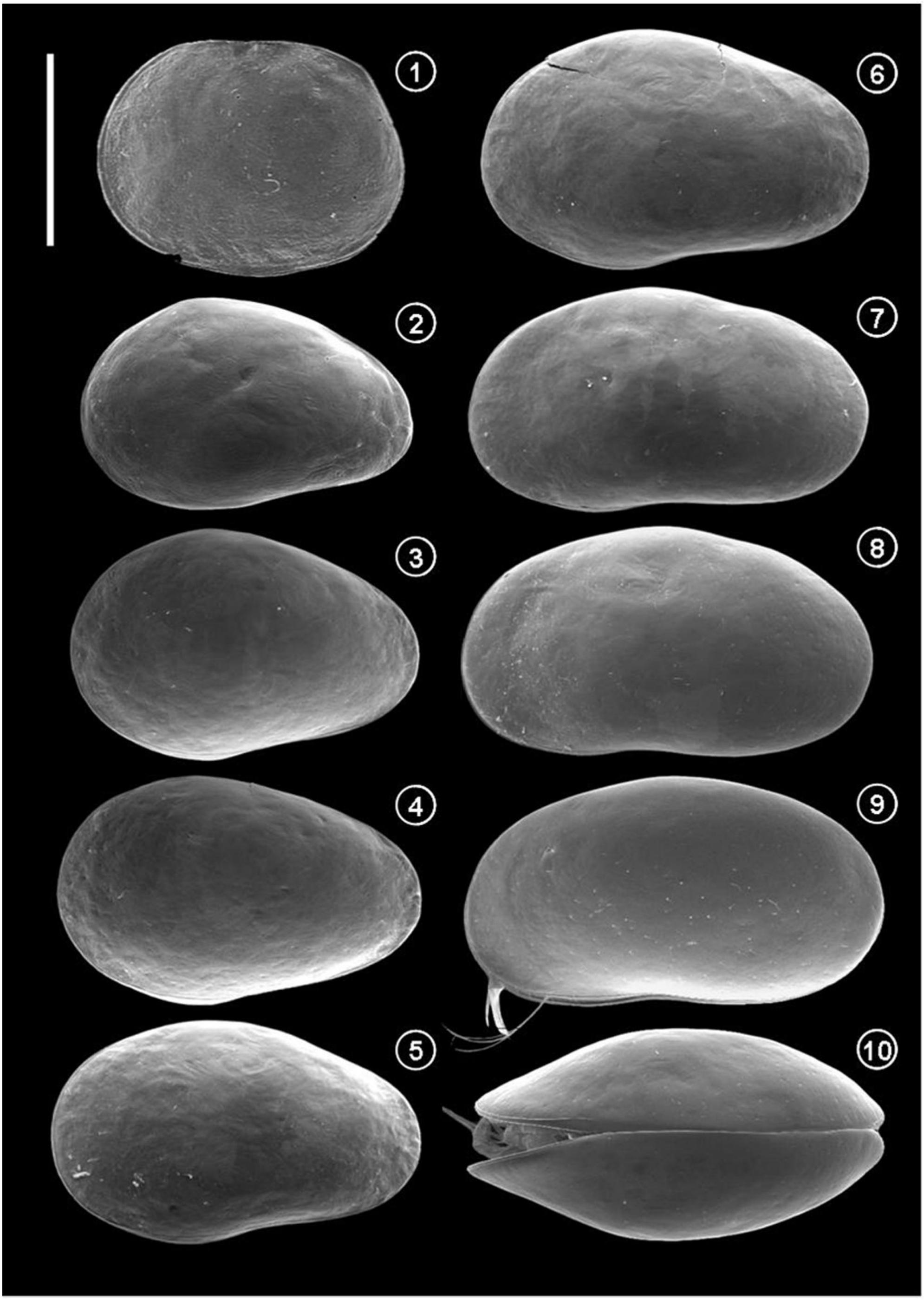

Fig. 3. External views of different instars of *H. bosniaca*. (1): A-8, left valve. Scale bar = 132 µm, (2): A-7, left valve. Scale bar = 139 µm, (3): A-6, left valve. Scale bar = 155 µm, (4): A-5, left valve. Scale bar = 197 µm, (5): A-4, left valve. Scale bar = 240 µm, (6): A-3, left valve. Scale bar = 290 µm, (7): A-2, left valve. Scale bar = 371 µm, (8): A-1, left valve. Scale bar = 495 µm, (9): Adult female, left valve. Scale bar = 632 µm, (10): Adult female, dorsal view. Scale bar = 632 µm.

The increase in size between two moults in general increases throughout larval development (Tab. 2). Consequently late instars are more readily separated from each other under the microscope than smaller ones.

Tab. 2. Average, median, minimum and maximum carapace length, number of individuals (N) measured for each instar, and moult increment for the median length of a given instar compared to the previous one.

INSTAR	N	Maximum (mm)	Minimum (mm)	Mean (mm)	Median (mm)	Moult increment % (median)
A	432	1.5	1.22	1.37	1.38	35.3
A-1	997	1.2	0.94	1.05	1.02	34.2
A-2	1153	0.9	0.67	0.77	0.76	31
A-3	606	0.7	0.5	0.58	0.58	26.1
A-4	561	0.51	0.4	0.46	0.46	24.3
A-5	189	0.4	0.33	0.37	0.37	27.6
A-6	88	0.33	0.25	0.28	0.29	16
A-7	4	0.26	0.21	0.24	0.25	13.6
A-8	2	0.23	0.21	0.22	0.22	15.8
EGG	5	0.19	0.18	0.19	0.19	-

This can also be seen if we plot the size (carapace length) frequency distribution for all individuals measured in pool X6. Several modes clearly correspond to late (older) instars. By contrast, juvenile bell-shaped distributions clearly overlap (Fig. 4). Despite the low number of individuals used in our growth experiments, the sizes obtained from lab-animals with known instar belonging allowed us to better establish size boundaries of the instars in general (Fig. 4).

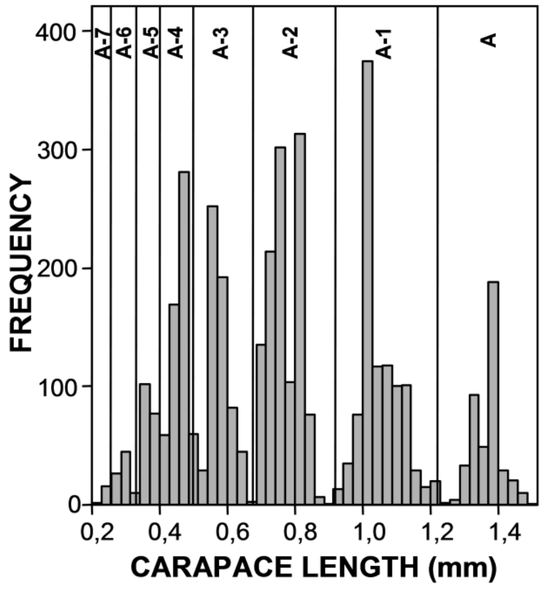

Fig. 4. Size frequency histogram showing the distribution of carapace lengths (CL) for pool X6. Vertical lines show the CL boundaries for the different instars, as obtained from laboratory cultures.

Upon comparing the carapace length of the individuals collected from pool X1 with those collected from X6, it can be seen that median length is always higher for those collected from X6 (Fig. 5).

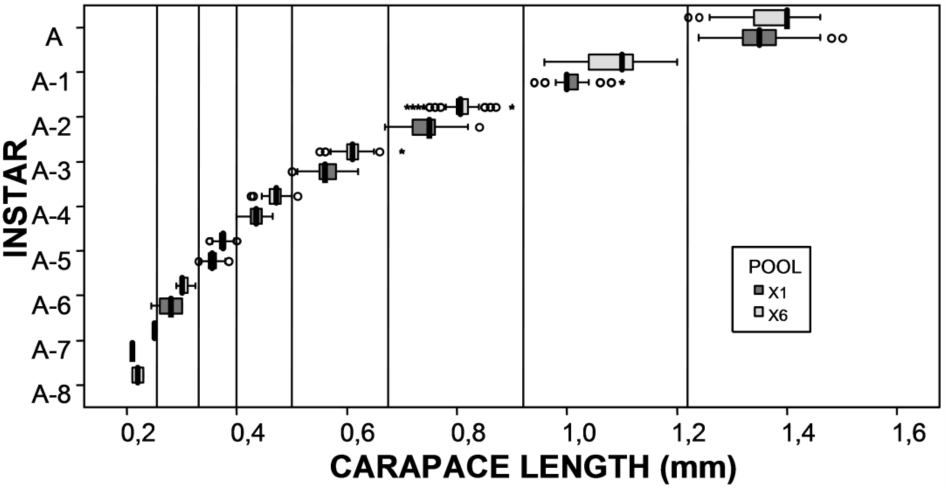

Fig. 5. Boxplots showing the carapace length (CL) distribution for the different instars, separately for pools X1 and X6. Vertical lines show CL boundaries obtained from laboratory cultures for each juvenile instar.

Population dynamics

Throughout the eight weeks of the study period (late November 2004 to late January 2005) there was a continuous increase in the proportion of older instars in both pools (X1 and X6). Additionally, a slight increase in the number of early juveniles was seen in pool X6 at the middle of the study period (Fig. 6).

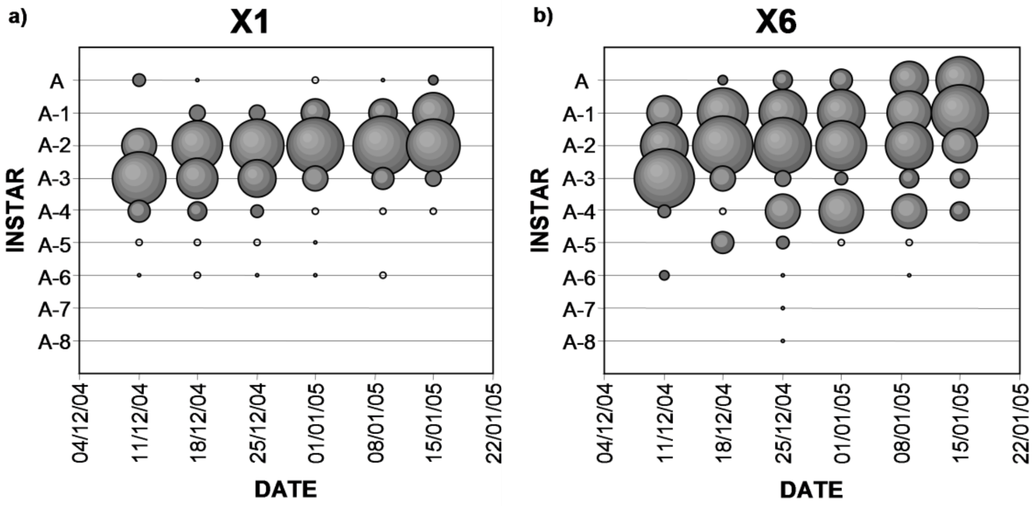

Fig. 6. Temporal changes in relative abundances of different instars of *H. bosniaca* in pools X1 (a) and X6 (b). Bubble size is related to percentage of individuals of a given instar in the sample.

Instar life spans

During the experimental cultures, the periodic checking of individuals allowed us to determine to some extent the time elapsed between two consecutive moulting events for each animal under study. In this way, we could calculate the life span of each growth instar and, consequently, of the whole lifecycle of an individual (excluding egg and A-8 life time) (Fig. 7). In general, the youngest juvenile instars lasted for about 50 hours each, whereas the oldest juvenile instar (A-1) lived for about 100 hours before moulting to adulthood. Adult individuals lived longer than 250 hours under the lab conditions used in our experiments. Three of the females that had reached sexual maturity each laid between 7 and 12 eggs.

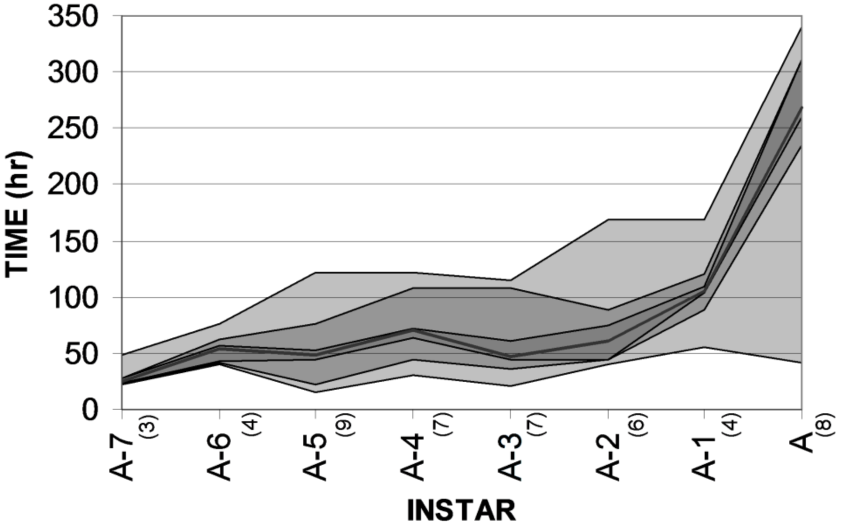

Fig. 7. Life span for each instar of *H. bosniaca* as calculated from laboratory experiments. The figure shows: i) minimum and maximum time span per instar (pale grey area), ii) time span range excluding outliers (intermediate grey area), iii) median time span per instar, including measuring error (dark grey area) and iv) median time span per instar (dark grey line). Number of measurements between brackets.

Discussion and conclusion

The eight pools in which *H. bosniaca* was found in Rafelguaraf were very similar in their habitat conditions. The environmental factors analysed, in spite of varying within a rather narrow range only, present fluctuations that are largely a result of the seasonality of the water body, for instance conductivity decreases after rainy periods to increase towards the end of the hydroperiod.

The high densities of *Heterocypris bosniaca* in this type of environment, together with the fact that this was the only ostracod present, clearly indicate that the species is tolerant to desiccation and fluctuating and unpredictable environments. From the fact that the nine sampling sites are all distributed over a 9 ha area, with a few of them being quite isolated from the others, *H. bosniaca* appears to have a good dispersal ability, at least at the small local scale.

Furthermore, the low oxygen concentrations observed at particular dates (*e.g.*, *c*. 2 mg L^{-1} at the end of the wet season in X6) indicates that the species is tolerant to hypoxia, a trait known from other members of the genus, so for instance *H. incongruens* (Margalef, 1953).

Similarly to other ostracod species (*e.g.* Smith & Martens, 2000), *H. bosniaca* presents moult increments that are progressively larger from early juvenile carapace length to adult size (increasing approximately at each moult from 14 to 35%). The differences in carapace length between individuals of the same instar living in X1 *versus* X6 are most likely attributable to the environmental differences between these two pools; environmental variables fluctuate less abruptly in X6, it contains a higher amount of organic matter derived from fig tree leaves and, in addition, harbours a population of anostracans that could serve as food to the ostracods. Thus the higher amount of food resources in X6 might explain the larger mean size of the ostracods it harbours.

The fact that the presence of the species in some of the studied pools can be dated back to ten years ago, when the first animals were collected and stored in a personal aquarium, suggests that *H. bosniaca* has been living in such temporary water bodies with success thanks to the high tolerance of its diapausing eggs to desiccation, because the pools dry up in summer for several months, and in some dry years they must have a very short hydroperiod. The appearance of a second cohort in X6 during the study period also suggests that the species is able to produce subitaneous eggs, in a similar way as its congener *H. incongruens* (Angell & Hancock, 1989).

The experimental results show that *H. bosniaca* has a relatively short life-cycle, as it takes A-7 juveniles only about 17 days to reach adulthood at temperatures above 20ºC. Latifa (1987) obtained similar results for *H. incongruens*: larval development stretched over 16.7 days at 20ºC and 12.5 days at 25ºC, respectively.

The largest difference observed between the two species relates to the life-span of A-1 juveniles. In *H. bosniaca* this instar lives almost twice as long as the previous instar, whereas in *H. incongruens* A-1 juveniles show a life-span similar to the earlier juvenile instars (Latifa, 1987). The latter author (*op. cit.*) also reported a clear shortening of the larval development with increasing temperatures. We did not check for this effect in the laboratory, but our field data indicate that the animals were taking longer from one moult to the next than under lab conditions. This is most likely due to the higher temperatures in the lab (20-27ºC) compared to those measured in the pools (17ºC).

The absence of males in the study area is not surprising, as geographical parthenogenesis is a somewhat frequent pattern in cypridid ostracods, and in the genus *Heterocypris* in particular (Horne *et al*., 1998).

The finding of high density populations of *H. bosniaca* demonstrates that the species has established stable populations in the area studied. The species is highly tolerant to desiccation, most probably through the production of resting eggs, and it is probably able to lay subitaneous eggs too. It seems also tolerant to some degree of fluctuations in water ionic and oxygen concentrations.

Parthenogenetic reproduction may explain long-distance dispersal in *H. bosniaca*. Indeed one single ovigerous female or even one single egg can give rise to a new population or even metapopulation as the one observed in the area investigated. Further studies are needed to understand the geographic origin and genetic structure of these populations.

Acknowledgements

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Capítulo II

Population dynamics and tolerance to desiccation in a crustacean ostracod adapted to life in small ephemeral water bodies

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Abstract

Given their small size, isolation and unpredictability, temporary rockpools present high environmental stress and impoverished communities of species that have adapted to such stressful conditions. Special adaptations of the invertebrates living in these habitats include tolerance to desiccation and fast ontogenetic development in order to maintain stable populations and face high risk of extinction. Dozens of small rockpools (mostly with Ø < 1 m) can be found in east Spain on limestone substrate, where the only known Iberian populations of *Heterocypris bosniaca* Petkowski *et al*. (2000), an ostracod species with geographic parthenogenesis, have been recently found. In this survey, two of these rockpools have been monitored during the main hydroperiod between the fall of 2005 and summer 2006 to test the ability of *H. bosniaca* parthenogenetic populations to face unpredictable hydroperiod dynamics. Pools were visited weekly, and limnological data and ostracod samples were obtained from either water or substrate in dry periods. Ostracod individuals were counted and assigned to growth instars to monitor population changes. In the laboratory, experimental cultures allowed the estimation of survival dependence on the substrate desiccation rate. Throughout the hydrological cycle studied, several hatching periods were observed, usually preceded by desiccation, followed by substrate hydration and water dilution by rain. The demographic changes observed indicate that *H. bosniaca* populations are able to persist in intermittently inundated environments and produce several generations per annual hydrological cycle. In addition, adult individuals were able to survive in the wet mud of dry pools for longer than five weeks. The experimental data suggest a lower average survival time when exposed to desiccation processes, and that the velocity of substrate water loss is a determining factor for the survival rate of ostracods resisting dry events in temporary ponds. As shown by ostracods' life histories in temporary aquatic environments undergoing unpredictable desiccation events, a combined strategy of adult tolerance to short periods of water scarcity and rapid hatching from resting egg banks can be advantageous for the monopolization of small-sized ephemeral habitats.

Keywords: Population dynamics, desiccation tolerance, ostracoda, temporary rockpool.

Introduction

Temporary pools are small-sized systems with particular environmental traits and functioning dynamics compared to other water bodies like lakes or rivers (Williams *et al*., 2003; Declerck *et al*., 2006). Although they are small, their special ecological setting helps them contribute significantly to regional biodiversity as they usually harbor taxa that are uncommon in other habitats (Eitam *et al*., 2004; Scheffer & Van Geest, 2006). The small areas of temporary pools involve high vulnerability to environmental degradation and local hydrological changes (Nicolet *et al*., 2004), resulting in impoverished communities which encompass species adapted to the particularly stressing conditions there (Bayly, 1997). Despite the scientific interest shown in temporary pools, their functioning is poorly known, although they are recognized as being very valuable water bodies for biodiversity conservation. Furthermore, they provide stepping stones for the spread of aquatic species among larger water bodies (Oertli *et al*., 2002; Nicolet *et al*., 2004).

The shorter the inundation period, the poorer the species richness of temporary pools (Jocqué *et al*., 2007). This is because extinction due to pool drying greatly affects community structure (Eitam *et al*., 2004). Despite this pattern, species respond individually to the environmental conditions in these habitats (Jeffries, 2003). Particular adaptations to life in temporary environments include migration to other habitats during the dry season or the survival during this period in arrested physiological stages, chiefly as dormant eggs (Wiggins *et al*., 1980; Wissinger, 1999). Apart from these traits, a fast developmental rate is essential in short hydroperiod pools (Boven, 2009; Jocque *et al*., 2010), and egg bank formation and erratic hatching are essential to help avoid extinction locally (Williams, 1998).

On the limestone rocky substrate of the Mediterranean shrublands of Racó de Rius (east Spain), we found 119 rainy rockpools over an area covering 65 ha. Of these, 35 harbor asexual populations of the ostracod crustacean *Heterocypris bosniaca* Petkowski *et al*., (2000) (Aguilar-Alberola & Mezquita, 2008a), which was recently discovered in the Iberian Peninsula (Aguilar-Alberola & Mezquita, 2005, 2008b). In 2004, we carried out initial sampling campaigns and selected two rockpools to start a monitoring survey on *H. bosniaca* populations (Aguilar-Alberola & Mezquita, 2008b). Surprisingly by the end of the hydroperiod we observed many individuals living in these rockpools which were already dry. They were grouped in compact packs below a mixture of pine needles and mud substrate, which shows an adaptive behavior in response to desiccation (Williams, 2006). Shortly after rehydrating a small sample of the substrate from these pools, hundreds of actively swimming ostracods could be seen. This amazing capacity to tolerate desiccation in mud may prove extremely beneficial to ostracods and other invertebrates living in such ephemeral and unpredictable environments (Delorme & Donald, 1969; Williams, 2006).

In order to understand how *H. bosniaca* population dynamics respond to short-term environmental changes that have a great impact on rockpools (*e.g.*, rain, temperature variability, desiccation), we initiated a full hydroperiod, high-resolution monitoring program which commenced after the summer of 2005 when all the previously studied ponds had been dry over a long period. In addition to field information, our aim was to test tolerance to the reducing water content in the substrate and the effect of the velocity of desiccation on the ostracod survival rate under controlled laboratory conditions, which could potentially allow us to decipher how some invertebrates can withstand repeated short desiccation events in small temporary pools.

Material and methods

Field sampling

The two studied rockpools, X1 and X6, are located in a limestone mountain area with Mediterranean shrublands in Rafelguaraf (Spain), and are separated by a distance of approximately 210 m (Fig. 1). Both pools dry up in summer for several months, and in dry years, they probably have a very short hydroperiod. The two pools differ in size as X1 is smaller (32 cm x 59 cm max. diameter, and max. depth of 13 cm) than X6 (92 cm x 120 cm, max. depth 20 cm), in illumination terms, and in some hydrochemistry and hydroperiod aspects (Aguilar-Alberola & Mezquita, 2008b).

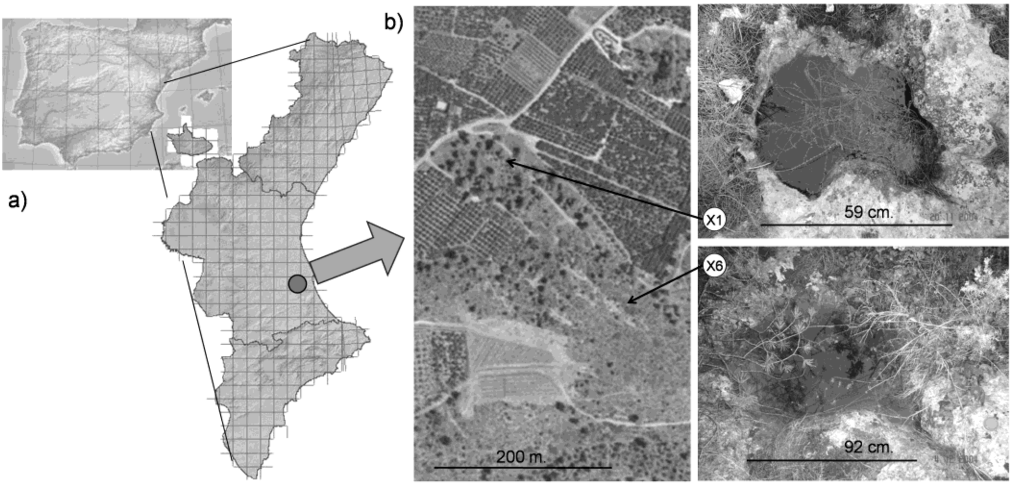

Fig. 1. (a) Location of the sampling area in the Valencian region of Spain, (b) aerial view of the sampling area and detail of the two sampled pools.

The organic substrate in rockpool X1 accumulates pine needles, while fig *(Ficus carica)* leaves are dominant in X6, which also harbors an anostracan, *Branchipus schafferi*, population. A previous work showed that the dominant ostracod species in these pools, *H. bosniaca*, has a relatively short life cycle in these environments (Aguilar-Alberola & Mezquita, 2008b), similarly to that of *Heterocypris incongruens* (Latifa, 1987). In this work, we once again found high density populations of this species, demonstrating that *H. bosniaca* has established stable populations in the study area. Aguilar-Alberola & Mezquita (2008b) discuss these and other initial observations on the hydrological cycle of these pools and the dynamics of their *H. bosniaca* populations.

In order to study a whole inundation period, we waited for a significant rainy event that would fill rockpools X1 and X6 (which had been dry since January 2005), and this event took place after summer 2005. We started sampling on the day immediately after the event, this being 18/09/05. From this date onward, we sampled both ponds weekly, even if they were dry (*i.e.*, without free water) until no living individuals were observed. The sampling campaign lasted 30 weeks and ended on 9/04/06. When free water was available, we measured water temperature, electric conductivity and pH using a Hanna Instruments® portable probe, and oxygen content with a WTW® oxymeter. At the same time, a water sample was obtained in a PE bottle to analyze chloride concentration and alkalinity using standardized volumetric tests (Aquamerck® kits 1.11106.0001 and 1.11109.0001, respectively). If no free water was present, none of these variables were measured. Daily information on maximum and minimum air temperatures and precipitation was obtained from the data available online on the Spanish Ministry of the Natural, Rural and Marine Environment's web site (2008) from the nearest meteorological observatory located in Carcaixent, Valencia. The field sampling area and the meteorological observatory are 8.6 km apart, and both sites belong to the same climatic region (Pérez, 1994). Therefore, no major differences in precipitation and temperature patterns are expected.

Ostracod samples were collected with a 100 mL, 5 cm Ø PE bottle which was directly introduced into the water of the pool and swept over the substrate and pool wall through an approximately 25-cm long transect. This method allowed us to sample enough individuals given the high densities of *H. bosniaca* in these pools, and was preferred to other alternatives because of the heterogeneous morphometry and small size of these systems. The obtained samples were preserved in 30% ethanol in the field and the preservative concentration was increased to 70% a few hours later. When ponds were dry, approximately half a bottle (100 mL, 5 cm Ø PE) was filled with substrate (mainly organic plant matter and mud) which was immediately filled completely with deionized water. In this way, living ostracods were seen swimming in the water column after only a few minutes. Half an hour later, the sample was preserved with increasing ethanol concentrations (15%, 30% and 70%) through progressive 1-h steps to facilitate the fixation of living ostracods with their valves wide open. This position allows easier differentiation by comparing the soft part remains of the living individuals caught in the samples from those already dead upon collection. We also collected five more similar substrate samples to be kept in hermetically sealed plastic bags until arrival at the laboratory where they were weighed and dried (under conditions close to natural drying: 30ºC, 120 h), and weighed again to calculate the percentage of water content in the substrate. The ostracod individuals collected in the samples were sorted and handpicked in the laboratory under a stereomicroscope. In each case, all individuals were assigned to belong to any of the 9 growth instars (A-8 to Adult) and were counted. To achieve this task with greater precision, at least 20 individuals per growth instar were initially measured with a minimum resolution of 20 µm.

Desiccation tolerance experiment

In order to evaluate the influence of substrate humidity on the survival of ostracods undergoing a desiccation process in their habitat, we carried out laboratory cultures with ostracods under two experimental treatments, fast and slow desiccation rates, in addition to a control group with no water loss. Cultures were placed in 50 mL PE bottles which were filled with 2.5 g of dry substrate from X6, plus 9.8 g of deionized water. In this experimental study, we used only individuals and substrates from pond X6 because it contained sufficient substrate. Pond X1 contained a smaller amount of substrate, which had been partly sampled already in the population dynamics study. So we decided to not use more bottom samples from this site to avoid major negative effects on its established community. A small amount (approx. 0.01 g) of dried *Spirulina* sp. was added as food at the beginning of the experiment following Schmit *et al*. (2007), and no more food was added subsequently. When the substrate was fully hydrated, 10 individuals (9 adult females +1 A-1 juvenile, all from X6) were added to the bottle. In total, 110 bottles made up the two treatments groups, each with 10 ostracods plus substrate and water. The 110 bottles were initially (starting on 18/5/2007) kept open at room temperature (21.5 ± 1.2ºC) for five days to lose water through evaporation at a similar rate until the water level reached the substrate level. As from day 6, the bottles were separated to form two groups of 55 replicates, and each group was arranged separately to follow a different desiccation rate: one of the groups was left exposed to air as before (the Fast Desiccation Group: FD), while the other was covered with a microperforated transparent plastic lamina (the Slow Desiccation Group: SD). Daily control of weight loss per bottle allowed comparisons to be made with the field data in order to adjust the SD group to follow a desiccation rate as close as possible to that observed in the field. Periodically, and starting on day 6, five randomly selected bottles per treatment group were rehydrated with deionized water (allowing a maximum of 11 of these periodical rehydrations per treatment) to check the number of surviving individuals. Initially for the SD group, rehydration selection took place every 5 days, later on every day, and finally every 12 h. Initially, this periodicity was every 1-2 days for the FD group to finally become every 12 h.

The experimental control group, *i.e.*, without desiccation (ND), was composed of 20 bottles; 10 with substrate, which were prepared in the same way as the experimental treatment groups as explained above, and 10 without substrate to check potential effects on survival. All these bottles were kept under the same conditions as groups FD and SD, but were periodically filled with deionized water to avoid significant water loss. The number of swimming ostracod individuals was checked daily by visual inspection.

The relationship between substrate water content and desiccation time for both the field and laboratory data was analyzed by linear regression. The comparison between the treatment slopes of this relationship was made using a general linear model with treatment taken as the fixed factor. We used Cox regression to check the effects of the different desiccation rates on ostracod survival and to test the hypothesis that faster desiccation may increase mortality rates. This type of survival analysis allows the use of predictor variables, which can be continuous and even time-dependent (Hosmer & Lemeshow, 1999; Norusis, 2004). In our case, the water content in the sediment was a time-dependent predictor variable. We used the SPSS software, v. 14.0, for all statistical analyses (Norusis, 2004).

Results

Habitat changes

Between the fall of 2005 and spring 2006, the two studied rockpools presented different hydroperiod dynamics (Fig. 2). After they filled in September 2005, both dried (no free water column) four weeks later to remain in this state for four additional weeks. Then both were refilled in November and X1 dried again on two occasions, December 2005 and February 2006, to remain dry for two and one week(s), respectively; X6 dried only once in December 2005, and this dry period lasted one week. Finally, X1 dried two weeks earlier than X6 in March, but both remained dry until the next rainy period in the fall (data not shown).

No major differences in the physico-chemical aquatic environment between the two studied pools were noted. The water temperature in both ponds remained low (around 10ºC) in winter (Fig. 2), but X1 presented slightly higher values than X6 in the fall. Conductivity, chloride concentration and alkalinity (Fig. 3) changed in

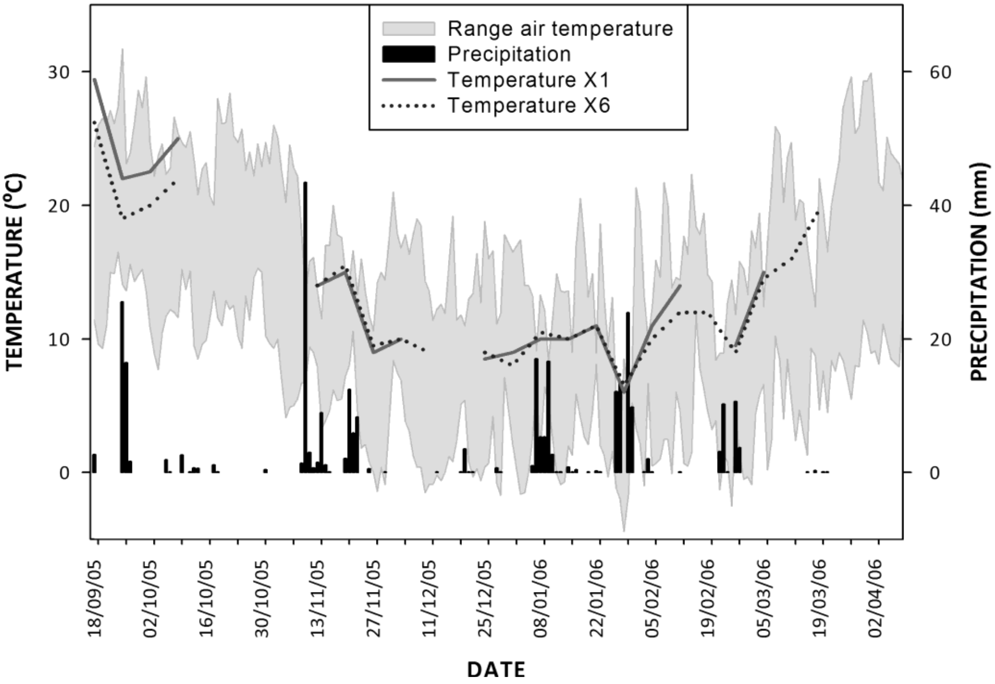

Fig. 2. Precipitation (bars) and air temperature range (grey filled area) in the weather observatory of Carcaixent during the study period. The water temperatures for rockpools X1 (continuous line) and X6 (dotted line) are also shown for the sampling periods when free water was available.

accordance with the hydroperiod; when they dried, the values of these parameters increased. The minimum electrical conductivity values recorded (183 and 244 µS cm^{-1} in X1 and X6, respectively) corresponded to sampling periods after rain events, whereas the maximum values (851 and 848 µS cm^{-1} for X1 and X6, respectively) were recorded at the end of the study period. The pH did not fluctuate considerably, although a slight trend toward more acidic values (from a maximum of 8.3 to a minimum of 6.7 in X1) was recorded at the end of the sampling period. Oxygen levels showed no clear pattern throughout the study cycle, and only a slight gradual decrease was observed at the end of the period to reach very low values in rockpool X6.

Table 1 shows the substrate water content (water weight/substrate dry weight) on the sampling dates when no free water column was present. In the first dry period, both pools behaved similarly in terms of substrate water content: they lost water in the first two weeks, but became wetter in the third week (which coincided with a rainy day, Fig. 2). Finally, they lost water again in the last week of this dry period. Toward the end of the hydrological cycle, both pools gradually lost their water content, and a minimum measured value of 0.29 was recorded in rockpool X1.

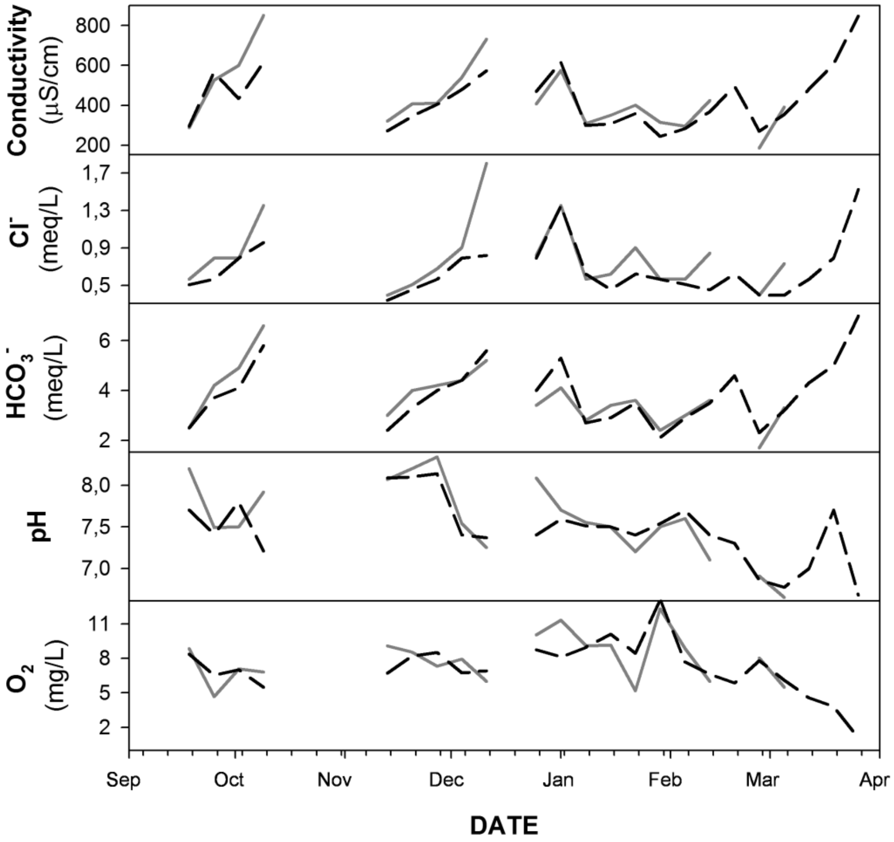

Fig. 3. Water abiotic parameters measured in rockpools X1 (grey line) and X6 (black dashed line).

Date	Substrate water content	
	X1	X6
16/10/2005	2.433 ± 0.368	2.577 ± 0.306
23/10/2005	1.260 ± 0.310	1.537 ± 0.195
30/10/2005	1.840 ± 0.370	1.853 ± 0.366
06/11/2005	1.235 ± 0.312	1.651 ± 0.168
11/12/2005	2.903 ± 0.465	
18/12/2005	1.904 ± 0.254	2.945 ± 0.602
19/02/2006	2.005 ± 0.345	
12/03/2006	2.610 ± 0.380	
19/03/2006	1.669 ± 0.069	
26/03/2006	1.238 ± 0.228	2.222 ± 0.381
02/04/2006	0.476 ± 0.023	1.685 ± 0.023
09/04/2006	0.292 ± 0.108	1.170 ± 0.298

Tab. 1. Average and standard deviation (5 replicates) of substrate water content (weight of water/weight of dry substrate) for the field samples taken in rockpools X1 and X6 when no free water column was available.

Population dynamics

In general, good discrimination and low overlapping are noted among instar sizes in *H. bosniaca* (Tab. 2) which, together with shape differences, allowed an analysis of changes in population structure over time. The temporal changes in the relative abundances of different *H. bosniaca* growth instars are shown in Figure 4 for both the studied rockpools. At the beginning of the hydrological cycle, the relative abundance of the largest sized instars in both rockpools quickly rose, reaching the maturity in about 21 days at 19°C (average of daily mean temperature from 11/9/2005 to 9/10/2005). The individuals in X6 developed more uniformly. Regarding each sampling date, most correspond to instars that are close in size; consequently after the third study week, the population was dominated by adult females (males were never observed in the area, as in previous studies). In rockpool X1, the growth pattern did not advance as sharply as in X6 because a delayed second hatching period occurred at this site (at *c*. 2/10/05), whose further development led to a wider distribution of size-class dominances.

Instar	Carapace lenght (mm)	*N*
A	1.39 ± 0.06	777
A-1	1.05 ± 0.06	1069
A-2	0.77 ± 0.05	1281
A-3	0.59 ± 0.04	871
A-4	0.45 ± 0.03	677
A-5	0.36 ± 0.02	664
A-6	0.30 ± 0.01	654
A-7	0.25 ± 0.01	712
A-8	0.21 ± 0.01	281

Tab. 2. Average and standard deviation of carapace length data for each developmental instar of *H. bosniaca* collected in rockpools X1 and X6 (pooled together) throughout the study period.

The first desiccation phase (4 weeks in October-November 2005) was the longest when ostracod individuals, mainly adults, were able to survive in the substrate for such a long period. At the mid-stage of this long desiccation period, a massive egg hatching coincided with the highest relative percentage of the first instar juveniles (A-8), which were very rare in the remaining sampling dates. Immediately after rainy events in early November, which refilled the pools, a similar instar growth pattern to that observed before the dry period was recorded. However, the growth rate then became slower if compared to the previous main cohort as significant numbers of adults were not detected until 63 days later for pool X6 (the average of daily mean air temperature for these days was 9.3°C) or 105 days in pool X1 (with an average temperature of 8.5°C). During this second major wet period, both pools dried again once (X6) or twice (X1), but these were short-duration desiccation events which lasted no longer than two weeks. By the end of this major wet period when no free water was present in X1 (mid-March 06) and X6 (end of March 06), the wet substrate samples collected still harbored (mainly adult) living ostracods for a few weeks.

Although our sampling method was not strictly quantitative, the data presented for the individuals collected per sample in Figure 4 indicate that densities were sometimes very high, attaining a maximum of 5003 individuals sampled on 22/01/2006 in X1 (with an approximate density of 67 ind. cm^{-2}). In general, we always collected a larger number of ostracods in X1 than in X6. At the beginning of the sampling period, maximum numbers were of a few hundred per sample, falling to a few tens when the rockpools were dry. However, densities were of several thousands in X1 after it refilled in winter.

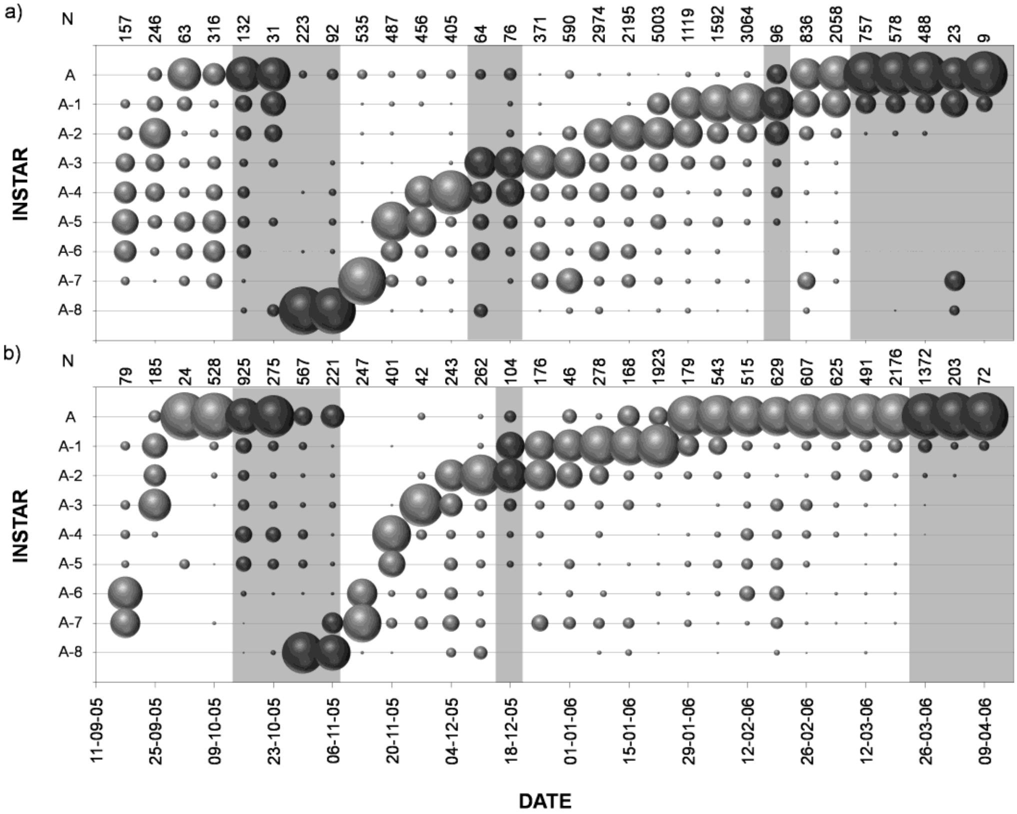

Fig. 4. Dynamics of *Heterocypris bosniaca* populations in rockpools X1 (a) and X6 (b) over an annual cycle. The bubble size represents the relative abundance of each developmental instar (*Y* axis) from the first juvenile instar (A-8) to the adult one (A) for each sample. The filled vertical rectangles indicate periods when the rockpools were dry (no free water column). The total number of individuals per sample is indicated at the top of each graph.

Experiments on desiccation resistance

Figure 5 depicts the substrate water content changes over time measured in X1 and X6 with both the field and experimental treatments. The slope corresponding to water loss during the FD treatment was kept as close as possible to the initial slope when free water evaporated from the water column. On the other hand, the regression slope for bottles undergoing the SD treatment changed from -0.2 to -0.1 at the point at which the water level reached the substrate level. This change in slope was kept as close as possible to the natural water loss rate observed in the substrate samples collected in the field during desiccation processes. The linear regression equations adjusted to the data are shown at Table 3. Despite a certain variability observed in the water loss slopes in the laboratory cultures, the slopes of the two treatments (FD, SD) differed significantly (*F*=2475, p<0.01). The slope of the regression line for water loss with time for the SD treatment was close to the slope calculated for the field data (for either X1 or X6). However, it differed significantly from the last two values (*F*=19.8; *F*=175.5, respectively, p<0.01 in both cases).

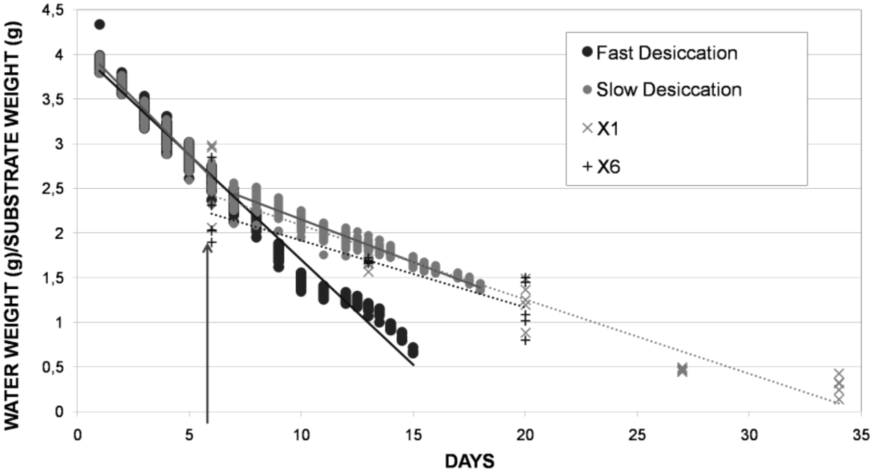

Fig. 5. Change in substrate water content (water weight/dry substrate weight) over time during the desiccation experiment for the two treatments (continuous lines) and during the natural desiccation process for the two studied rockpools, X1 and X6 (dotted lines). The arrow indicates the beginning of the two different treatments.

Group	Linear regression equation	R^2	*p*-value
FD	W = 2.707 – 0.206 t	0.954	$p < 0.01$
SD	W = 2.664 – 0.100 t	0.953	$p < 0.01$
X1	W = 2.506 – 0.083 t	0.915	$p < 0.01$
X6	W = 2.293 – 0.075 t	0.747	$p < 0.01$

Tab. 3. Linear regression equations for the relationship between water content and time, adjusted to the data obtained since no free water column was available in the experimental cultures (FD: fast desiccation, SL: slow desiccation) or in the field (pools X1 and X6).

Ostracod survival during the experiment (Fig. 6) was similar for the two control treatments (no desiccation), with or without substrate, and no significant differences were noted between both groups. However, differences were seen between the SD and FD desiccation treatments and also with the control groups. The Cox regression survival analysis performed with the time-dependent variables shows that time, substrate water content and interaction all significantly affect survival. Indeed, survival lowers with time (age effect), but increases with higher substrate water contents. In addition, survival also lowers with a higher water loss rate. Therefore, the highest survival in desiccation treatments was observed for the highest substrate water content and the lowest water loss rates (SD treatment) where ostracods can survive for 17 days, as opposed to a survival of 14.5 days (the maximum observed) with the FD treatment.

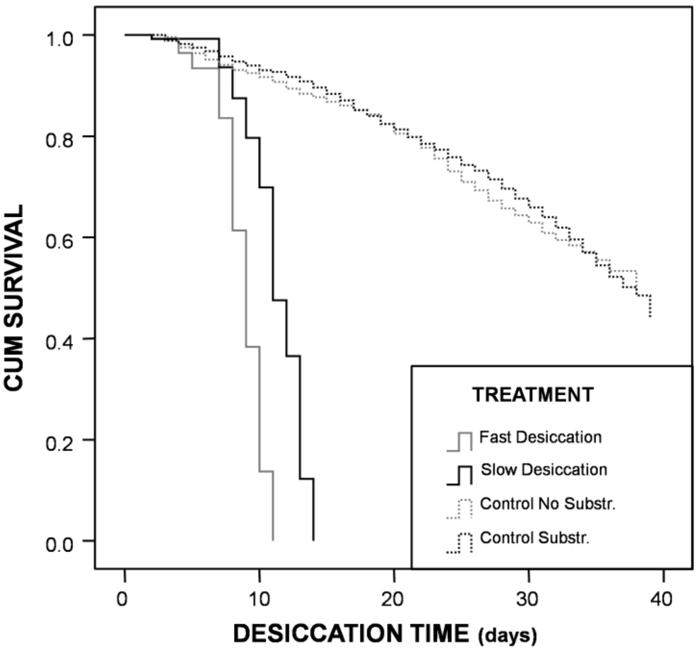

Fig. 6. Survival function for the control (dotted lines) and the desiccation (continuous lines) experimental ostracod groups.

Discussion and conclusion

The two temporary rockpools studied are very similar in terms of the physical and chemical properties of their water despite their differences in size, illumination and surrounding vegetation. Hydroperiod variability is the most important difference noted between them in terms of effects on system functioning, which clearly derives from differences in pool size (surface area, depth) (Hulsmans *et al.*, 2008). Increased conductivity, chloride content and alkalinity recorded toward the end of wet periods are caused by evaporation, whereas dilution occurs after rain, and these factors vastly affect small temporary pools like those studied herein (Williams, 2006). The progressive lowering of pH during the hydroperiod is probably due to leaf litter decomposition, humic acid and tannins production (Bonner *et al*., 1997), reduced O_2 photosynthetic production, plus the dominance of respiration processes. Dark-colored water is more typical of X6, which received a higher input of plant material, not only pine needles, but also big *F. carica* leaves, which decomposed faster than pine remains.

Unlike other studies that reveal a clear succession of dominant invertebrates in temporary ponds (*e.g.*, Lahr *et al*., 1999), including ostracod species (McLay, 1978), the only dominant ostracod species found throughout the study period in both X1 and X6 was *H. bosniaca*. The densities of this dominant species differed between the two studied sites, and the higher density was recorded in the smaller rockpool (X1). This may be due to the physical substrate structure as the bottom of X1 was totally covered with a dense mat of pine needles that notably increased the available surface for benthic ostracod oviposition and protection. Although the fig leaves in X6 initially provided increased available surface, they rapidly decomposed unlike pine needles. Another possible negative effect of the presence of fig leaves on ostracod density could relate to the release of chemicals into the water by these leaves.

The presence of *H. bosniaca* in these temporary environments indicates its ability to produce desiccation-resistant eggs, which is also the case with many other crustaceans (Alekseev, 2007), and is particularly common in ostracods of the genus *Heterocypris* (Meisch, 2000). These crustaceans keep a propagule egg bank in the substrate when pools dry (Wissinger, 1999), and individuals can remain in this arrested state for several months until the next rain period arrives (Geiger, 1998; Bilton *et al*., 2001). Most *H. bosniaca* hatching events occurred after the rockpool filled, but some others were detected during wet periods. This suggests that this species can also produce subitaneous eggs, similar to its congeneric cosmopolitan species *H. incongruens* (Angell & Hancock, 1989). However as observed in X6 on 12/02/2006, hatching events during wet periods mainly take place after some minor rainfall. It is therefore likely that pool water dilution acts as a hatching stimulus in *H. bosniaca*, as it does in other invertebrates (Bonner *et al*., 1997). In this sense, it is remarkable to observe a massive hatching event inside the substrate after heavy rainfall in late October, although no free water was present in the pools.

With the studied population cycle, we observe how the individual development rate varied among different cohorts. At the beginning of the study period (late summer-early fall) with high temperatures, it was much faster than in the cohorts that developed in late fall or winter months with low temperatures. As with other ostracods from temporary pools (Ganning, 1971; Latifa, 1987), temperature is a key factor for the development rate. The time needed by *H. incongruens* to complete development in lab cultures was 18 days at 20°C (Latifa, 1987), very close to the 21 days at 19°C observed during the first main cohort development of *H. bosniaca* in the field. However, *H. incongruens* needed 81 days at 10°C while *H. bosniaca* reached maturity in 63 days at an average temperature of 9.3°C in pool X6 during the second main cohort growth period. These differences in developmental rate could be due not only to species-specific physiological traits but also to the high variability of water temperature in the field, which can provide short daily periods with water temperature warmer than the average, facilitating growth more effectively than the negative effects of reduced temperatures. Future experiments to evaluate the growth rate of *H. bosniaca* under controlled temperatures could clarify this issue.

In a study on the population dynamics of *Heterocypris salina*, Ganning (1971) found that each periodical sample was dominated by just one or two instars. *H. bosniaca* shows a similar pattern, although there are a few samples dominated by more than two instars. This can be seen in Figure 4 during the first four samples in pond X1, where the third and fourth samples show the appearance of further instars due to new hatching periods. This is a difference with *H. salina*, because in this species second cohorts never developed as simultaneously as the first (Ganning, 1971).

During the 25-27-week study period, *H. bosniaca* showed a clear bivoltine life history pattern, similar to that found by Ganning (1971) on *H. salina* in brackish rockpools, but further minor hatching events occurred sparsely over the main cohorts' development periods in *H. bosniaca*, which led to the overlap of different cohorts (the best example can be seen in Fig. 4, which occurs in pond X1 from de day 2/10/2005). This bivoltine (or broadly multivoltine) strategy, together with parthenogenesis reproduction, makes *H. bosniaca* a pioneer r-strategist in the framework of ostracod ecological life cycles (Ganning, 1971; Geiger, 1998; Williams, 2006), as is mostly the case of other members of the genus.

Our results on the seasonal dynamics of parthenogenetic populations of *H. bosniaca* might not be directly extrapolated to other geographic areas where bisexual populations are found (Israel, the Balkans: Petkowski *et al*., 2000; Martens *et al*., 2002) as these might behave differently. In the geographic parthenogen ostracod *Eucypris virens*, Martins *et al*., (2008) have shown that parthenogenetic individuals developed equally fast as those from bisexual populations, but, on average, parthenogenetic resting eggs hatched earlier and more synchronously than sexual resting eggs. If *H. bosniaca* followed the same pattern of development as *E. virens*, we should expect differences in population dynamics if the studied pools contained sexual populations because this hatching pattern should not produce massive blooms of eggs hatching spaced in time, but cohorts that overlap more gradually; moreover in each sample, we might found more variability in the proportions of various growth instars found. In the future, it would be desirable to test if this was confirmed in sexual populations in other areas.

Generally, in ostracod life history strategies, diapausing eggs are the main growth phase considered to be desiccation-resistant, and juvenile or adult individuals are rarely taken into account (but see Delorme & Donald, 1969; Horne, 1993). In our experimental study on desiccation resistance, we conclude (as Horne did in 1993) that substrate water content is a determining factor for the ostracods buried in the sediment. In addition, not only the total water content, but also the water loss rate, influences survival rates. In his experiments with *Candona patzcuaro*, Horne (1993) observed how the juvenile individuals buried in the mud survived for more than one year, yet later on these individuals took more than one day to leave their diapausing state. *H. bosniaca* individuals seem to survive a much shorter time, but start swimming a few minutes after adding water to the substrate. Our field data suggest that both the adults and juveniles of this species can survive in substrate for more than one month (depending on water loss), while nothing is known about longer periods with the free-living ostracod instars of the same genus. As we explained in Material and methods section, we used only individuals from pond X6 in the experimental study on desiccation resistance because it contained sufficient substrate, while pond X1 may have been negatively affected if we collected the same amount of substrate. Given its smaller size, pond X1 undergoes more desiccation events and, in this sense, can be considered a more stressful environment than pond X6. Consequently, ostracods may adapt better to desiccation and may present genetic and/or ecological differences between the two ponds. It would be interesting to test in the future whether or not there are any differences in the genetic structure of both populations, and if these relate to the possible differences in their adaptation to habitat variability.

Diapausing eggs are considered the key propagule stage for aquatic invertebrate dispersal through vectors such as wind (Vanschoenwinkel *et al*., 2008a) or vertebrates as they are able to tolerate adverse conditions and allow the quick colonization of new habitats (Zadereev, 2007). As we have seen, *H. bosniaca* juvenile and adult individuals can tolerate certain periods with no free water available and, therefore, they (and not only their eggs) can potentially be transported by vertebrates. Mammals and birds have been observed directly or indirectly when the studied rockpools have been used for drinking or bathing purposes (particularly wild boar); therefore, these animals can act as dispersal vectors among the pools in the area and in those located further away (Vanschoenwinkel *et al*., 2008b). Both mammals and birds are known to readily disperse ostracods over relatively long distances and in significant amounts (Figuerola & Green, 2002; Figuerola *et al*., 2003; Green & Figuerola, 2005; Green *et al*., 2008).

In small temporary aquatic environments, resource limitations and habitat unpredictability impose strong selection pressures, leading to impoverished invertebrate fauna composed of highly specialized small body-sized species (Collinson *et al*., 1995) that develop dense populations in the absence of big predator taxa (Jocque *et al*., 2010). These include anostracans, cyclopid copepods, cladocerans and ostracods, among other groups. Many ostracod species have strategies to survive desiccation in the long term (diapausing eggs). In *H. bosniaca*, the combination of this long term strategies, and over short periods (juveniles and adults protected by a closed carapace), besides parthenogenetic reproduction, quick eggs hatching and short growth time to complete development, may offer ecological advantages to allow the rapid colonization and monopolization of small temporary water bodies.

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Capítulo III

Breaking the temperature-size rule: Thermal effects on growth, development and fecundity of a crustacean from temporary waters.

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Abstract

The temperature-size rule (TSR) is a well-established phenomenon to describe the growth response of ectotherms to temperature by which individuals maintained at low temperatures grow more slowly, but attain a larger size upon maturity. Although there are adaptive and non-adaptive theories about the plasticity of body size in response to temperature, these cannot be applied to all ectotherms, and little is known about the changes in growth and development rates through ontogeny. The ostracod species *Heterocypris bosniaca*, an inhabitant of freshwater temporary ponds, was used to examine the growth and development rates of its nine growth stages and female fecundity at four different temperatures (15ºC, 20ºC, 25ºC and 30ºC). The development rate of this species accelerates with increasing temperature, reaching a maximum value at 25ºC. The growth factor has a reverse-TSR in younger instars, and the typical TSR is followed only in the last two moults, resulting in non-monotonic response of adult size to temperature. Fecundity (total offspring per female) was not directly related to adult size and was generally higher at lower temperatures. Our results agree with recent research showing that the TSR may vary during ontogeny, and may not be a general trend in ostracod species from temporary waters.

Keywords: Development, fecundity, growth, temperature-size rule, intermoult period, Ostracoda.

Introduction

The age and body size of individuals are probably the most important features in the life history of organisms. Both are critical because they affect all aspects of life, from anatomical and physiological to ethological and ecological (Forster *et al.*, 2011; Walters & Hassall, 2006). Therefore, understanding the mechanisms regulating the development and body size of organisms is of vital importance in ecology.

Given the absence of thermoregulatory mechanisms in ectotherms, growth and development strongly depend on temperature. Most studies have shown that ectotherms grow faster at higher temperatures, but that attained size is smaller than at lower temperatures (Atkinson, 1994; Atkinson & Sibly, 1997; Hartnoll, 2001). This is a widely documented phenomenon, called the Temperature-Size Rule (TSR). However, it is not clear what general physiological mechanisms might regulate it (Kingsolver & Huey, 2008) and whether or not the same pattern is always shown during ontogenetic development (Forster *et al.*, 2011). In addition, it is unclear whether this response is an adaptation, a product of physiological limitations (Walters & Hassall, 2006), or a combination of multiple factors (Angilletta *et al.*, 2004). Initial attempts to explain the TSR include the models of Von Bertalanffy (1960) and Perrin (1995) that postulated that anabolism is less thermally dependent than is catabolism. A related remarkable hypothesis is the “maintain aerobic scope-regulate oxygen supply” (MASROS) by Atkinson *et al.* (2006), that point out that metabolic rate increases more dramatically with temperature than does oxygen supply. This situation can lead to oxygen limitation at warmer temperatures that would be more likely to occur in larger individuals. Alternatively, Angilleta and Dunhan (2003) proposed that the TSR could arise from a constraint on growth rate that arises late in ontogeny, *i.e.* decreasing the thermal optimum for growth efficiency with increasing body size. Despite the TSR being generally applied widely, some species do not follow it, either because they do not show variations in body size or because they reach larger sizes at higher temperatures (Hartnoll, 1982; Atkinson, 1995). Walters and Hassall (2006) suggested that ectotherms that obey the TSR can be identified as having a higher minimum temperature threshold for development rate than for growth rate. However, more recently Zuo *et al.* (2012) proposed an energetic fractionation model to account both for TSR and reverse-TSR depending on imbalances in the energy allocated to somatic growth or to maintenance and development. The diversity of TSR trends are important when considering that variations in body size are among the most sensitive indicators of minor changes in environmental temperature (Hoog & Williams, 1996) and are considered a universal ecological response to climate change in aquatic systems (Daufresne *et al.*, 2009).

Discontinuous growth of crustaceans is divided into two components (Hartnoll, 1982): increase in body size (moult increment in absolute terms and the growth factor in relative ones) and time from one moult to the next (intermoult period). Ostracod growth is characterised by a series of moults until the final ecdysis, during which sexual maturity is reached. There are many ecological factors that can affect growth and development (Alcorlo *et al*., 1999; Hartnoll, 2001; Juárez-Franco *et al*., 2009; Hart & Bychek, 2011), but temperature is considered the dominant one in ostracods (Schreiber, 1922; Kesling, 1951). In these organisms, the developmental rate decreases with lower temperatures in natural populations and under laboratory conditions (Ganning, 1971; Latifa, 1987; Roca & Wansard, 1997; Mezquita *et al*., 1999), while adult size does not present a clear monotonic response to temperature (Martens, 1985; Liberto *et al*., 2013). This scenario demonstrates that ostracods undergo accelerated development with increasing temperatures, but the TSR does not always apply to this group's body size, as is also the case for other crustaceans (Hartnoll, 2001).

Heterocypris bosniaca is a poorly known species of ostracod. It was discovered in 2000 in Bosnia (Petkowski *et al*., 2000) and since then it has only been reported in Israel (Martens *et al*., 2002) and Spain (Aguilar-Alberola & Mezquita, 2008). In previous studies on its population dynamics in two small temporary rockpools in eastern Spain we observed that the species has a multivoltine life cycle adapted to the unpredictability of these stressful environments (Aguilar-Alberola & Mesquita-Joanes, 2011). The main dry phase occurs in summer and the resting egg bank enables the continuity of the species in the rockpools. We also observed variations in the intermoult period among different cohorts; they developed faster at higher temperatures in fall than during cold winter months, which match the expected relationship between development rate and temperature within the TSR framework. For this reason, we decided to analyse the response of the species under controlled laboratory conditions in detail to check if warmer temperature had the effect of development acceleration observed in natural populations and whether or not this ostracod species followed the TSR pattern.

This paper analyses the effects of temperature on development rate, size changes at each developmental instar and adult fecundity and survival of a multivoltine temporary water ostracod. This allows evaluating the validity of the TSR through ontogeny in aquatic ectotherms with determinate growth, but also to relate differential temperature effects on growth and development with fitness components such as fecundity and survival, in order to test if the assumptions *Bigger is better* and *Hotter is smaller* (Kingsolver & Huey, 2008) hold in this group of organisms adapted to quickly changing environments.

Material and methods

The ostracods used for this study were collected in a small (92 x 120 cm, max. depth 20 cm) temporary pond in eastern Spain (rockpool “X6” in Aguilar-Alberola & Mezquita, 2008; coord. 39º02'51"N 0º26'14"W). In the previous field study by Aguilar-Alberola and Mesquita-Joanes (2011), the main hydroperiod of this pond lasted for 23 weeks, from September 2005 to March 2006. During this period water temperature measured during the day averaged 13.7ºC, ranging from a minimum value of 6.5ºC to a maximum of 27ºC, but the average air temperature in the area was 11.4ºC, varying between -4.4ºC and 31.7ºC. Individuals (all parthenogenetic females) were captured using a small hand net, and were transported to the laboratory and divided into four 1.5 L aquaria with filtered water from the pond to set stock cultures. The four aquaria were kept in growth chambers at constant temperatures of 15ºC, 20ºC, 25ºC and 30ºC each and under a photoperiod of 12:12 h. Cultures were provided with food every 2-3 days, which alternated between insect homogenate and dried *Spirulina* sp. in all the experimental phases. The water level was kept constant by the addition of distilled water when required. Under these conditions, stock cultures were maintained until ostracods had acclimatised to the temperature and a new cohort of individuals had appeared in the aquaria.

In order to determine the intermoult period at different temperatures, the ostracods belonging to early developmental instars were individually isolated in 2 mL multi-well plates and checked every 12 h (the first two juvenile instars A-8 and A-7) or 24 h (instar A-6 to adult) for survival and moulting. Due to the fragility and short duration of stage A-9 (no longer than 2 min at 25ºC; Aguilar-Alberola & Mesquita-Joanes, 2013), the present survey does not take this stage into account. With each observation event, individuals’ status (alive/dead) was checked and detached valves were sought to determine on which the day the moult (ecdysis) occurred. All the valves and dead individuals were removed from the multi-well plates and they were measured by stereomicroscope observations to calculate the growth factor (growth rate between the instar carapace length after moulting compared with length of previous instar) and the instar to which they belonged. This enabled the duration of all the instars to be calculated, except A-8 because the day they hatched was unknown. Due to the high mortality of isolated individuals, new multi-well cultures had to be prepared with ostracods belonging to older instars in order to obtain a minimum of 20 data per instar and temperature. In total, we used between 200 and 300 individuals at each temperature. All the individuals in multi-well plates used for periodic observations were picked from the stock cultures grown at the same temperature tested.

In order to determine i) fecundity (number of eggs or offspring per female), ii) maturation time (from adulthood to the first egg laid), iii) egg development time (from laying to hatching) and iv) duration of the A-8 instar, two types of experimental treatments with adult females were performed. One group consisted of adult females, which were isolated individually in one well until death (the fixed well adults or “fwA” group). It was not possible to count the number of eggs in this treatment because the species lays eggs attached in clutches at the bottom of wells, which overlap. However, we were able to count the number of offspring which eventually hatched per female in this treatment. In another treatment, isolated adult females were transferred to a new well just when freshly laid eggs were observed (the transferred adults or "tA" group). This different treatment was necessary in order to estimate the number of eggs laid daily, the proportion of eggs hatched over a 40-day period without dehydration since they were laid, and the time required for hatching. The isolation of some of the recently hatched individuals allowed us to estimate the duration of instar A-8. Comparison of adult instar duration and the total amount of offspring hatched between both adult female groups was assessed to evaluate the potential effects of female handling and mother presence on adult survival (adult life span) and fecundity (final offspring).

The main life history traits (instars' time span, time to first egg laying, egg development time, number of offspring of fwA and tA females) were first analyzed applying a Levene's test to check homogeneity of variance compared among temperature treatments. Then, according to the absence of homocedasticity, the differences in median values for these traits among treatments were tested by Kruskal-Wallis nonparametric tests. When significant differences were obtained, the Bonferroni-corrected Mann-Whitney test was used for pair-wise comparisons. This test was also applied to check for any statistical differences for the duration of adult females and the number of offspring between treatments fwA and tA for each temperature in order to analyse if female extra handling and mother absence had significant effects on adult survival and fecundity. In order to standardise the data of number of eggs and hatchlings per female, these values were divided by the number of fertile days for each female (time since the first egg was laid until death). Variations in both total carapace length and its relative increment were analysed by ANOVA. When the Levene's test indicated that the variance between temperature treatments was homogeneous, the Bonferroni-corrected t-test was used for pair-wise comparisons, whereas the Games-Howell test was utilised when variances were not homogeneous. Following Liberto *et al*. (2013), we used a sigmoid regression model to estimate the relationship between size and time, as this model offered the best significant fit in ostracods according to these authors. The expression of this model was $y=a/(1+\exp(-(x-b)/c))$, where y = ostracod length (mm), x = time (days) and a, b and c are the parameters relating to the shape of the curve. To build these models, as some individuals of late instars were obtained from the stock cultures (and consequently their age was unknown), we calculated their approximate age by summing the mean life span of each previous stage plus or minus a random value of the standard deviation. The relationship between female size and fecundity was tested by linear regression. For this analysis we used the number of offspring per female in the fwA treatment (as they experience reduced handling compared to tA females) as dependent variable and shell length as independent factor, and used the data from all temperature treatments pulled together. All the statistical analyses were performed with software packages SPSS v.15 (Norusis, 2004) and PAST v.2.14 (Hammer *et al*., 2001).

Results

As postembryonic development advanced in *Heterocypris bosniaca*, the duration of juvenile instars progressively prolonged at all the temperatures tested from instar A-8 (with a median intermoult period of 0.84 days at 20ºC) to instar A-1 (6.49 days at 20ºC; see Fig. 1). The Kruskall-Wallis analyses always showed significant differences when comparing the duration of each stage among the different culturing temperatures (for the nine comparisons the value of the H statistic ranged between 62.16 and 132.7; H(A-8)=77.06, H(A-7)=94.23, H(A-6)=105.8, H(A-5)=82.54, H(A-4)=62.16, H(A-3)=65.59, H(A-2)=68.55, H(A-1)=94.24, H(A)=132.7. In all cases, d.f.=3 and $p<0.001$). The longest recorded intermoult period and adult life-span corresponded to 15ºC, and a clear pattern of reduced time with increasing temperature was observed (Figs. 1 and 2), although the duration of some instars at 25ºC and 30ºC was similar and showed no significant differences (Fig. 1). At all the temperatures tested, except at 20ºC, significant differences were found in adult instar duration when comparing the two types of handling treatment carried out; transferred adult females (tA) lived for shorter time spans than fixed well adult females (fwA) (Fig. 2).

Due to the high instar-specific mortality rate (on average 21% of individuals died before moulting from one instar to the next) recorded in isolated individuals (Tab. 1), no single ostracod completed the entire development cycle in our experiment. For this reason, a large number of individuals at different instars was needed to obtain the amount of data required for robust statistical analyses. When throughout its development an individual died, the time elapsing between the last moult and its death (Tab. 1) could be very short (1 or 2 days) or could be extended to take considerably longer than the time usually required for the next successful moult (*i.e.* specific-instar mean duration, Fig. 1). Regarding adult individuals, on average 39% of the adult females died without laying a single egg (Tab. 1).

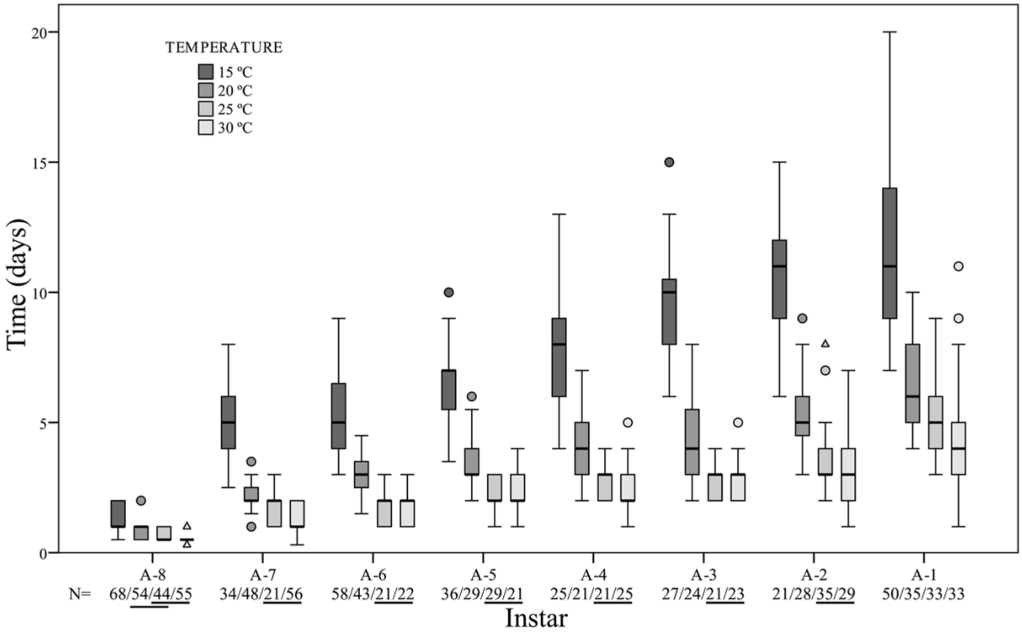

Fig. 1. Intermoult period of *H. bosniaca* juvenile instars (A-8 to A-1) tested at different temperatures. The number of data (N) is shown at the bottom. Lines below N values link groups with no significant differences according to Mann-Whitney U-tests with Bonferroni correction (p>0.05).

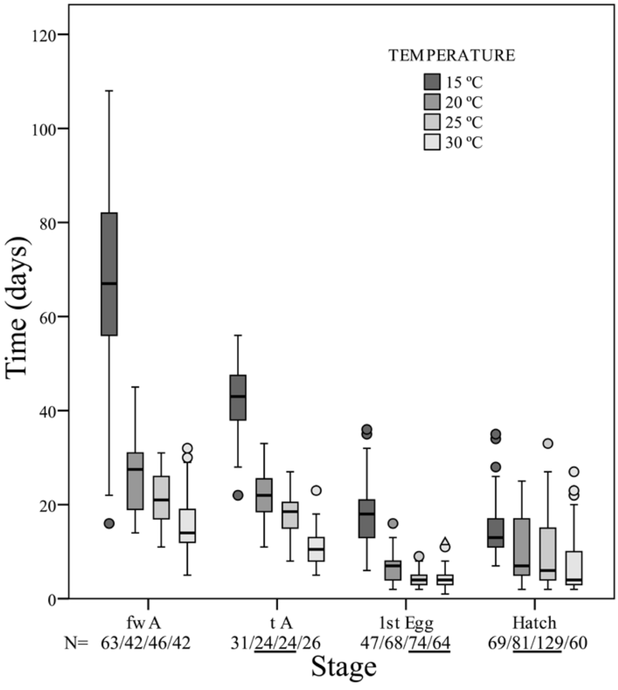

Fig. 2. Adult life span at two treatments (fwA and tA), time required to lay the first egg since moulting to adulthood (“1st egg”), and development time of the eggs (“Hatch”). The number of data (N) is shown at the bottom. Lines below N values link groups with no significant differences according to Mann-Whitney U-tests with Bonferroni correction (p>0.05). fwA = fixed-well adult females; tA = transferred adult females.

Tab. 1. Time (days) elapsed from moulting to death (MAX, MEDIAN and MIN), number of deaths recorded (N) and the proportion that they represent of the total individuals that have reached this instar (instar-specific mortality rate, %DEATH). For the adult instar, the data refer to females that did not lay any eggs.

		INSTARS								
		A-8	A-7	A-6	A-5	A-4	A-3	A-2	A-1	A
15º	MAX	-	20	17	17	31	21	31	45	50
	MEDIAN	-	10	11	6	10	7	19	18	23
	MIN	-	1	1	1	1	1	1	1	1
	N	0	28	34	30	26	14	17	13	61
	%DEATH	0	21.4	32.4	33.7	34.7	25.9	20.7	8.2	39.4
20º	MAX	-	4	8	11	18	21	19	37	21
	MEDIAN	-	2	3	4	9	5	10	13	14
	MIN	-	2	1	1	4	1	2	1	1
	N	0	7	17	28	8	15	15	21	32
	%DEATH	0	8.1	21.1	47.4	11.1	23.7	18.6	16.4	32.7
25º	MAX	6	8	7	10	14	12	17	24	18
	MEDIAN	2	4	4	3	2	4	4	8	5
	MIN	2	1	1	1	1	2	2	1	1
	N	5	26	21	28	25	12	10	45	34
	%DEATH	1.9	28.8	24.7	37.3	38.5	17.7	10.5	28.3	32.7
30º	MAX	-	3	8	11	10	8	8	15	18
	MEDIAN	-	2	3	4	3	3	3	5	4
	MIN	-	1	1	2	1	1	1	1	1
	N	0	5	55	13	26	20	14	28	74
	%DEATH	0	5.0	52.0	13.3	35.6	26.5	17.7	16.0	52.1

The time required by a female to lay the first egg since reaching adulthood decreased with increasing temperature (Fig. 2), although there were no significant differences between these times recorded at 25ºC and 30ºC. The variability in the species' fecundity was broad (Fig. 3) with up to a maximum number of 78 offspring per female at 20ºC. According to the linear regression results, we found no significant relationship between female size and fecundity (considered as the number of offspring per female in the fwA treatment; F=1.22, d.f.=1, 203, p>0.05). Significant differences between temperature treatments were observed for the number of eggs laid per female (for tA. H=61.61, d.f.=3, p<0.001), or per female per fertile day (H=57.65, d.f.=3, p<0.001) and for the number of offspring per female (for fwA. H=36.64, d.f.=3, p<0.001) or per female per fertile day (H=41.41, d.f.=3, p<0.001). Maximum values for these response variables were recorded at 20ºC and 25ºC, except for offspring per female, with maximum values at 15ºC (Fig. 3). The Kruskall-Wallis analysis found no significant differences among temperatures in the number of offspring per female (H=2.31, d.f.=3, p>0.05) or per female per day (H=2.58, d.f.=3, p>0.05) in the tA group (Fig. 3). It was not possible to compare the number of eggs laid by females in both treatment types. However when comparing the number of offspring per female at each temperature, significant differences were found (H(15ºC)=54.8, H(20ºC)=22.77, H(25ºC)=30.8, H(30ºC)=12.59. In all cases, d.f.=1 and p<0.001), with higher values for the fwA group compared to the tA group (Fig. 3).

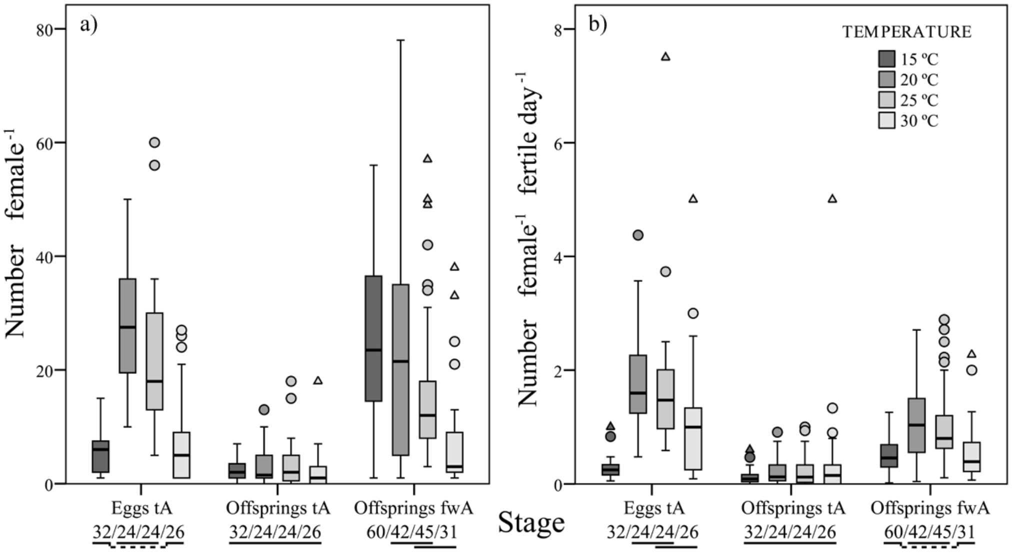

Fig. 3. Total (a; number female^{-1}) and standardised (b; number fertile day of each female^{-1}) number of eggs and offspring of the two adult treatments (fwA and tA) of *H. bosniaca* cultured at different temperatures. The number of data (N) is shown at the bottom. Lines below N values link groups with no significant differences according to Mann-Whitney U-tests with Bonferroni correction (p>0.05).

Regarding the mean number of eggs laid per adult female (Fig. 4), they produced the largest number of eggs at the beginning of laying periods (<1 egg day^{-1} at 15ºC, 3 eggs day^{-1} at 20 and 25ºC and 1.5 eggs day^{-1} at 30ºC). The proportion of these eggs that hatched at any moment during the 40-day observation period since they were laid (black bars, Fig. 4) was very low and no clear pattern of subitaneous *vs.* diapausing egg laying or hatching was found.

The minimum egg development time (*i.e.* the time elapsed from laid to hatched) was 2 days at 20ºC, 25ºC and 30ºC, whereas it increased to 7 days at 15ºC (Fig. 5). At all the temperatures tested, the distribution of egg development time showed several peaks, although the most important was the first, which reached a maximum of between 15% and 25% of the eggs hatched on a single day. These peaks appear earlier at warmer temperatures, about 3-5 days after laid at 20-30ºC but eggs develop slower at 15ºC, where the main hatching peak occurs 12 days after being laid.

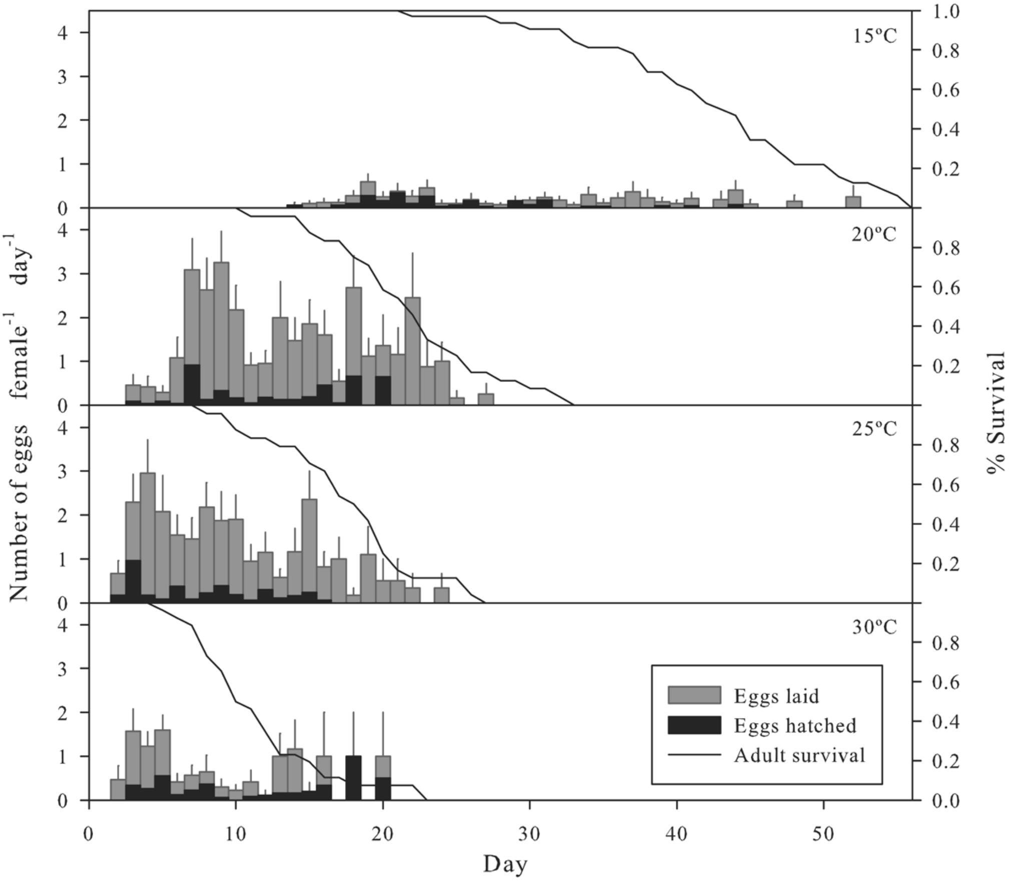

Fig. 4. Average daily eggs laid per female (grey bars with standard error represented by lines) and proportion of those that eventually will hatch at any time during a 40-day observation period since they were laid (black bars). The proportion of surviving adult females is also shown (black line).

Individuals' length varied with water temperature, but no constant pattern throughout development was seen (Fig.6). In early instars (A-8, A-7), larger individuals were those cultured at lower temperatures, but size did not significantly differ among temperatures for instar A-6 (Tab. 2). In older instars (A-5 to Adult), the individuals reared at 15ºC were the smallest for each instar. The longest carapace lengths were attained at 30ºC in instars from A-5 to A-2. However by the end of development (instars A-1 and Adult), the largest individuals were those cultured at 25ºC, whereas the individuals grown at 30ºC were smaller in size.

The variation observed in the growth factors shows that the largest relative increase in size occurred for the moults from A-3 to A-2, but most notably from A-2 to A-1, with values increasing by up to 40% in length (Fig. 6). In general, the individuals reared at 25ºC show the largest growth factors during early developmental

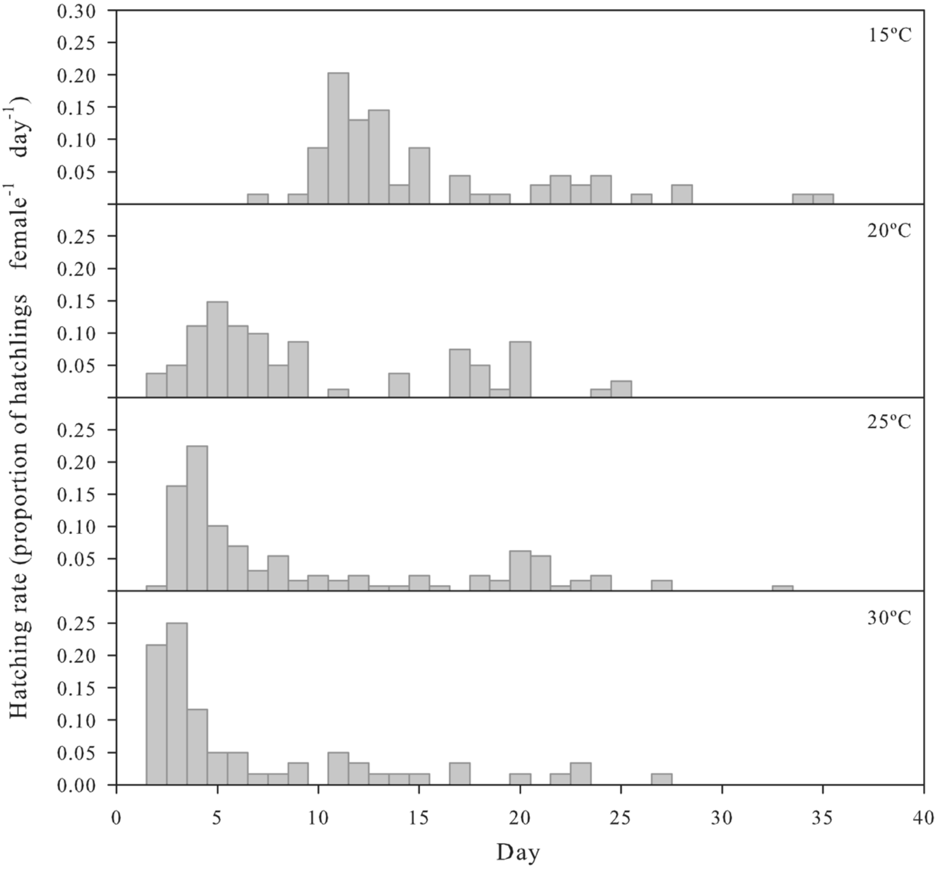

Fig. 5. Distribution of egg development time (days from laid to hatched) over a 40-day observation period since they were laid (day 0).

instars. However from instar A-2 to adulthood, the temperature response seemed more gradual for the range used in these cultures, showing the largest relative increases at lower temperatures, although they did not suffice to reach the longest adult lengths.

Figure 7 shows the size-age distribution at the four tested temperatures. The dispersion of data is smaller at the beginning of the development and increases with time and larger individual sizes. The sigmoidal regression models were significant and presented good fit according to the coefficient of determination (at 15ºC $R^2=0.96$, F=3736.22, d.f.=2, 335; at 20ºC $R^2=0.94$, F=2632.25, d.f.=2, 307; at 25ºC $R^2=0.97$, F=3540.25, d.f.=2, 244; at 30ºC $R^2=0.94$, F=2183.51, d.f.=2, 275; in all cases $p<0.0001$). The increase in growth rate with temperature is smaller at warmer conditions, changing approximately from 0.059 mm day^{-1} at 25ºC to 0.064 mm day^{-1} at 30ºC and from 0.018 mm day^{-1} at 15ºC to 0.040 mm day^{-1} at 20ºC (values estimated from the regression models for the growth from A-4 to A-1, where the slope is maximum and more linear).

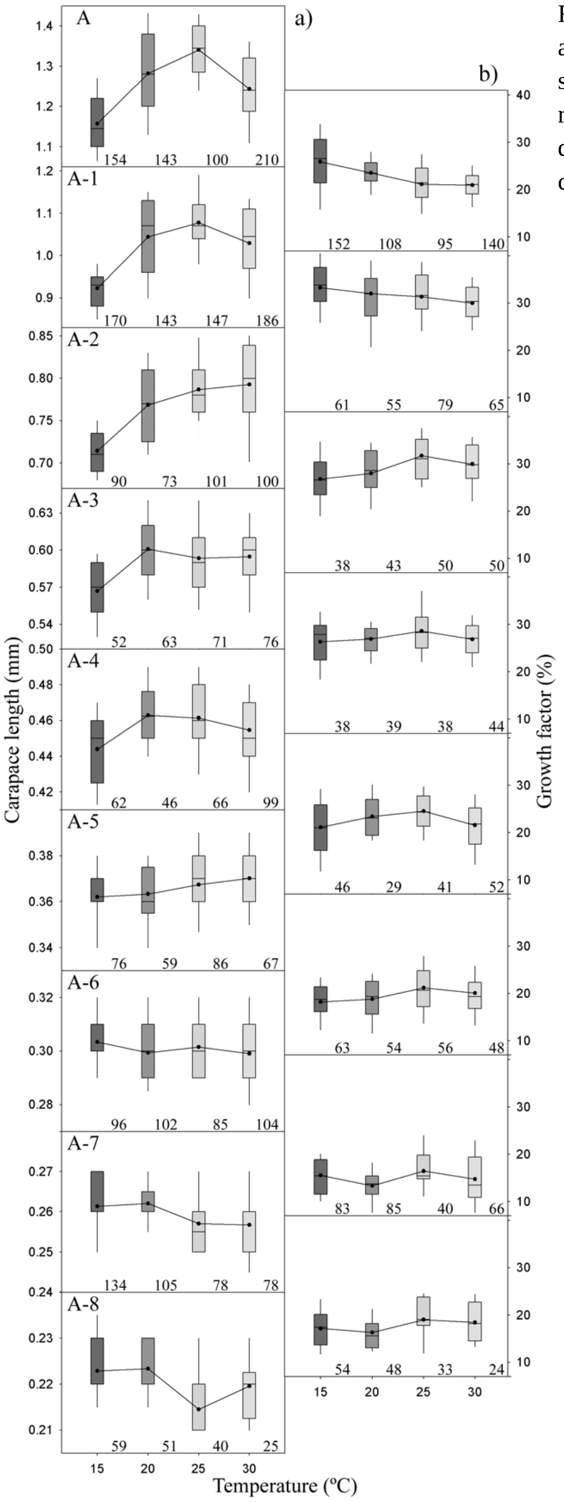

Fig. 6. Length (a) and growth factor (b) for all the instars at the four temperatures studied. The points connected by lines represent the mean value and the number of data is shown at the bottom right for each category.

Tab. 2. Significance values obtained by comparing the length (top right triangular matrix) and growth factor (lower left triangular matrix) among all pairs of experimental temperatures using Bonferroni-corrected t-tests. Pairs of temperatures showing significant differences (P<0.05) are in bold.

Inst.	Temp.	15ºC	20ºC	25ºC	30ºC
A	15ºC	-	**0.000**	**0.000**	**0.000**
	20ºC	**0.003**	-	**0.000**	**0.004**
	25ºC	**0.000**	**0.009**	-	**0.000**
	30ºC	**0.000**	**0.001**	1	-
A-1	15ºC	-	**0.000**	**0.000**	**0.000**
	20ºC	0.689	-	**0.006**	0.491
	25ºC	0.128	0.928	-	**0.000**
	30ºC	**0.000**	0.35	0.594	-
A-2	15ºC	-	**0.000**	**0.000**	**0.000**
	20ºC	1	-	**0.041**	**0.016**
	25ºC	**0.001**	**0.021**	-	0.817
	30ºC	0.091	0.767	0.838	-
A-3	15ºC	-	**0.000**	**0.000**	**0.000**
	20ºC	1	-	0.913	1
	25ºC	0.503	1	-	1
	30ºC	1	1	1	-
A-4	15ºC	-	**0.000**	**0.000**	**0.019**
	20ºC	0.305	-	1	0.208
	25ºC	**0.022**	1	-	0.309
	30ºC	1	0.753	0.08	-
A-5	15ºC	-	1	0.197	0.016
	20ºC	1	-	0.783	0.104
	25ºC	**0.006**	**0.057**	-	1
	30ºC	0.282	1	1	-
A-6	15ºC	-	0.143	1	0.092
	20ºC	**0.004**	-	1	1
	25ºC	0.769	**0.007**	-	1
	30ºC	0.783	0.318	0.388	-
A-7	15ºC	-	0.881	**0.007**	**0.001**
	20ºC	1	-	**0.001**	**0**
	25ºC	0.174	0.021	-	0.996
	30ºC	1	0.384	1	-
A-8	15ºC	-	1	**0.000**	0.408
	20ºC		-	**0.000**	0.256
	25ºC			-	0.05
	30ºC				-

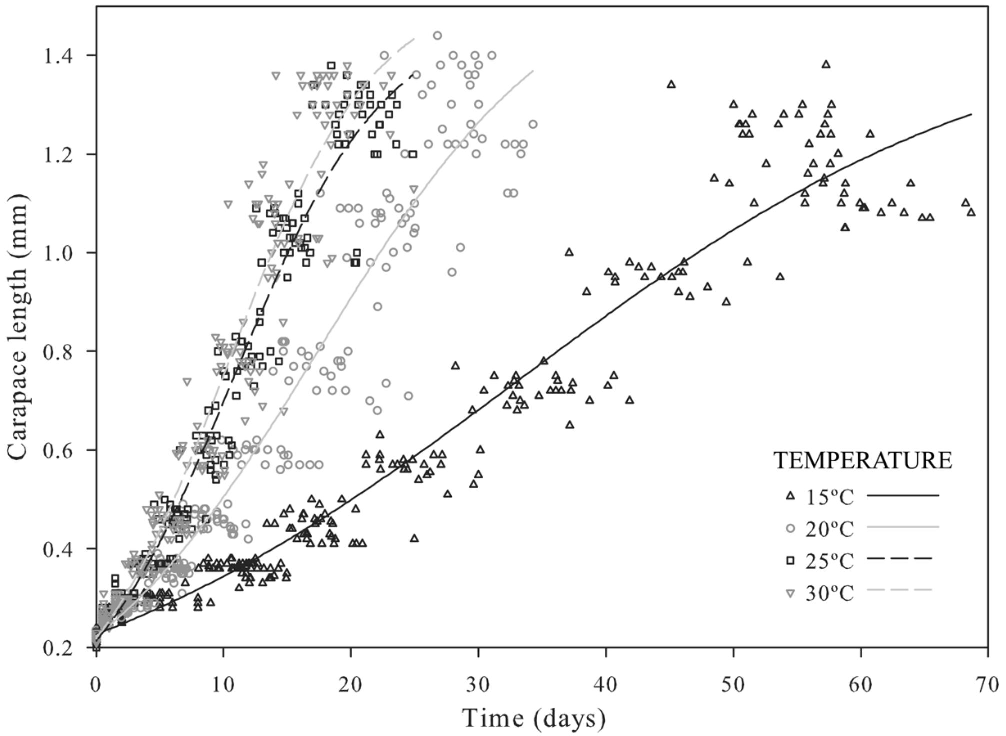

Fig. 7. Relationship between carapace length and age in *Heterocypris bosniaca* at four culture temperatures. Lines correspond to the sigmoid growth models fitted to the data.

Discussion

Similarly to other species of ostracods (Latifa, 1987; Roca & Wansard, 1997) and most ectotherms (Atkinson, 1995), the developmental rate of *H. bosniaca* is strongly influenced by temperature, so that its developmental stages' intermoult periods reduce with increasing temperatures. Yet this increase in the development rate reduces with higher temperatures, until a point is reached where non-significant differences are observed for most stages between 25ºC and 30ºC (the only exception being instar A-1). Hartnoll (2001) stated that the intermoult period in crustaceans always decreases with temperature, while mortality increases. However, in *H. bosniaca*, the maximum developmental rate is attained at around 25ºC, and despite mortality being high in the cultures, no clear pattern is observed. Therefore, these crustaceans can show an optimal temperature for development at between 25ºC and 30°C.

During a previous population dynamics study of *H. bosniaca* in natural ponds, about 21 days at an average air temperature of 19ºC (with a maximum of 31.7ºC and 7.7ºC minimum) were required to complete the species' development (Aguilar-Alberola & Mesquita-Joanes, 2011), while 30.1 days at constant 20ºC are needed in the cultures carried out in this experiment. In the field, a second cohort which appeared during winter months needed 63 days to complete development at a mean air temperature of 9.3ºC (with a maximum of 24.5ºC and -1.7ºC minimum). In the laboratory, we did not test temperatures in this low range because the intermoult period of individuals lasted too long in preliminary cultures at 10ºC and instar-specific mortality was very high. At 15ºC, however, an average of 58.1 days is required in our experiment to attain adulthood. Hartnoll (1982) indicates that the intermoult period in crustaceans is always likely to be longer under experimental conditions than in the wild due to the possible disruptive effects associated with the trauma of capture. In addition, the temperature in natural ponds varies on a daily basis; therefore, daytime temperatures above the mean can be achieved for some hours, which may favour development (Worner, 1992) and moulting processes (Mezquita *et al*., 1999), unlike laboratory conditions where individuals remain at a constant temperature. However, under natural conditions, the individuals need to withstand cold winter night temperatures that could be potentially lethal due to diurnal temperature increase (Sweeney & Schnack, 1977). Temperature fluctuations are part of the scenario in which natural selection operates to optimize the response of the species and promote high genetic variability (Sweeney & Schnack, 1977; Rossi & Menozzi, 1990).

In a similar study with the congeneric *H. incongruens*, Pfau (1982) shows that the time required from hatching to reach adulthood is 32, 20 and 12 days at 15ºC, 20ºC and 25ºC respectively, and Latifa (1987) records similar values (35, 18, 14 and 10 days at 15ºC, 20ºC, 25ºC and 30ºC respectively). When compared with the growth rate of *H. bosniaca* (58, 30, 21 and 19 days for the same culturing temperatures), we observe that the developmental time is longer in *H. bosniaca* at all the temperatures studied. Although *H. bosniaca* shows greater resistance to certain water pollutants (Aguilar-Alberola & Mesquita-Joanes, 2012), the shorter developmental time recorded in *H. incongruens* may be the reason why it is one of the most cosmopolitan species in temporary waters (Fryer, 1997).

The variability in fecundity (total number of eggs or offspring per female) in ostracods in general (Cohen & Morin, 1990), and in freshwater species in particular, is vast; between 8 and 290 eggs according to Geiger (1998). The intraspecific variability in *H. bosniaca* is also high, with values ranging from 1 to 78 offspring for females reared at the same temperature. *Heterocypris bosniaca* lays eggs attached in clusters and rarely lays them freely on the sediment, similarly to other freshwater ostracods from temporary waters (Havel & Talbot, 1995; Rossi & Menozzi, 2012). When *H.*

bosniaca females begin egg laying, the process usually lasts several days, unlike *H. incongruens*, which lays eggs during 24-hour periods (clutches) separated by periods which may last 9 days with not laying at all (Havel & Talbot, 1995). In other crustaceans, such as copepods, the egg production rate has been shown to increase with temperature until it reaches a maximum value, and it decreases with further temperature increases. This suggests that there is an optimal temperature range for egg production and that it is species-specific (Ara, 2001; Holste & Peck, 2006; Neila *et al*., 2012). Notwithstanding some differences in adult survival and offspring per female depending on different handling treatments (also noticed for other ostracods such as *Eucypris virens* by Rossi & Menozzi, 2012), females of *H. bosniaca* reared at 15ºC (and to a lower extent 20ºC and 25ºC) show the highest total offspring, implying that this is the optimal temperature range for fecundity. However, the optimal development rate and the shorter egg development time are attained between 20ºC and 30ºC. Maturing fast at high temperatures is advantageous in small temporary ponds that can suffer rapid desiccation at warm periods, even if fecundity is reduced compared to colder periods.

The growth factors observed for *H. bosniaca* do not show the same temperature response throughout the ontogeny. In the first two moults (A-8 to A-6), relative growth follows no clear pattern, but from here to instar A-2, it shows a reverse-TSR pattern; *i.e.*, a warmer temperature results in increased growth factors, except at 30ºC, when growth is reduced compared to 25ºC. The last two moults follow the pattern expected for the TSR (greater relative length increase at lower temperatures). These results are consistent with recent research which conclude that crustaceans change the temperature dependence of growth during ontogeny (Forster & Hirst, 2012). As in *H. bosniaca*, these authors observe a reverse or no TSR in the early developmental stages of *Artemia franciscana* and other crustaceans, while the TSR is established only in more mature stages. In the anostracan *Artemia franciscana*, growth presents a different response to temperature depending on whether individuals are in the process of adding thoracic segments and appendages (early development stages) or abdominal segments (late stages). With *H. bosniaca*, various appendages are added and modified through different moults, except for the last two moults in which the main change consists in the emergence and maturation of sexual organs (limbs undergo minor changes; Aguilar-Alberola & Mesquita-Joanes, 2013), precisely when the growth factor follows the TSR.

Final adult size in the focus organism follows a reverse-TSR between 15ºC and 25ºC but the expected TSR pattern between 25ºC and 30ºC. According to Walters and Hassall (2006) the reverse-TSR is expected in terrestrial univoltine ectotherms such as grasshoppers adapted to warm seasons. Contrary to this expectation, we found this reverse pattern to be dominant in an aquatic multivoltine organism enduring wide temperature variations through its life-cycle. In ectotherms, following a reverse-TSR is expected to be more energy costly at high temperatures than growing according to TSR, taking into account energy allocation between somatic growth and development (Zuo *et al*., 2012). These authors suggest, though, that a reverse-TSR should be expected in cases when it is advantageous to develop at cold temperatures, for instance in seasonal environments, such as those experienced by the studied temporary pond ostracods in Mediterranean climates, where rockpools fill with water during cold periods. Still, the absence of a monotonic response of adult size *vs*. temperature calls for further discussion: are temporary pond ostracods taking advantage of lower energy costs of a TSR at the highest temperature range (>25ºC), but adapted to follow a reverse-TSR in colder periods? Further hints on why this group of crustaceans do not follow the general TSR might come from life-history theory, by relating growth and development to fecundity as a surrogate for fitness. In this framework, the *Bigger is better* assumption (Kingsolver & Huey, 2008) does not seem to apply to this system which lacks a significant relationship between adult female size and fecundity. Indeed, attaining bigger size might even be worse in temporary ponds were visual predators might reduce the survival of larger individuals faster than smaller ones. These predators (mainly insects larvae) take longer to develop than their ostracod preys, and ponds might last longer in cold periods, when it is probably more advantageous to be as small as possible to escape from predation pressure (Williams, 2006; Schmit *et al*., 2012). Under this framework, natural selection might favour energy expensive reverse-TSR in cold periods (with longer hydroperiods and higher predation on bigger organisms), but to follow a less costly TSR pattern at shorter hydroperiods under warm temperatures, with reduced predation pressure.

Conclusion

Temperature has a strong effect on the development rate of the ostracod crustacean *Heterocypris bosniaca*. The intermoult time in any postembryonic stage and life span notably reduces with increasing temperatures, thus supporting a general physiological effect of temperature on development in crustaceans, as a general rule for ectotherms. However, no clear monotonic pattern of reduced growth with increasing temperatures is seen, which contrasts with the expected TSR found in many ectotherms. Our results challenge the generality of the TSR and support the hypothesis that increased body size in relation to temperature is variable through ontogeny, possibly in relation to adaptive constraints. Similarly to the findings of Forster and Hirst (2012) for other crustacean groups, the ostracod *H. bosniaca* follows a reverse TSR, or no TSR, during early juvenile instars, and a TSR pattern is observed only in late development stages and just at the highest temperature range for final adult size.

In addition to development and growth, the effects of temperature on reproductive output are also vitally important. Despite vast variations in fecundity at any temperature, the per capita rate of egg laying and number of offspring are higher at intermediate-low temperatures, but egg development is notably delayed at the lowest temperatures.

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Capítulo IV

Acute toxicity tests with Cadmium, Lead, Sodium Dodecyl Sulfate, and *Bacillus thuringiensis* on a temporary pond ostracod

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Abstract

A complete assessment of effects of pollutants in the environment requires the use of a wide assemblage of test organisms. Crustaceans have been widely used in aquatic environments, but within this group, ostracods have been overlooked, even though they are one of the commonest groups of animals in temporary waters. Within this framework, static acute toxicity bioassays were conducted with heavy metals Cd and Pb, the surfactant SDS and spores of the bacteria *Bacillus thuringiensis* var. *israelensis* using adult parthenogenetic females of the temporary pond ostracod *Heterocypris bosniaca*. Experiments were run at 20°C and in a 12 by 12 h photoperiod. The toxicity tests results are expressed as LC_{50} and EC_{50} at 48 h and 96 h using probit analysis. The sensitivity of *H. bosniaca* at 48 h decreased in the following order Cd > Pb > SDS > *Bti*, while Pb and SDS exchanged their positions at 96 h. Comparison of the LC_{50} values for this species with information available in the literature reveals that it is generally more resistant than other crustaceans.

Keywords: *Heterocypris bosniaca*, lead, cadmium, SDS, *Bt* toxin

Introduction

Growing economical development of societies worldwide has contributed to an increase in global pollution in terms of both the quantity and variety of pollutants. Rivers, lakes, ponds and seas are recipients of large amounts of waste by either direct discharge or other indirect ways. In order to measure toxicity in aquatic environments, a wide variety of organisms have been used. Given their diversity and abundance, crustaceans have been the most widely used, especially copepods, anostracans and cladocerans (Markich *et al*., 2002; Sánchez-Bayo, 2005). Ecotoxicological studies based on ostracod crustaceans are not so common, even though these organisms can play an important role in determining the structure of small aquatic systems (Diner *et al*., 1986) and are sensitive test organisms for pollution studies (Havel & Talbott, 1995).

Heterocypris bosniaca is a freshwater ostracod living in small water bodies around the Mediterranean (Petkowski *et al*., 2000; Martens *et al*., 2002; Aguilar-Alberola & Mezquita, 2008). In the shrubland area known as Racó de Rius (València, Spain) *H. bosniaca* is the commonest ostracod species found in 35 out of the 119 small rainy temporary rockpools located over an area of *c*. 65 ha (Aguilar-Alberola & Mesquita-Joanes, 2011). Although the type of environment this species inhabits is not as directly exposed to pollution as with rivers, lakes and seas, given the ponds' small size, they are very vulnerable to pollutants because here these can reach very high concentrations. Barry and Logan (1998) showed how the microcosm of temporary ponds is a useful system for assessing ecosystem-level toxicants.

Major sources of pollution in small ponds come about through precipitation and runoff passing over wastes near them. In the case of precipitation, because of the worldwide use of fossil fuels and nearby roads passing, some heavy metals like lead or cadmium can accumulate in the water and sediment of temporary ponds (Nriagu & Pacyma, 1988). In addition, and as a special case for runoff passing over wastes, we can find lead pellets that spread out due to hunting in mountain areas where temporary rockpools inhabited by *Heterocypris* species can be found. We proposed to study these pollutants because heavy metals are considered the lethalest toxicants (Goodyear & McNeill, 1999), are not affected by chemical or biological degradation, and are bio-accumulative.

Throughout history, people have used a large variety and quantity of synthetic insecticides in attempts to eliminate pests. More recently, the search for pesticides that only affect the target pest and not other species present in the environment has led to the use of "biopesticides" such as the *Bacillus thuringiensis* (*Bt*) toxin, which has a low ecological impact and is at low risk for aquatic environments (Swadener, 1994;

Kreutzweiser *et al*., 1996). *Bt* var. *israelensis* (*Bti*) is a specific toxicant for aquatic diptera, but it has been shown to also affect other organisms (Brazner & Anderson, 1986; Mulla, 1991); indeed, the DNA of both *Bt* and *Bt corn* is so persistent that it can be detected at long distances in runoff areas (Douville *et al*., 2007; Rosi-Marshall *et al*., 2007).

Surfactants are common industrial and household wastewater components, and are the most widespread xenobiotics in aquatic environments (Jeŕábková *et al*., 1999). Among them, sodium dodecyl sulfate (SDS) is the most widely used in personal hygiene, found at high concentrations in sewage outlets of cities. Consequently, even though surfactants are less lethal than heavy metals or insecticides, and are not bio-accumulative, their common and widespread release into natural habitats make them usual and dangerous pollutants. In fact some of their biological effects are considered to be as strong as those of pesticides or heavy metals (Guilhermino *et al*., 2000).

Due to the negative effects that the above-described chemical and biological products can have on aquatic environments, and given the limited information available about their impact on ostracods we propose to assess the response of one species of the *Heterocypris* genus to cadmium, lead, SDS, and *B. thuringiensis* spores. In this way, we expected to extend knowledge on the use and applicability of ostracods, *Heterocypris* in particular, to ecotoxicological assessments, as previously demonstrated for other members of this genus (see Chial & Persoone, 2002a, b). In addition, with this work we aim to analyze the potential effects of pollutants in the distribution and ecology of an ill-known ostracod species that has been recently discovered from temporary habitats in the eastern Iberian Peninsula, where only asexual populations are found, suggesting that this is an area of recent colonization and expansion in the Mediterranean biogeographical context.

Material and methods

Individuals of *H. bosniaca* were collected by hand nets from a small (92 × 120 cm, max. depth 20 cm) temporary pond located at Racó de Rius (39°02′51″ N 0°26′14″ W, Rafelguaraf, E. Spain). In order to avoid interference with possible pollutants in pond water, artificial hard water was prepared following USEPA (2002) (final water pH=6.3 and EC=0.29 mS cm^{-1}, very similar values to those found in the studied pools after a rainy period). Individuals belonging to the last two instars of development (adult and A-1 subadult juveniles) were maintained in this water for several days in acclimation at a constant temperature of 20°C and in a 12:12 h photoperiod. On the first day, dried *Spirulina* and insect homogenates were introduced as food to these ostracod stocks; no more food was added when the animals were transferred to the acute toxicity culture plates or for the remainder of the experiment.

Stock solutions of pollutants were prepared using lead nitrate salts ($Pb(NO_3)_2$ Sigma-Aldrich, ≥ 99% purity) and cadmium sulfate ($CdSO_4 \cdot 8/3\ H_2O$ Fluka, ≥ 99%) for heavy metals. The SDS detergent (Sigma-Aldrich, 99%) and the commercial strain of *Bacillus thuringiensis* var. *israelensis* Vectobac 12AS (1200 *Bti* ITU mg^{-1}) were also used, and were all diluted with the artificial hard water. To assess the LC_{50} of the different pollutants, preliminary tests were conducted to establish the range of concentrations used in the experiment. Once the LC_{50} ranges at 48 h and 96 h were obtained, we prepared 11 concentrations of each pollutant by serial dilutions.

Multiwell plates with 5 mL of the prepared solution plus 10 adult (female) individuals of *H. bosniaca* were used to perform the bioassays. Tests were carried out under static conditions. For each concentration, four replicates were used and 12 replicates were employed for the controls. Each well was observed every 24 h until all the individuals died. In each observation event, we counted the number of immobile (not swimming, but with some movement of appendages) and dead individuals (no movement was noted during an observation period of 10 seconds or longer). Dead individuals were removed from the well. As this species laid eggs glued in clutches, we could not well estimate the number of eggs per individual because many of them were hidden in the clutch by the more external ones. For this reason we only counted the number of offspring per well which appeared during the experiment.

The data from each bioassay were analyzed by probit analysis (Finney, 1971). The median lethal concentration (LC_{50}) and the median effective concentration (EC_{50}) with their 95% CI were calculated using the EPA-Probit program, v.1.5 (USEPA, 1994).

Results

The results of the toxicological tests showing the effects on survivorship and immobilization of *H. bosniaca* through time are shown in Figures 1-4. In general, survivorship shows a sigmoid pattern, indicating that at the beginning and at the end there is a poor response of the individuals, being more sensitive in the central days of each culture. This pattern calls for taking more attention to toxicity effects of pollutants on survivorship on a longer temporal scale than simply considering LC_{50} and EC_{50} values obtained with short term tests.

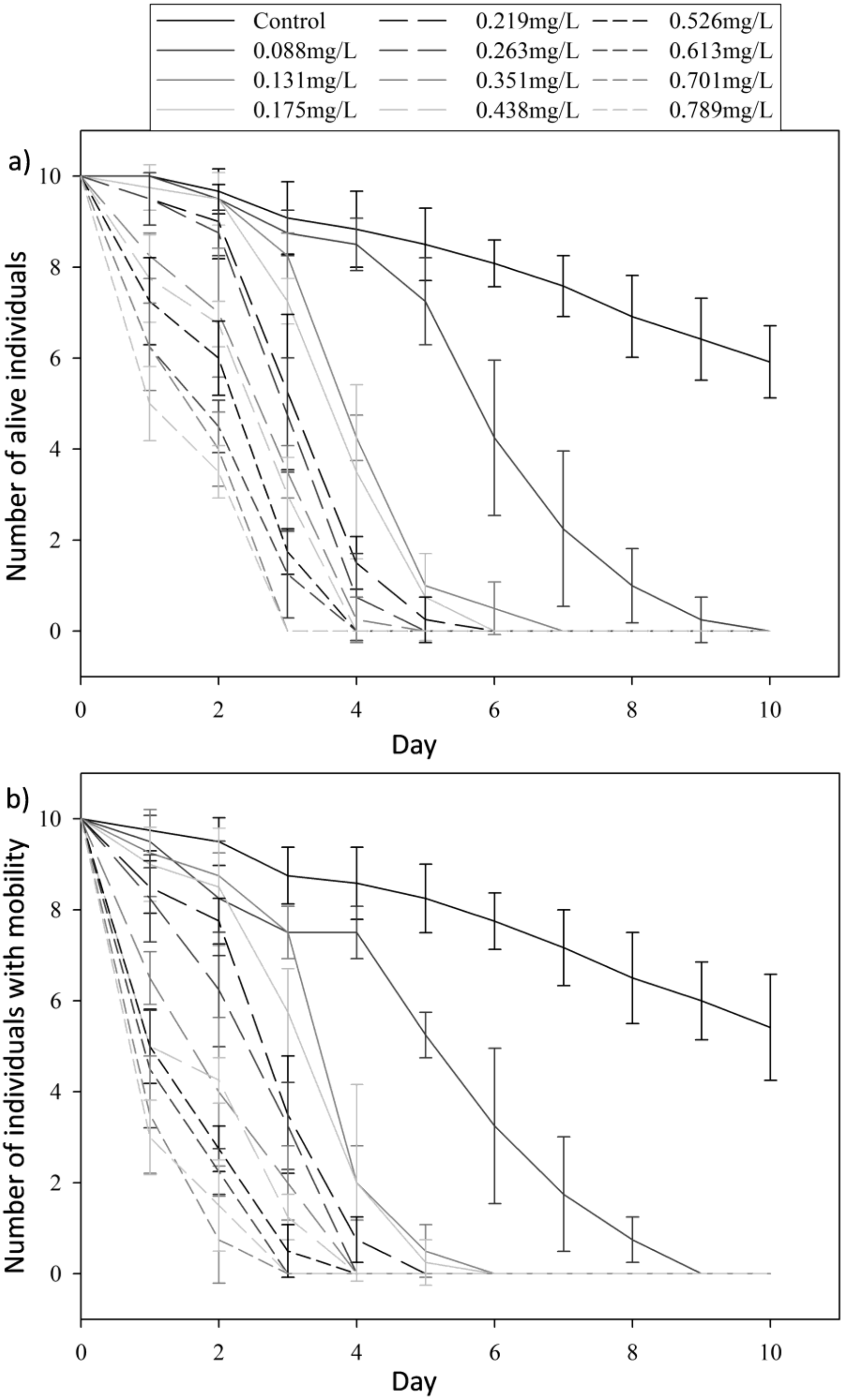

Fig. 1. Number of alive (a) and actively moving (b) individuals during the experimental treatment with Cd. Curves represent the average value of the four replicates and the vertical bars show ± 1 standard deviation.

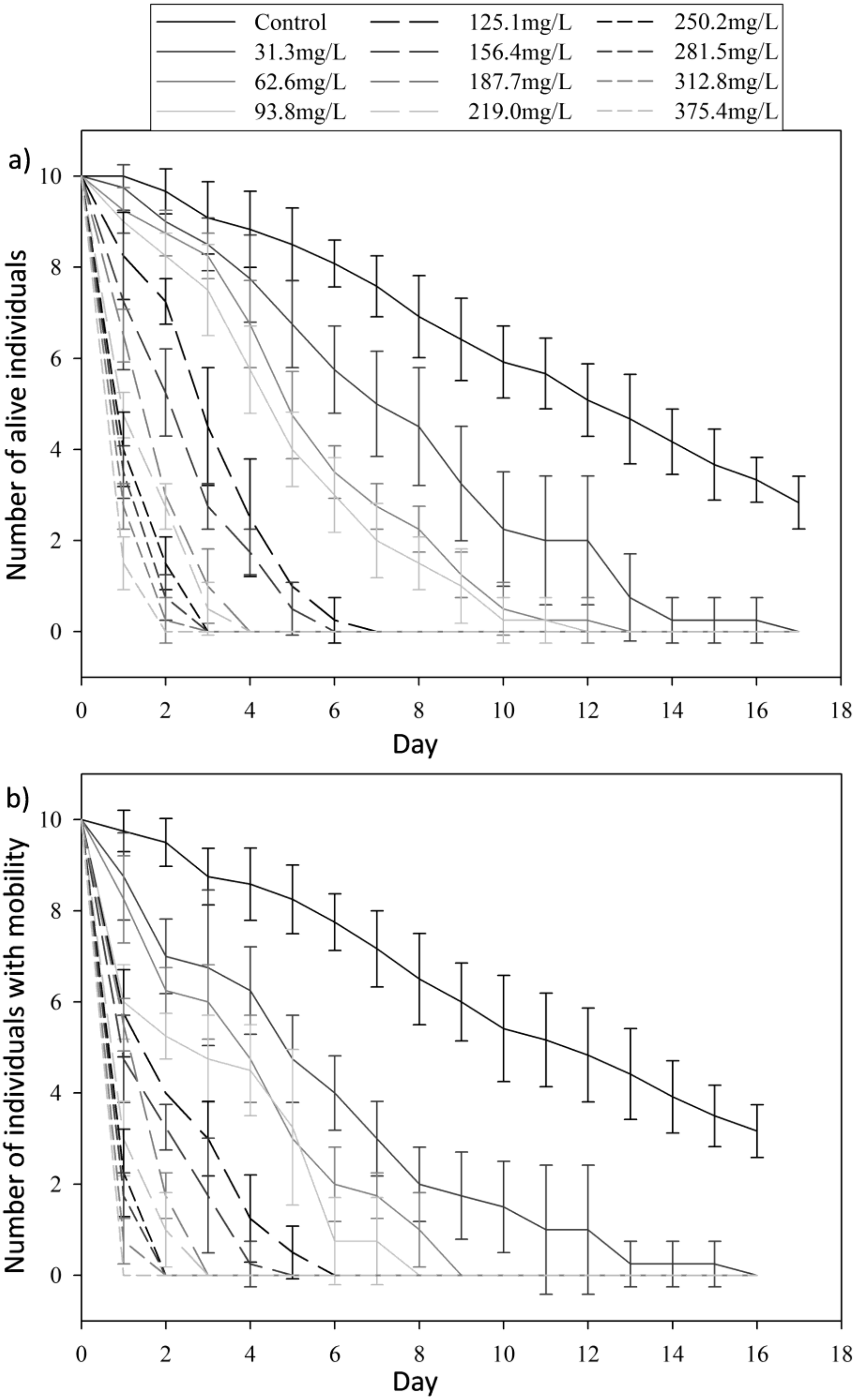

Fig. 2. Number of alive (a) and actively moving (b) individuals during the experimental treatment with Pb. Curves represent the average value of the four replicates and the vertical bars show ± 1 standard deviation.

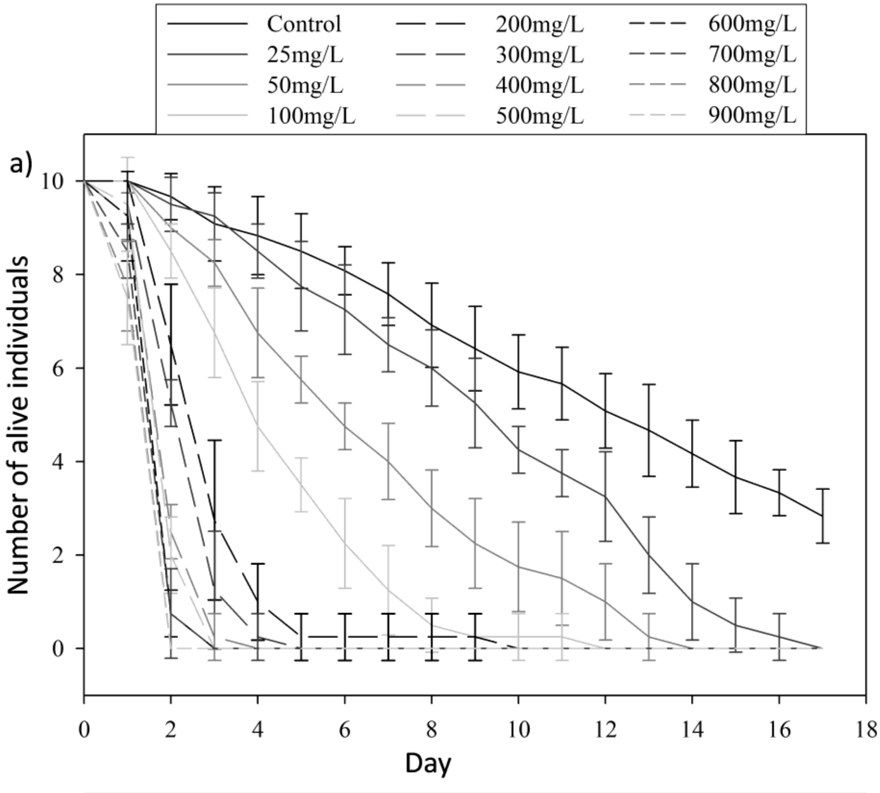

Fig. 3. Number of alive (a) and actively moving (b) individuals during the experimental treatment with SDS. Curves represent the average value of the four replicates and the vertical bars show ± 1 standard deviation.

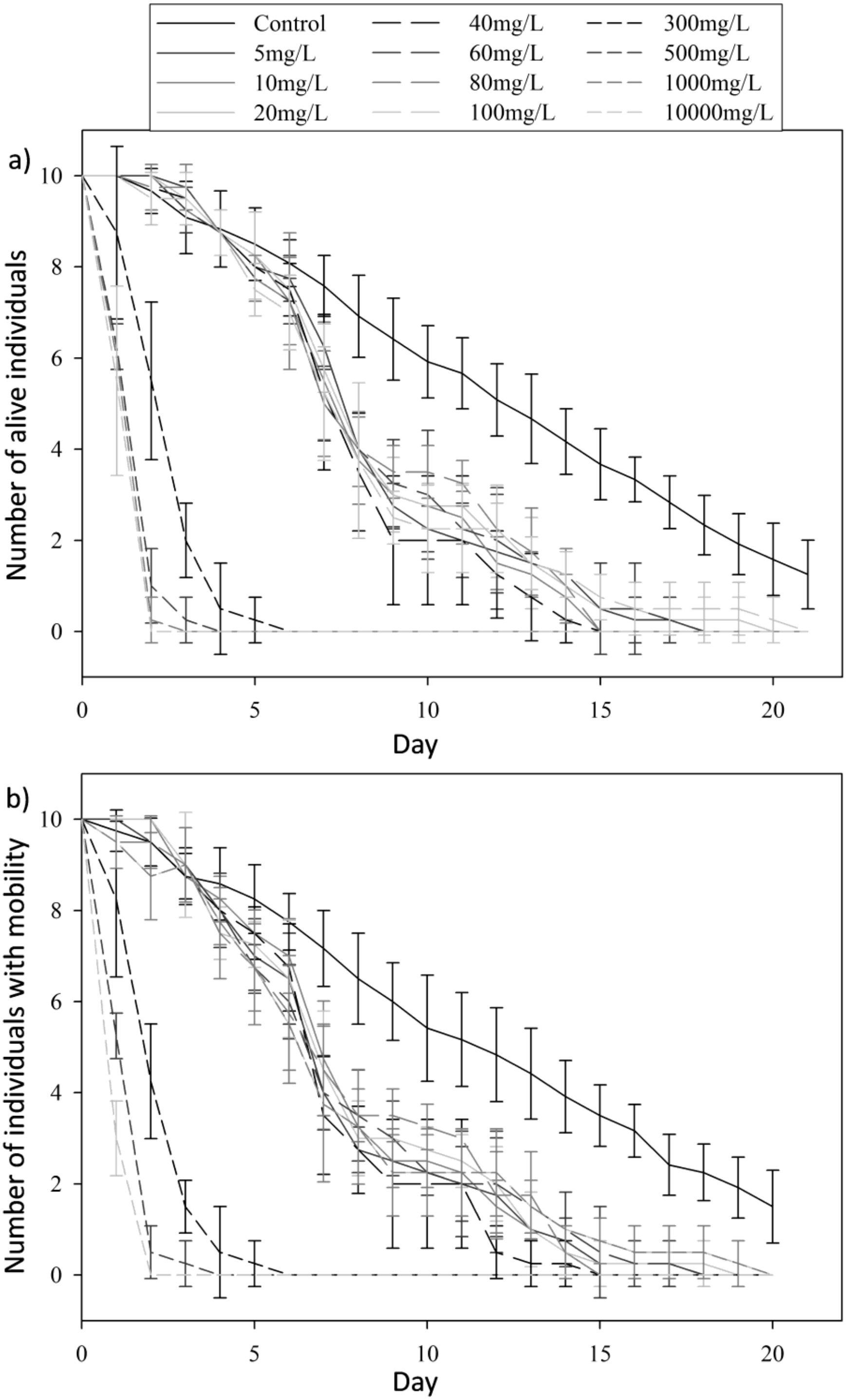

Fig. 4. Number of alive (a) and actively moving (b) individuals during the experimental treatment with *Bacillus thuringiensis* var. *israelensis* (*Bti* toxin). Curves represent the average value of the four replicates and the vertical bars show ± 1 standard deviation.

For heavy metals and SDS, a relationship was found between the increasing concentration of pollutants and the higher impact on ostracods' response (either immobilization or death) (Figs. 1-3). For *Bti*, increasing concentrations above 300 mg L^{-1} also showed the expected pattern of higher negative effects on ostracods, but with unexpected results at lower concentrations: during the first three days of treatment, mortality with *Bti* was similar (or even lower) than in the control treatment. After day 4, mortality under the effects of *Bti* was higher than for the control treatment, although a clear relationship between concentration and the biotic response of individuals was not observed (Fig. 4).

Tab. 1. Acute toxicity values of the four tested products on the ostracod *Heterocypris bosniaca*.

Pollutant	LC_{50} - EC_{50} (mg $Liter^{-1}$)*			
	48h			
	LC_{50}	95% CI	EC_{50}	95% CI
Cd	0.61	0.54 - 0.70	0.37	0.32 - 0.41
Pb	162.88	148.3 - 175.73	91.27	59.27 - 116.87
SDS	280.00	238.55 - 316.54	157.27	125.57 - 186.29
Bti	298.75	245.02 - 347.85	238.86	196.36 - 281.60
	96h			
Cd	0.15	0.13 - 0.16	0.12	0.10 - 0.13
Pb	104.83	91.64 - 115.47	80.44	66.77 - 91.43
SDS	94.02	73.71 - 113.16	66.11	50.82 - 80.61
Bti	N.A.	N.A. N.A.	169.11	119.34 - 210.27

*LC_{50} and EC_{50} values at 48 h and 96 h with corresponding 95% confidence interval (CI). LC_{50} value at 96 h for *Bti* has not been calculated due to lack of data for 0-100% mortality (N.A. Not Available).

The EC_{50} and LC_{50} values obtained for each toxic product are presented in Table 1. The relative sensitivity of *H. bosniaca* to the four pollutants studied was Cd > Pb > SDS > *Bti* at 48 hours, whereas lead and SDS exchanged their position at 96 hours. Figures 1-4 allow a comparison to be made between observable effects on activity and individuals' survival. For example, during the first two days of SDS treatment, most individuals were still alive, even at high concentrations, but many were immobile. The differences between LC_{50} and EC_{50} values indicate this effect more precisely. In this case, the largest differences between LC_{50} and EC_{50} were observed in the treatments with SDS and the two heavy metals, whereas the *Bti* treatment showed no much difference (but note that at 96 h, the data did not allow the calculation of LC_{50} and EC_{50} with the probit method). The differences between the effects of pollutants on immobilization and mortality are depicted in Figure 5. In the

Pb and SDS treatments, the increase in concentrations revealed a similar pattern of the effects on both mobility and survival, with parallel lines in both cases. On the other hand, the negative effects (either on activity or survival) of Cd at 96 h were more intense (higher slope) with a higher concentration than at 48 h. However, the opposite can be said of *Bti* treatment, where ostracods apparently suffered less negative impacts from increased concentrations at a more prolonged exposure (96 h) than with a shorter exposure time (48 h) (Fig. 5).

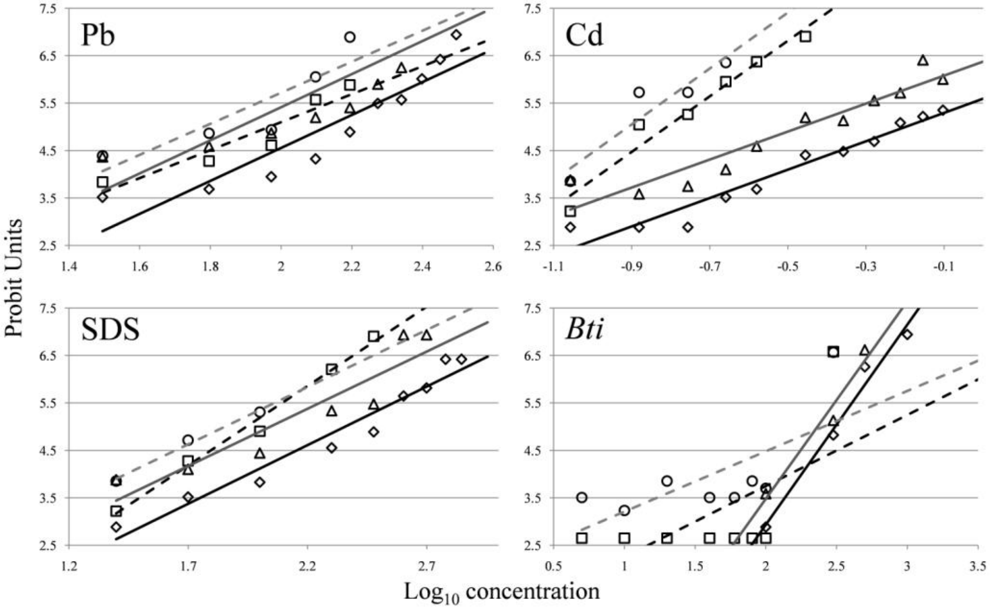

Fig. 5. Regression lines for the concentration effect on mortality (LC_{50}) and immobilization (EC_{50}) of *H. bosniaca* after exposure to the four tested products (Cd, Pb, SDS and *Bti*) at 48 h and 96 h.

During daily observations, dead individuals were removed from cultures. In most cases, individuals were inflated and acquired a reddish-brown color with Cd treatment. With the control and *Bti* treatments, dead ostracods always disappeared because they were eaten by the living individuals, which only left valves as remains.

As all the individuals used in the experiment were parthenogenetic females, they could reproduce; indeed, we observed egg production in many cases (except for the wells where mortality took place very quickly). We also observed new offspring in any pollutant treatment, but only for low concentrations; however, the most abundant offspring appeared in the *Bti* treatment. In the SDS and Cd treatments, we saw a single offspring at 25 and 0.088 mg L^{-1}, respectively (corresponding to an offspring of $2.3 * 10^{-4}$ and $1.3 * 10^{-4}$ individuals per female and day, respectively). In the Pb treatment, two eggs hatched at 31.3 mg L^{-1} (2.6 * 10^{-4} ind. f^{-1} d^{-1}), while a total offspring of 63 juveniles spread over all the concentrations (68.1 * 10^{-4} ind. f^{-1} d^{-1}) in the *Bti* treatment, but mainly at *Bti* concentrations of 40 mg L^{-1} and 100 mg L^{-1}. In the control treatment wells, 41 juveniles were observed in total (126.5 * 10^{-4} ind. f^{-1} d^{-1}).

Discussion and conclusion

As expected, both with heavy metals (Cd, Pb) and SDS, ostracods responded by presenting negative effects in proportion to the concentration of pollutants in the cultures; however, the response to *Bti* concentrations resulted in an unexpected pattern. The presence of bacteria spores always had negative effects on *H. bosniaca* long-term survival, even at the lowest concentrations used (5 mg L^{-1} = 6000 International Toxic Units -ITU-), and with effects similar to higher concentrations of up to 100 mg L^{-1}. These are striking results because most studies show no negative impact of *Bti* on non target aquatic organisms (Caquet *et al*., 2011, and references therein). This finding therefore suggests having to test *Bti*'s potentially negative effects on the ostracod species in both the field and the laboratory. On the other hand, there appears to be a positive response of short-term ostracod survival (2-3 days) to the presence of *Bti* spores in comparison to control treatments. It may be speculated that ingestion of spores by ostracods may result in better short-term survival. There is no published evidence for the potential ingestion of *Bti* spores by ostracods, but other crustaceans have been shown to eat these spores with no short-term (1-24 h) (Brazner & Anderson, 1986) or long-term (5 weeks) (Su & Mulla, 2005) effects.

As expected, the concentrations needed to show negative effects on individuals (either LC_{50} or EC_{50}) are lower at longer exposure times (values lower at 96 h than at 48 h), and the slope of the relationship between concentration and LC_{50} or EC_{50} is similar for both the exposure times for Pb and SDS, but not in the treatments with Cd and *Bti*. With cadmium, both LC_{50} and EC_{50} seem to increase more quickly (greater slope) at the longer exposure time of 96 h. This is most probably related to the highest Cd toxicity if compared to Pb and other pollutants (Khangarot & Das, 2009). With *Bti*, the graph is distorted by the peculiar effect that it has on the studied species, as previously discussed. Obtaining both LC_{50} and EC_{50} values is important when there are large differences between them. Then, EC_{50} is the most representative index because, even when alive, ostracods remain immobile, and this situation means greater vulnerability to potential predators in ponds (Sánchez-Bayo & Goka, 2006).

As we observed, in all treatments live offspring appeared, although in the Cd, Pb and SDS treatments in very small amount. We did not analyze this topic in detail, because at the beginning of the experiment we did not consider the idea that individuals treated with pollutants were able to lay viable eggs. These are interesting results, but we cannot know if the reduction in the number of offspring in the treatments is due to a reduction in female fertility, reduced viability of eggs or variations in subitaneous to resting eggs ratio. More experiments are needed to study more deeply this subject.

The results of the present work provide new data on the toxicity of Cd, Pb, SDS and *Bti* in aquatic invertebrates, and allow a comparison to be made of the studied ostracod's sensitivity in relation to those of other species for the same toxicants (Tab. 2-3). *Heterocypris bosniaca* shows high resistance to pollutants when compared with other organisms, except for ostracods *Cypris subglobosa* and *Diacypris compacta* in the case of Cd (Brooks *et al*., 1995; Khangarot & Das, 2009). It presents even higher tolerance (at least one order of magnitude) to Cd and Pb than its congener *H. incongruens* (Tab. 2, López-Gutiérrez, 2010). The high resistance showed to Pb and (to a lesser extent) Cd may be due to the benthic lifestyle of the species (and most freshwater ostracods); sediments tend to have higher concentrations of heavy metals than water (Chial & Peersone, 2002a; Hoffman *et al*., 2002), so that benthic organisms are expected to be adapted to these higher densities of pollutants and consequently appear as more resistant than planktonic organisms present in the pond. In addition, comparisons on the sensitivity of ostracods are made on the adult instar because each growth stage may have different sensitivity, as is the case of *H. incongruens*, which increases resistance to heavy metals as the development progresses (López-Gutiérrez, 2010).

To date, nothing has been known about the effects of *Bti* on ostracods, although it has been shown that this toxin has little or no significant negative impact on other crustaceans, such as cladocerans, amphipods and notostracans (Brazner & Anderson, 1986; Su & Mulla, 2005). When the LC_{50} value of *Bti* in *H. bosniaca* is compared with those of target (Culicidae; Russell *et al*., 2003) and non target (Chironomidae; Ali *et al*., 1985) aquatic insects (dipteran larvae), it is clear that ostracods are much more tolerant in the short term (Tab. 3). However, and as we have shown before, ostracods cultured in the presence of the *Bti* toxin present a poorer long-term survival than those cultured in clean artificial water. Consequently, even relatively low concentrations of *Bti* in field applications might have negative effects on ostracods; therefore, more tests should be carried out to study the impact on natural populations because biotic and abiotic factors can affect these results (Nayar *et al*., 1999).

Tab. 2. Acute toxicity LC_{50} or EC_{50} (*) values for different crustaceans.

Pollutant	Species		LC_{50} - EC_{50}* (mg $Liter^{-1}$)		Ref.
			48h	96h	
Cd	*Heterocypris bosniaca*	Ostracoda	0.610	0.150	This work
	Heterocypris incongruens	Ostracoda	0.053		López-Gutiérrez, 2010
	Chlamydotheca incisa	Ostracoda	0.307		Liberto, 2010
	Strandesia bicuspis	Ostracoda	0.039		Liberto, 2010
	Stenocypris major	Ostracoda	0.051	0.013	Shuhaimi-Othman *et al.*, 2011
	Cypris subglobosa	Ostracoda	0.821 *		Khangarot & Das, 2009
	Diacypris compacta	Ostracoda		4.340	Brooks *et al.*, 1995
	Daphnia magna	Cladocera	0.572		Pérez & Beiras, 2010
	Tigriopus brevicornis	Copepoda		0.048	Forget *et al.*, 1998
	Artemia sp.	Anostraca	1.500		Gajbhiye & Hirota, 1990
	Neomysis awatschensis	Mysidacea		0.118	Tian *et al.*, 2002
	Siriella amata	Mysidacea	0.344	0.099	Pérez & Beiras, 2010
Pb	*Heterocypris bosniaca*	Ostracoda	162.880	104.830	This work
	Heterocypris incongruens	Ostracoda	2.701		López-Gutiérrez, 2010
	Stenocypris major	Ostracoda	2.886	0.526	Shuhaimi-Othman *et al.*, 2011
	Cypris subglobosa	Ostracoda	40.190 *		Khangarot & Das, 2009
	Diacypris compacta	Ostracoda		3.100	Brooks *et al.*, 1995
	Artemia sp.	Anostraca	1.400		Gajbhiye & Hirota, 1990
SDS	*Heterocypris bosniaca*	Ostracoda	280.000	94.020	This work
	Chlamydotheca incisa	Ostracoda	48.238		Liberto, 2010
	Strandesia bicuspis	Ostracoda	40.888		Liberto, 2010
	Cypris subglobosa	Ostracoda	2.050 *		Khangarot & Das, 2009
	Daphnia magna	Cladocera	14.380		Villegas-Navarro *et al.*, 1997, 1999, 2001
			27.343		Pérez & Beiras, 2010
	Tigriopus fulvus	Copepoda		7.420	Mariani *et al.*, 2006
	Artemia parthenogenetica	Anostraca	12.200		Nunes *et al.*,2005
	Neomysis awatschensis	Mysidacea		11.600	Tian *et al.*, 2002
	Siriella amata	Mysidacea	11.612	8.479	Pérez & Beiras, 2010

Our results on the high tolerance to pollutants by *H. bosniaca* might be extrapolated to animals from other populations of this species in the area of study, and probably also to others present in the eastern Iberian Peninsula, given the potential low genotypic variability in a parthenogenetic ostracod. However, it has been shown that different clonal lineages of the same species in other crustaceans may present different tolerances to pollutants (Haap & Köhler, 2009) and, consequently, our data should be taken with caution and more tests are needed using individuals from other populations in order to confirm or not the pattern observed for this species.

In general, *Heterocypris bosniaca* is a highly resistant species to the pollutants studied, even if compared to its congener *H. incongruens*. This resistance could offer advantages over other species invading new small temporary environments, which might undergo wide hydrochemical variations throughout the hydroperiod, particularly if there is some kind of pollution. Nonetheless, we do not expect this to be a key factor for the moment because its potential competitor *H. incongruens* is more widely distributed, while *H. bosniaca* shows a much more localized distribution (Fryer, 1997; Aguilar-Alberola & Mesquita-Joanes, 2011), but due to the continuous increase of anthropogenic pollutants in the environment, it is expected that high resistance could be very beneficial in the future for the extinction avoidance of metapopulations of the species with such traits, and to facilitate its further spread.

Tab. 3. Susceptibility of dipteran larvae to various preparations of *Bacillus thuringiensis* var. *israelensis* (*Bti*) at 48 h*.

Species	LC_{50}		*Bti* formulation	Potency	Ref.
	(mg L^{-1})	(ITU L^{-1})		(ITU L^{-1})	
Heterocypris bosniaca	298.750	358500	VectoBac 12AS	1200	This work
Culex sitiens	0.019	57	VectoBac WG	3000	Russell *et a*l., 2003
Culex annulirostris	0.004	12	VectoBac WG	3000	Russell *et al.*, 2003
Culex quinquefasciatus	0.005	15	VectoBac WG	3000	Russell *et al.*, 2003
Aedes aegypti	0.002	54	VectoBac WG	3000	Russell *et al.*, 2003
Ochlerotatus notoscriptus	0.015	45	VectoBac WG	3000	Russell *et al.*, 2003
Ochlerotatus vigilax	0.013	39	VectoBac WG	3000	Russell et al., 2003
Chironomus crassicaudatus	4.980	4980	IPS-78	1000	Ali *et al.*, 1981
	12.500	12500	ABG-6108	1000	Ali *et al.*, 1981
Chironomus decorus	4.660	4660	IPS-78	1000	Ali *et al.*, 1981
	14.710	14710	ABG-6108	1000	Ali *et al.*, 1981
Chironomus salinarius	4.460	15610	Bactimos	3500	Ali *et al.*, 1985
	5.400	10800	VectoBac	2000	Ali *et al.*, 1985
	14.630	21945	Teknar	1500	Ali *et al.*, 1985
Glyptotendipes paripes	13.590	13590	ABG-6108	1000	Ali *et al.*, 1981
	8.310	8310	IPS-78	1000	Ali *et al.*, 1981

* Dipteran larvae belong to the third and/or fourth instar of development. Values are also offered in International Toxicity Units (ITU) per mg against *Aedes aeypti* for the best comparison.

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Capítulo V

Ontogeny of *Heterocypris bosniaca* (Ostracoda: Cyprididae): Description of postembryonic instars and rediscovery of the neglected A-9 stage

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Abstract

Despite being the most common and widespread family of Ostracoda in fresh water, the Cyprididae are still poorly known with respect to species ontogeny. The full series of eight juvenile instars has been described in detail for just five of the approximately 1000 living species, and only in one case did this include the earliest A-9 stage. We aimed to fill this gap with a morphological study of the post-embryonic development of *Heterocypris bosniaca* Petkowski, Scharf and Keyser, 2000, a species with unusual morphological traits within the genus, including putatively paedomorphic characters such as valves without tubercles and the narrow calcified inner lamella on both valves. Inside the egg, the poorly known stage A-9 (prenauplius) develops and starts the moulting process. After the egg breakage, the juvenile instar A-8, still inside the A-9 exoskeleton, emerges passively from the egg and finishes the moulting process to a free A-8 instar in less than two minutes. The prenauplius stage A-9 has two pair of limbs; the first are the antennules, while the second correspond to the fused group of the antennae and mandibles, observed by checking the homology by formation. This is apparently the first time that such fusion of the second and third pairs of appendages is described in crustaceans. The later juvenile instars (A-8 to A-1) and the adult stage of *H. bosniaca* follow a growth pattern that is similar to the rest of the Cyprididae, and particularly close to other members of the genus, but notably differing in the development of the maxillular *Zahnborsten* and the segmentation of the endopod of the fifth limb.

Keywords: Morphology, ontogeny, Ostracoda, paedomorphosis, prenauplius.

Introduction

Ostracodes have determinate growth, *i.e.*, with a terminal ecdysis. A maximum of nine postembryonic stages (from the recently hatched “A-8” to the adult “A”) has usually been reported. [N.B.: Although the terms “instar” and “stage” can be considered synonymous (Williamson, 1982; Cohen & Morin, 1990; Ferrari & Dahms, 2007), we use “stage” here to indicate each of the main divisions of the life cycle, and “instar” as a stage of development between two moults, following Carlson (1983), Fink (1983) and Lincoln *et al*. (1998)]. However, Roessler (1982b, 1982c, 1998) found an earlier stage in some species belonging to four different genera of Cyprididae: *Heterocypris, Chlamydotheca, Strandesia* and *Potamocypris.* He called it the “prenauplius” or A-9 stage, and described it for three species: *Heterocypris bogotensis* Roessler, 1982a, *Chlamydotheca unispinosa* (Baird, 1862), and *Chlamydotheca incisa* (Claus, 1892), out of which the full description of all juvenile instars (Roessler, 1982b) was only provided for *H. bogotensis*. This finding challenged the well-established “fact” concerning the common and maximum number of juvenile stages in podocopid ostracodes is eight (Cohen & Morin, 1990), and up to now, no other author has reported this early stage in any other species of ostracode. This is not surprising, given the low number of works studying ostracode post-embryonic development in detail.

In the literature there are detailed descriptions of the juvenile instars of many of the superfamilies of Podocopa, including Bairdioidea (Smith & Kamiya, 2002), Cytheroidea (Smith & Kamiya, 2003, 2005), Darwinuloidea (Smith & Kamiya, 2008), Terrestricytheroidea (Horne *et al*., 2004), and Cytherelloidea (Okada *et al*., 2008). Among other works, Maddocks (2000) reviewed the chaetotaxy and ontogeny of the podocopid antennule, Smith and Tsukagoshi (2005) conducted a detailed study of the antennule of various genera, Kaji and Tsukagoshi (2010) studied the antenna and Smith *et al*. (2005) analysed the development of the maxillular branchial plate. More specifically for Cypridoidea, some detailed morphological studies focus on body parts like the antenna in the last instars of the species of *Sclerocypris* (Martens, 1987), the cleaning limb of *Herpetocypris chevreuxi* (Sars, 1896) (Broodbaker & Danielopol, 1982), the fifth limb ontogeny and function (Kaji, 2010), or changes during development in the carapace shape of *Eucypris virens* (Jurine, 1820) (Baltanás *et al*., 2000). Other studies are based on the ontogenetic study of all the limbs in particular species such as *Cypridopsis vidua* (Müller, 1776) (Kesling, 1951), species of genera *Stenocypris*, *Potamocypris*, and *Cypridopsis* (Ghetti, 1970) and species of genus *Heterocypris* including *H. salina* (Brady, 1868) (Kubanç *et al*., 2007), *H. bogotensis* (Roessler, 1983), *H. incongruens* (Ramdohr, 1808) (Schreiber, 1922), and sometimes also carrying out descriptions of the carapace, like in *E. virens* (Smith & Martens, 2000).

Although there are a notable number of studies on the ontogeny of ostracodes, the morphology of younger stages is still not very well known, especially regarding the A-9. In the present paper we report on *Heterocypris bosniaca*, Petkowski, Scharf and Keyser, 2000. This is a medium-sized species of ostracode living in temporary ponds where it may form populations with high densities (Aguilar-Alberola & Mesquita-Joanes, 2011). Bisexual populations have been found in Bosnia and Israel (Petkowski *et al*., 2000; Martens *et al*., 2002), but in Spanish ponds only parthenogenetic females are present (Aguilar-Alberola & Mezquita, 2008). The species is relatively easy to maintain in laboratory cultures, and its ability to produce desiccation-resistant eggs makes it easy to get new individuals by drying eggs and rehydrating them some days after. The relatively large body size allows easy dissection and study of the limbs.

By analysing the morphology of the valves, the complete chaetotaxy of the limbs, and the whole body of each postembryonic stage from egg to adult, we aim to provide a detailed description of the ontogeny of this species and look at the juvenile condition of potentially paedomorphic adult traits. Furthermore, we aim to compare this detailed information with other species, thereby identifying morphological differences among them, along with conserved developmental features. These may be useful for the analysis of phylogenetic morphological trends in Cypridoidea. We also focus particularly our study on the morphology of the hatching stage in detail because of the implications it can have on the phylogeny of ostracodes.

Material and methods

Several hundred individuals of *H. bosniaca* were collected alive from a small (92 × 120 cm, max. depth 20 cm), temporary rock pool in Racó de Rius (Rafelguaraf, València, 39°02'51"N, 0°26'14"W), named “X6” in a previous study on the ecology of the species carried out by Aguilar-Alberola and Mezquita (2008). Living ostracodes were kept at a constant temperature of 25°C and a photoperiod of 12:12 hours in a culture chamber at the laboratory. Food was supplied regularly every two days alternating micronized *Spirulina* and animal protein (homogenates of insects). The ability of *H. bosniaca* for producing resting and subitaneous eggs allows the continuous reproduction and a constant supply of juvenile instars and adults for study. After removing the right valve of selected individuals of each stage, they were mounted in glycerine on glass slides, covered with cover slides and sealed with clear nail varnish. These specimens were used to draw the whole body, with the soft parts in the original position. In other individuals, the appendages were dissected using needles, and mounted using the same procedure. Dissected limbs, carapaces and valves with their corresponding undissected bodies were drawn using a camera lucida.

Observations of hatchlings (as A-8 individuals inside A-9 exoskeletons; see below) are complicated because of their small size, fragility and short time duration. In order to find this ephemeral stage is necessary to have a large number of eggs and carefully observe the hatching process. Because *H. bosniaca* lays eggs glued in clusters, preferably on rough surfaces (personal observation), we established a separate culture in polyethylene squared recipients with one of its interior walls previously sanded to stimulate the egg laying on its surface, and then we added adult females from the previous cultures. In a few days clusters of eggs could be seen, which were removed with a pipette and placed in petri dishes for its observation. We recorded a digital video using a compact camera attached to the stereomicroscope ocular in an attempt to register hatchings from these clusters. Some of these eggs (either hatched, or unhatched) were critical point dried, coated and mounted for SEM observation and photographed. Other clusters were placed in 2.5% sodium hypochlorite for a few minutes to oxidize the outermost egg layer, following a procedure used with anostracan cysts (Sorgeloos *et al*., 1977) until its interior structure and contents could be observed. After this treatment, some eggs suffer an artificial hatching process and we then used these individuals to take pictures and make drawings of the A-9 (and inner A-8) limbs with the camera lucida. The released exuviae of the A-9 stage individuals were also drawn to appreciate with better detail their external structures, because when ostracodes hatched, the structures belonging to instar A-8 are visible by transparency inside the A-9 cuticle and complicate the observation of A-9 morphology.

The species was identified according to Petkowski *et al*. (2000). Chaetotaxy of the limbs follows the model proposed by Broodbaker and Danielopol (1982), revised for the antennae by Martens (1987), and consider the antennule uniramous (Smith & Tsukagoshi, 2005; Boxshall *et al*., 2010). Roessler (1998) proposed the term “caudal process” to define the posterior elongated structure of the A-9 stage, but this was not very appropriate because the term was already used to describe the posterior extension of the ostracode carapace, therefore, in order to avoid any possible confusion we suggest to use the term “Roessler Protruding Bursa” (RPB), to honor the author that first described it and at the same time providing indications of its function and structure.

Other abbreviations used herein to account for morphological traits are as follows: An1 = antennule; An2 = antenna; Md = mandible; RLO = rake-like organs; Mx = maxillula; L5 = 5th limb; L6 = walking leg; L7 = cleaning limb; CR = caudal ramus; CS = caudal seta; LV = left valve; RV = right valve.

Results

The following section provides firstly a brief description of the egg, the hatching process and changes in the general body plan until reaching adulthood, including both carapace morphology and soft body parts. Readers interested in detailed shell morphology of all instars of the species can look at SEM photographs of the valves previously published in a paper by Aguilar-Alberola and Mezquita (2008). Afterwards, we offer a description of the chaetotaxy of each limb separately, which can be seen with more detail from the provided illustrations.

The eggs of *H. bosniaca* (Fig. 1) are usually glued over plant remains and mineral substrate, and are rarely found free on the sediment. The colour varies from white to orange through shades of yellowish, but when dehydrated they acquire a reddish appearance. Its shape is ellipsoidal, measuring 140-160 μm long and 100-120 μm wide. When dried, their central area deflates acquiring a general shape reminiscent of a red blood cell or a deflated soccer ball. In matured eggs containing high amounts of pigment, the embryo eye can be seen as a dark orange spot. When the eggs with the naupliar eye formed were treated with hypochlorite, the RPB of stage A-9 and An1, An2 and Md of instar A-8 become visible by transparency of the inner membrane of the egg and most of the structures of stage A-9 (Fig. 1).

We obtained video recordings of two hatching events. The time between the first evidence of a crack in the egg shell and the complete emergence of the individual inside was 6 min 1 s in the first case and 2 min 14 s for the second individual. The process of emergence occurs passively by swelling of the RPB, which presses the anterior chamber of the A-9 stage (enclosing the A-8 body) from the back, and therefore protruding it out from inside the egg. These hatching individuals (consisting of the instar A-8 inside their A-9 exoskeleton, Fig. 2a) remained immobile for 7 s and 1 min 31 s respectively until their cuticle split to release the instar A-8 juveniles, leaving the A-9 exuviae (Fig. 3) behind and swimming away.

Stages of Development (Figs. 1-6)

We could not observe the A-9 stage previously to the development of the A-8 instar, because the early A-9 stage only exists inside the egg. However, we can appreciate its external morphology from its exoskeleton and from the ephemeral, recently-hatched individuals. The frontal part corresponds to the anterior chamber which contains the A-8 instar inside (Figs. 2, 3). It is oval in shape and slightly smaller than the egg, about 130-140 μm long and 100-105 μm wide (Figs. 1-4). It has two pairs of rudimentary appendages: no clear division into podomeres is observed, the setae have a rounded distal tip and they do not seem to have much mobility. The first

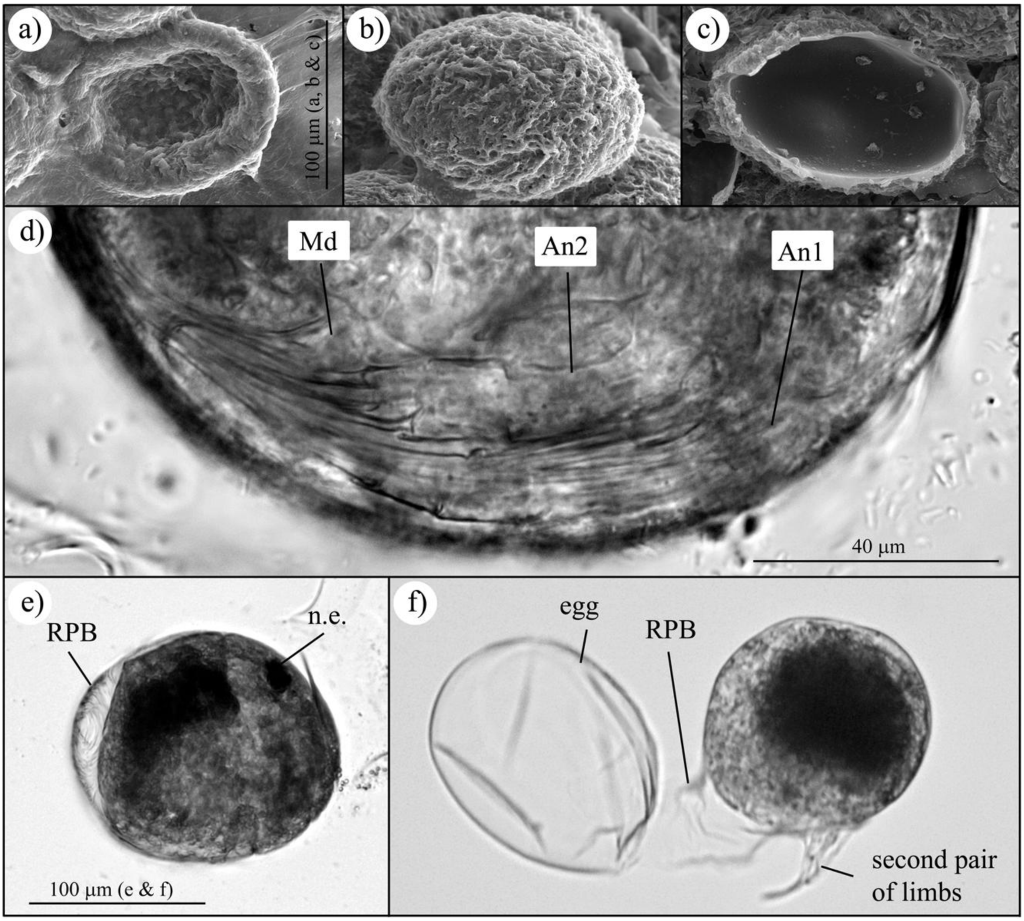

Fig. 1. (a-c) SEM pictures of eggs of *H. bosniaca*. (a) dried egg looking sunk and shaped like a red blood cell, (b) normal hydrated egg, (c) hatched egg. (d-f) stereomicroscope pictures of eggs treated with hypochlorite, (d) detail of the egg bottom in lateral view in which the limbs of instar A-8 can be seen by transparency through the egg cuticle, (e) lateral view of an egg with the naupliar eye (n.e.) and the Roessler Protruding Bursa (RPB) formed, (f) artificial hatching obtained after treatment. An1 = Antennule; An2 = Antenna; Md = Mandible.

pair of appendages corresponds to the An1 (well-developed A-8 An1 appendages are observed inside); the base of this structure seems to be covered with a membrane, within which a short seta appears, together with two longer setae protruding out of the membrane (Fig. 3). The second pair of limbs arises from a very wide basis and dorsally displays a bulge with a terminal claw. In the mid of the limb, a curved seta directed perpendicular to main axis protrudes, embracing both limbs from ventral to dorsal position. Close to this embracing seta, another short straight seta appears distally (Figs. 2-3). The distal margin of this appendage ends with three short setae arising together (fused in a shared trunk) and split in three parts at about one third of the appendage length from the distal tip. This second pair of limbs corresponds to the

Md (outermost area in ventral view ending with the embracing seta) together with the An2 (central zone of the structure and the rest of setae) of instar A-8 as seen in the hatching stage (Fig. 2). The carapace is not well differentiated, with only a small fold that originates near the An1 and extends toward the rear end coinciding with ecdysial lines. The RPB is transparent and, when swollen, longer than the anterior chamber (Fig. 2), reaching a length of 220 µm. In the cuticle left after moulting, the membrane that separates the RPB from the anterior chamber is clearly observed (Fig. 3).

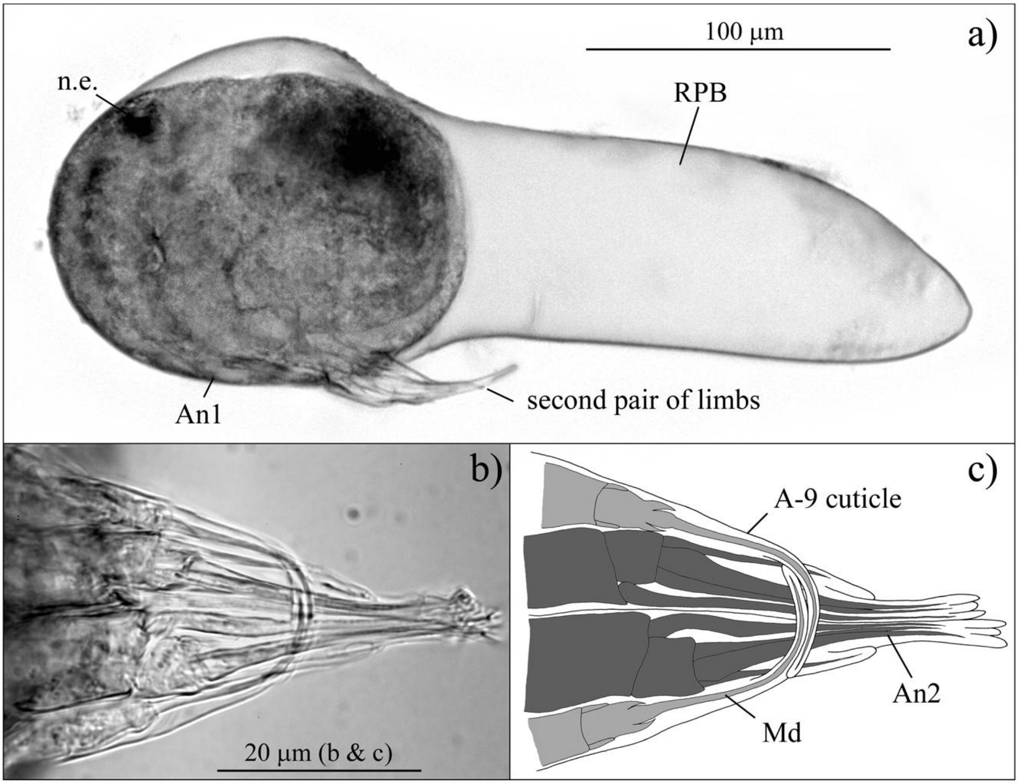

Fig. 2. (a) lateral view of a hatching stage. At this stage of development the instar A-8 is located inside the anterior chamber of the A-9 stage, which has a pair of antennules (An1) close to the body and a second pair of limbs that protrude from behind them. The transparent Roessler Protruding Bursa (RPB) is fully inflated at the rear of the anterior chamber. (b-c) detail of the second pair of limbs in ventral view; outermost part is the cuticle of stage A-9 and inside it has the antennae (An2) and mandibles (Md) of instar A-8, (b) stereomicroscope picture, (c) explanatory drawing.

The A-8 instar in *H. bosniaca* has a rounded bivalved carapace in lateral view (Figs. 5-6), 200-220 µm in length and 130-150 µm in height (Figs. 4-5). The carapace is poorly calcified and flexible, and muscle scars are not visible. The calcified inner lamella is narrow and remains so for the rest of the development. The surface of each carapace valve is ornamented with small holes and grooves and has three normal pores

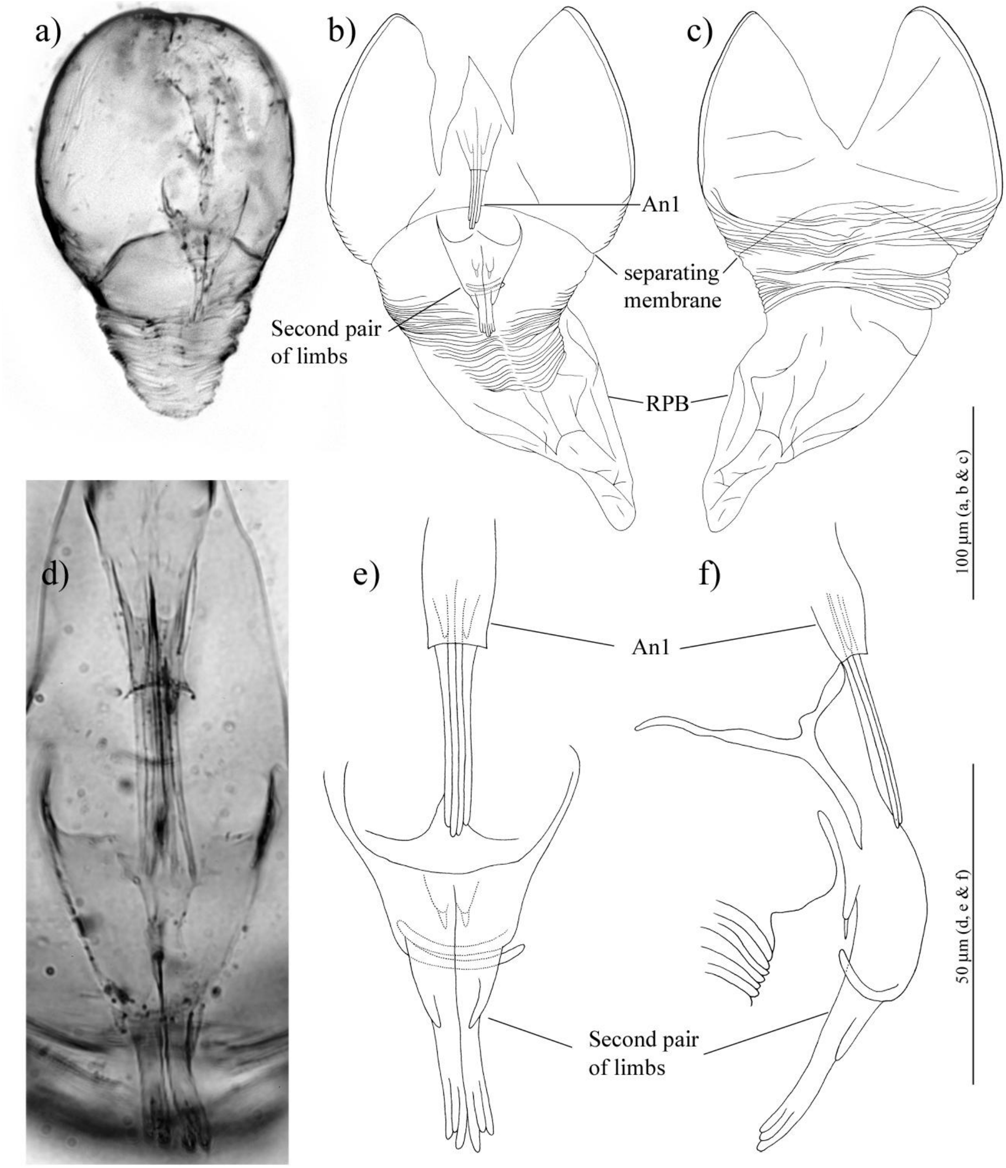

Fig. 3. Exuviae of stage A-9 (a-c) and Fig. details of its limbs (d-f). (a) stereomicroscope picture of exuvia in ventral view, (b) drawings of the complete exuvia in ventral view, (c) the same in dorsal view, (d) microscope picture of the detail of the limbs in ventral view, (e) drawing of the detail of the limbs in ventral view, (f) the same in lateral view. An1 = antennule. RPB = Roessler Protruding Bursa.

with setae. The eye is shaped like an orange bulb in the anterodorsal region of the body. This instar has three pairs of functional limbs (An1, An2 and Md), and a pair of structures that seems to be the pre-Anlagen of the Mx (Fig. 6). The posterior region of the body has a hirsute caudal seta pointing rearwards.

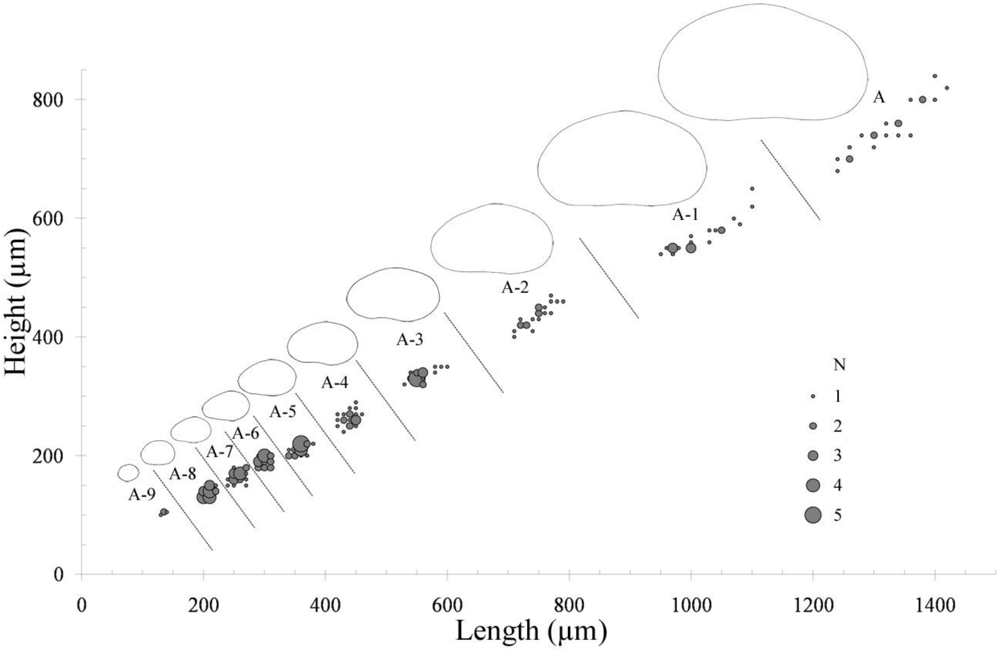

Fig. 4. Carapace sizes of the growth stages of *H. bosniaca*.

Instar A-7 has an elongated carapace in lateral view (240-270 µm long and 150-180 µm high), with the posterior region more pointed than in the previous instar and with a bulge in the ventral area close to the mouth. LV overlaps RV. The carapace is more strongly calcified than in the previous instar, with the calcareous inner lamella already defined. The ornamentation of the valves is less marked than in A-8, and has 11 normal pores on each valve (Fig. 5) with the same layout as in other species of *Heterocypris*. Muscle scars are present in the posterior-central region of the valves, but not yet fully defined. The eye has acquired a dark colour that is maintained until adulthood. This instar has five pairs of limbs (An1, An2, Md, Mx Anlagen, and Cr) and a hirsute caudal seta pointing ventrally behind the Cr (Fig. 6).

Instar A-6 has a carapace of 280-310 µm in length and 170-200 µm height (Figs. 4-5). Its shape in lateral view is very similar to that of the previous instar. Muscle scars are well defined and it is possible to recognize the two biggest mandibular scars anteroventral to the four smaller adductor scars (2 + 4). This instar has the same number of limbs as the previous one but a more fully developed Mx (including a branchial plate) and a bulge on each side between Mx and Cr that may be the L5 pre-Anlagen (Fig. 6).

In instar A-5 the carapace is 340-380 µm long and 200-220 µm high. Its shape is very similar to that of the previous instar but it has begun to widen slightly

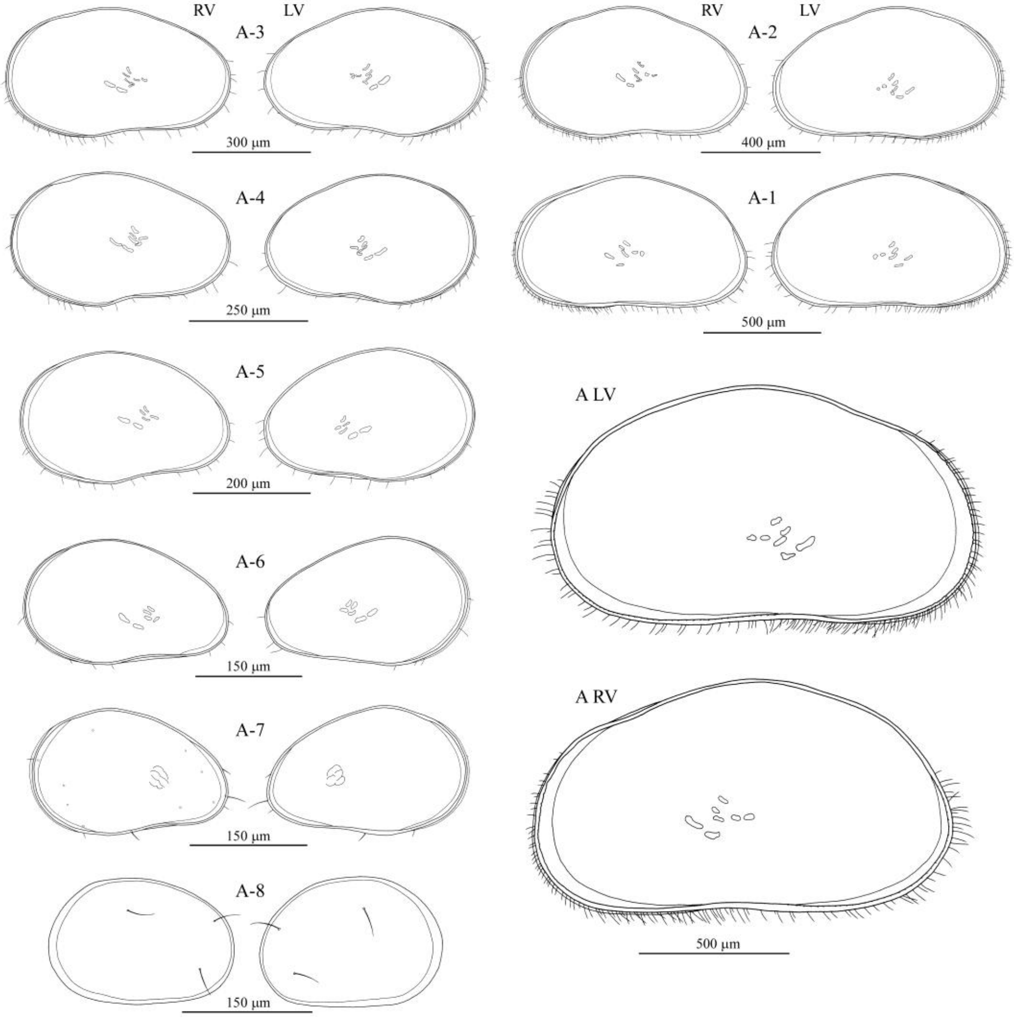

Fig. 5. Inner view of right and left valves of juvenile and adult female stages with their muscle scars of *H. bosniaca*; position of the normal pores shown in the right valve of A-8 and A-7. RV = right valve; LV = left valve.

posteriorly. The muscle scars have changed slightly but their disposal remains 2 + 4. This instar continues with five pairs of functional limbs together with the L5 Anlagen (Fig. 6).

Instar A-4 has a carapace length of 420-460 µm and height of 240-290 µm. The muscle scars have shifted towards the centre of the carapace and a new adductor scar has appeared (2 + 5) (Fig. 5). The posterior region of the carapace has continued to widen. L5 now has the appearance of walking leg and the L6 Anlagen have appeared for the first time (Fig. 6). Cr has and adult-like appearance.

Fig. 6. Lateral (right side) view of juvenile instars (except A-9) and the adult of *H. bosniaca* with right valve removed to see the position and layout of the limbs in the context of the whole body.

In instar A-3 carapace length attains 530-600 µm, and height 320-350 µm. The shape of the carapace is very similar to that of the previous instar but relatively more elongated. The muscle scars are now near the centre of the carapace and a new adductor scar has appeared (2 + 6) (Fig. 5). L5 now looks like a maxilliped, L6 is developed as a walking leg, and the Anlagen of L7 have appeared (Fig. 6). This instar thus has all the pairs of appendages, although not yet fully developed, typical of the adult.

Instar A-2 has a carapace length of 710-800 µm and a height of 400-470 µm (Fig. 4). The posterior side of carapace is wider than before, forming a bulge in the dorsalcentral margin. The muscle scars are located in the centre of each valve, unchanged in number (2 + 6). L7 has developed as a cleaner limb and, therefore, all eight pairs of limbs are now functional (Fig. 6).

Instar A-1 has a carapace length of 950-1100 µm and a height of 540-650 µm. Due to the increased widening of the dorsal side, the dorso-central bulge is not as marked as in A-2. The number of muscle scars is reduced to 2 + 5, the same as in the adult (Fig. 5). The gap between L7 and Cr has increased due to the appearance of the sexual organs, which are not yet fully developed.

The adult female carapace length is 1240-1440 µm, and its height is 680-840 µm. The posterior part of the carapace is further widened, having become higher than the anterior part, and the bulge of the buccal area is not as bulky as before. There is greater separation between L7 and Cr than in the previous instar due to the maturation of the sexual organs.

Antennule (Figs. 2, 3, 7)

The An1 of stage A-9 has been described previously (Figs. 2-3). After leaving the prenaupliar sheath, the A-8 An1 consists of five podomeres (Fig. 7). The first two are semimerged and the most distal three podomeres are rectangular in lateral view. The third podomere has a single dorso-apical seta and one very short spine on the ventral side; the fourth podomere has a long and a medium-length setae; and the last podomere already has the complete set of setae displayed at maturity that consists of two long setae, a medium-length seta and aesthetasc y_a.

In instar A-7 the first two podomeres are stronger than in the previous instar and the two setae on the fourth podomere are now long and with the same length.

The An1 of instar A-6 is very similar but with an additional seta on the apical ventral position of the fourth podomere.

In instar A-5 the first two podomeres are large and subrectangular, the second of which bears one small seta on the dorso-apical edge and a protuberance at the ventral margin that corresponds to the Rome organ; the spine presented previously on the third podomere is now transformed into a short seta; on the fourth podomere the (A-6) short seta is now long and a new short one has appeared.

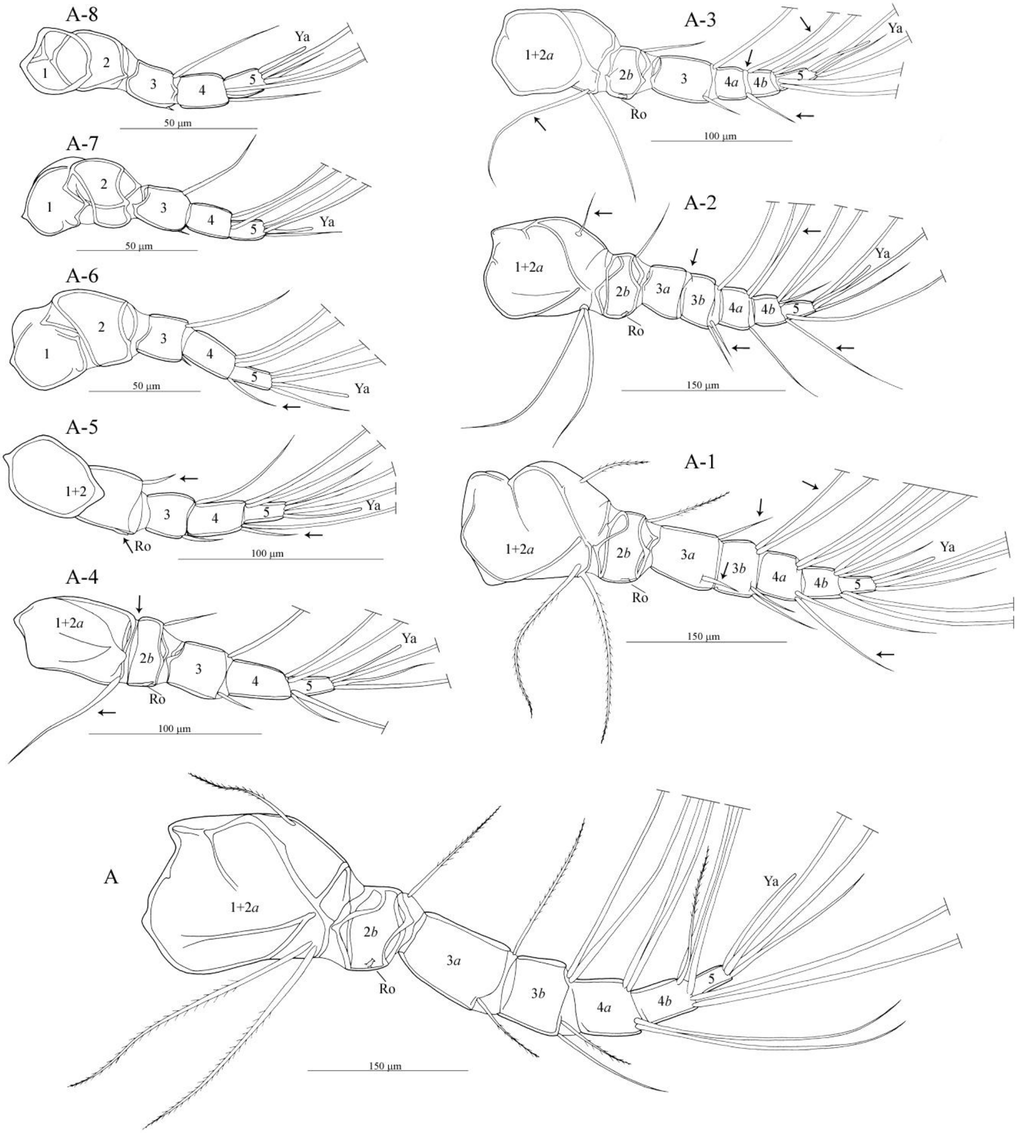

Fig. 7. Antennule of juvenile and adult stages of *H. bosniaca* in lateral view.

In instar A-4 the An1 consists of six podomeres. The second podomere is now clearly divided into two podomeres, one of which become fused with the first and now constitutes a larger subquadrate basal podomere (corresponding to podomeres 1 and 2*a* according to the nomenclature of Smith and Tsukagoshi (2005), with a ventral medium length seta, and a smaller distal subrectangular podomere (2*b*). The Rome organ is now bottle-shaped.

The An1 of instar A-3 now consists of seven podomeres (the two most proximal ones fused), resulting from the division of the fifth podomere into two smaller quadrate podomeres (now called podomeres 4*a* and 4*b*). Podomeres 1 + 2*a* are broad shaped, supporting two long subapical setae on their ventral edge (only one in the previous instar); podomere 2*b* has the Rome organ widened at the apex, and will remain in this shape up to adulthood; podomere 3 is more elongated; podomere 4*a* bears two apical setae, one long dorsal and one short ventral.

In instar A-2 the An1 has eight podomeres, derived from the split of podomere 3 to form two quadrate podomeres. Podomere 1 + 2*a* has an additional short seta on its dorsal part; podomere 3*b* has an extra short seta on the ventral edge; the segment 4*a* has now two long setae on its dorsal margin; podomere 4*b* has a new medium length seta on its ventroapical edge.

In instar A-1 all the setae present on podomeres 1 + 2*a*, and 2*b* are hirsute. On segment 3*a* two new short setae appear, one dorsal and one ventral; podomere 3*b* has a new long dorsal seta but it is still shorter than the other setae; podomere 4*a* has an additional medium-length seta on its ventral margin; podomere 4*b* has now a more elongated ventral medium-length seta.

In adults, the setae present on podomere 3*a*, and also the ventral seta of podomere 3*b* and the short seta of podomere 4*b* are hirsute. The shorter dorsal seta of podomere 3*b* becomes more elongated and attains the same length as the other dorsal seta of this segment (Fig. 7).

Antenna (Figs. 2, 3, 8)

The An2 of stage A-9 has been described previously (Figs. 2-3). In instar A-8 the An2 consists of a protopod, an endopod and a small exopod (Fig. 8). The protopod has two podomeres, both with a seta protruding from the ventroapical corner. The seta at the proximal segment is short and the seta at the distal one is long. The endopod is made up of three podomeres; the first one has a long seta and anlage of aesthetasc Y protruding together from its ventro-apical margin; the second podomere is subrectangular and holds the large dorso-apical claw G_3, the aesthetasc y_2 and one dorso-apical spine that corresponds to the anlage of claw G_1; the terminal podomere has one dorsal spine, claws G_M and g and the bifurcating aesthetasc y_3. The exopod is situated on the apical outer face of the protopod and made up of a small base with one dorsal plumed seta and two ventral shorter smooth setae.

In instar A-7 a second seta appears on the ventral part of the first podomere of the protopod. Claw g, on the last podomere of the endopod, is now thinner and transformed into a seta. The exopod dorsal seta is now more elongated and smooth, and the remaining two setae are much shorter; this morphology of the exopod is maintained until the adult form.

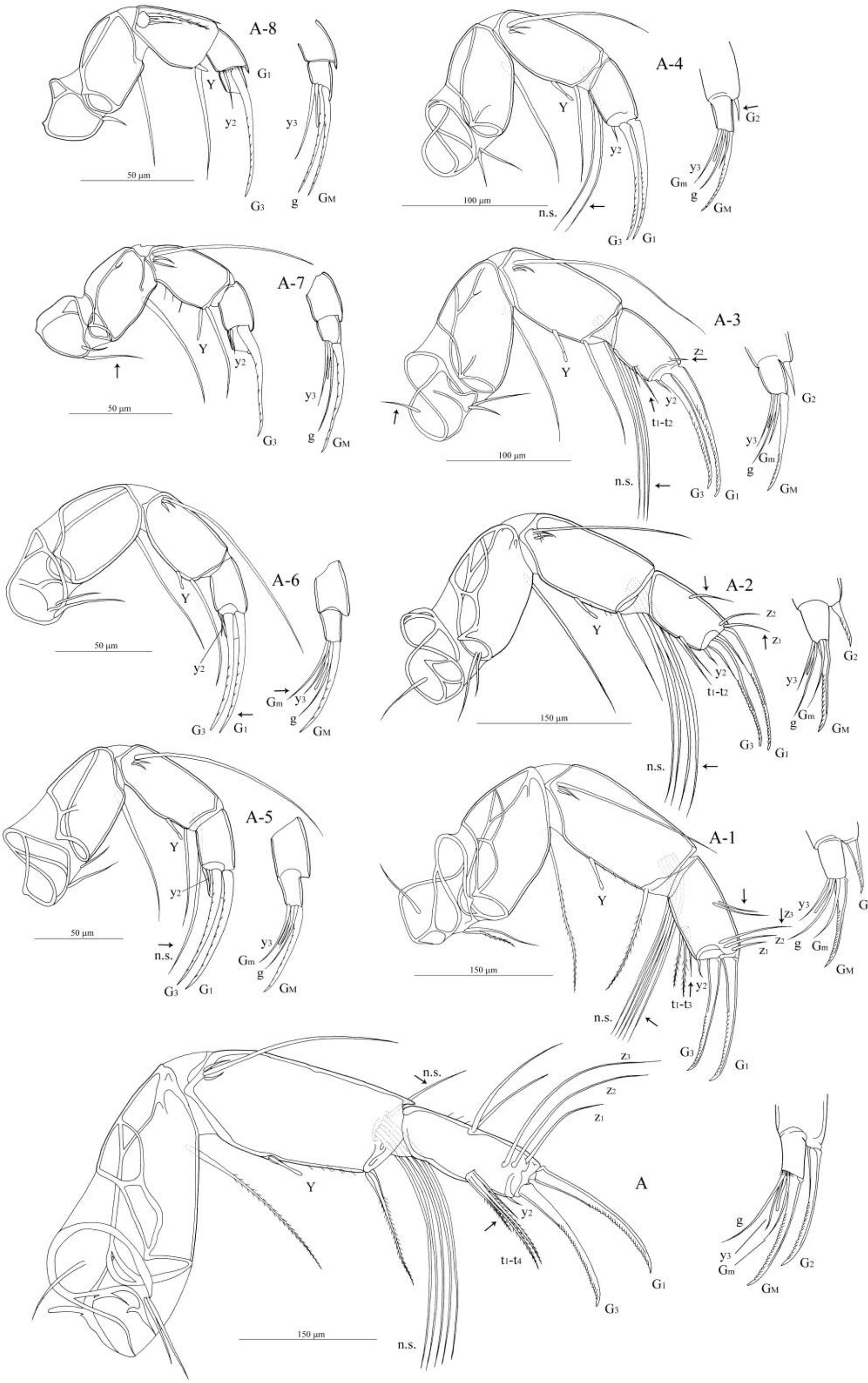

Fig. 8. Antenna of juvenile and adult stages of *H. bosniaca* in lateral view.

In instar A-6 claw G_1 develops on the penultimate podomere. In the last podomere a new seta appears corresponding to G_m, and this structure will not change further during development.

The first natatory seta appears on the inner margin of the first endopodal podomere in instar A-5.

In instar A-4 the An2 shows two natatory setae, and the second endopodal podomere shows a new terminal seta on the dorsal margin that corresponds to the primordium of claw G_2.

In A-3 a new seta appears on the dorsal margin of the first segment of the protopod; the first endopodal segment has now three natatory setae; the t_1 and t_2 setae appear on the mid-ventral margin of the second podomere, and the short apical seta z_2 on its dorsal edge.

Instar A-2 has now four swimming setae on the An2; the second endopodal podomere holds setae z_1 and z_2 on its dorsal subapical zone and a medial seta on the mid of the dorsal margin. Claw G_2 has now a dentate margin.

Juvenile instar A-1 has five long natatory setae (as in the adult stage). The second endopodal podomere has three ventral t-setae (t_1-t_3, but now t_1 and t_2 are hirsute), a new z-seta (z_3), and a new medio-dorsal seta.

In the adults a short natatory seta appears dorsally on the inner face of the first endopodal podomere; the second endopodal podomere has now a t_4 seta present and a well developed G_2 claw.

Mandible (Figs. 2, 3, 6, 9)

The Md of stage A-9 has been described previously (Figs. 2-3). After the first moult, four podomeres can be seen in the A-8 mandibular appendage (Fig. 9). The first podomere is elongated and corresponds to the protopod, on which a strong endite stands out projecting from the protopod anteroventral edge into the mouth. The protopod has a strong long seta on its inner margin and a bifurcated seta at the apical zone. The exopod (respiratory plate, Fig. 6) is composed by two long setae, protruding from the dorsal margin of the protopod. The endopod consists of the following three podomeres; the first segment carries a set of three short setae on the distal margin; the second segment holds one ventroapical and one dorsoapical seta; the last segment has two short setae at the apical region and between them a long claw curved forward with a thickening at the base.

In instar A-7 the third pair of appendages develops into well-differentiated mandibles with coxa and palp. The coxal endite bears apical teeth and a subapical seta. The first palp segment (protopod) is large, with well-developed S1 and S2 setae, a long simple seta and holds the α-seta on its ventral margin; these structures remain basically unchanged to adulthood. The endopod presents three segments, but the

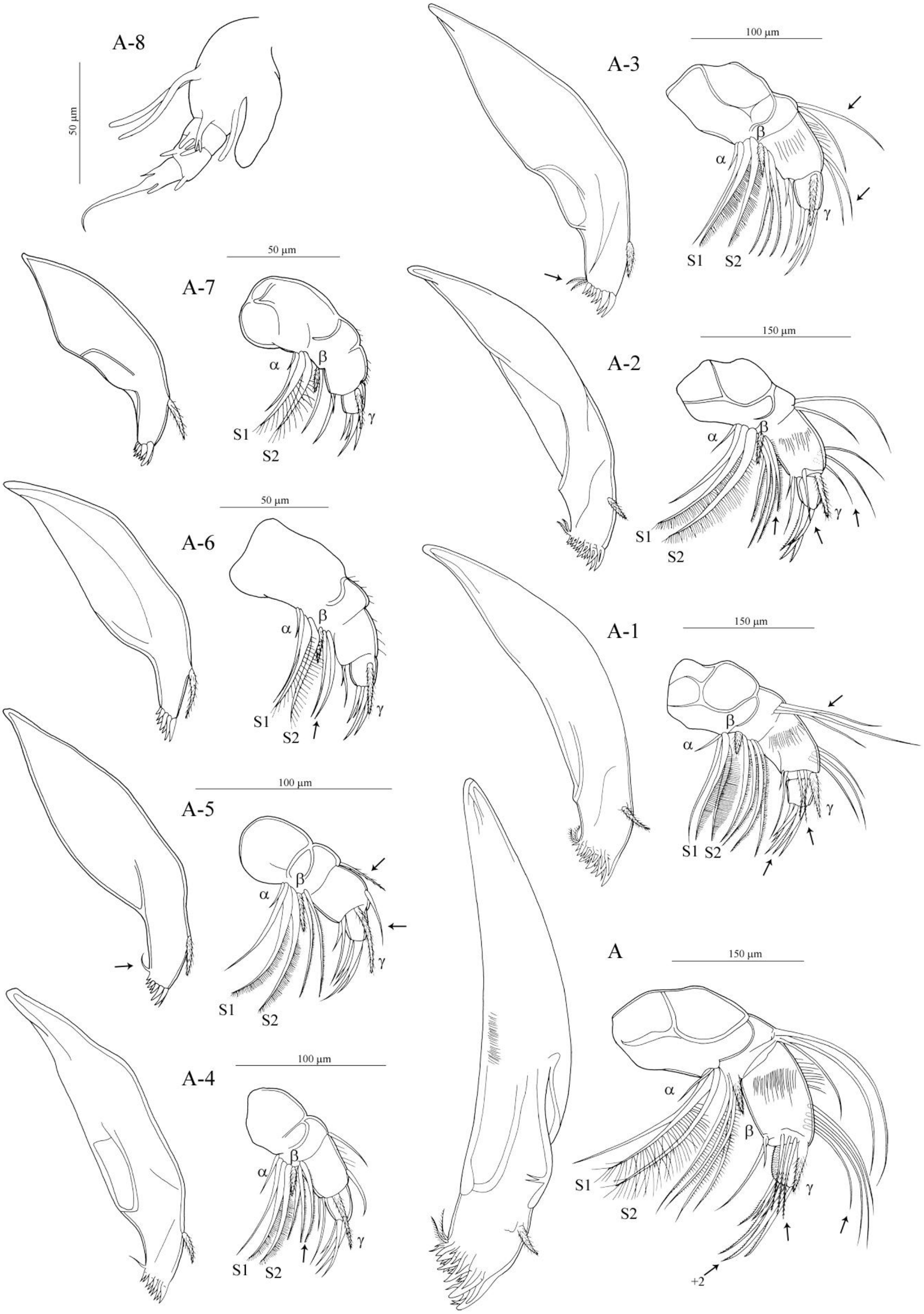

Fig. 9. Mandible of juvenile and adult stages of *H. bosniaca* in lateral view, branchial plates not shown (these can be seen in Fig. 6).

separation between them is not complete or conspicuous. The first segment has a long ventral seta together with the *β*-seta; the second podomere holds the *γ*-seta on its anterior margin and two ventral setae; the terminal segment has four apical claws.

In instar A-6 the Md has a new ventral seta on the first endopodal podomere.

In the next instar (A-5) a new hirsute seta appears on the dorsal margin of the first endopodal podomere (Fig. 9) and the setae on its ventral edge are now plumose; a new subapical smooth seta is now present on the dorsal part of the second podomere. The respiratory plate has a new seta, shorter than the two older setae (Fig. 6). The coxal endite has a new seta near the teeth on the ventral side of the coxa.

The endopod of instar A-4 has an additional seta within the *β*-group at the apex of the first podomere and the seta on the opposite (dorsal) side is now smooth. The three setae of the respiratory plate are now all of similar length.

The mandibular palp of instar A-3 has a new dorsal seta on the first endopodal segment and another new subapical seta on the second podomere (Fig. 9). The coxa has an extra seta added to the endite and the two setae present are now plumose. The coxal structure will remain unchanged to adulthood. The branchial plate (exopod) has a new seta (Fig. 6).

Instar A-2 has a new plumose seta within the *β*-group; the second endopodal podomere has a new subapical dorsal seta and another one on its inner face (close to the *γ*-seta) (Fig. 9). The exopod (respiratory plate) has an extra seta (Fig. 6).

Instar A-1 has an additional long seta on the outer margin of the first endopodal segment; the second podomere has a new seta in the *γ*-group, forming a group of two hirsute setae on the inner side; the final podomere has an extra claw.

In adults a new seta appears within the *γ*-group and another in the subapical group of setae on the dorsal edge of the second endopodal podomere, now composed by four thin setae. Two new short setae appear on the terminal podomere margin (Fig. 9) and two additional setae appear on the ventral margin of the respiratory plate (Fig. 6).

Rake-Like Organ (Fig. 10)

The RLO has a very similar shape since its first appearance in instar A-6 to adult. In instar A-6 it shows 11 denticles, and this figure progressively reduces throughout development to a final number of 8 denticles in adults.

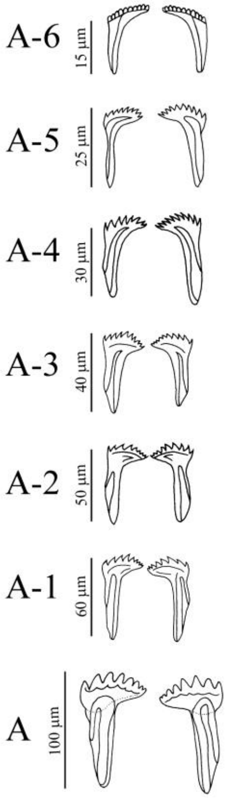

Fig. 10. Rake-like organs of juvenile and adult stages of *H. bosniaca*.

Maxillula (Figs. 6, 11)

Instar A-8 has a bulge behind the mandible, which corresponds with the maxillular pre-Anlage. After moulting (instar A-7), the structure becomes more apparent and has four different horizontally oriented lobes, the second of which has a terminal pinnate extension.

In instar A-6 the maxillula is better developed, consisting of an endopod (maxillular palp), three elongated endites (Fig. 11), and an exopod (branchial plate; Fig. 6). The endopod has two apical claw-like setae and a stout subapical dorsal seta. Its third endite has two apical setae and a rounded prolongation; the second endite has a smooth and curved *Zahnborste*-like claw accompanied by a dorsal seta of similar length; and the first endite has three terminal setae all of the same size (Fig. 11). The branchial plate is elongated, with seven setae pointing rearwards (Fig. 6).

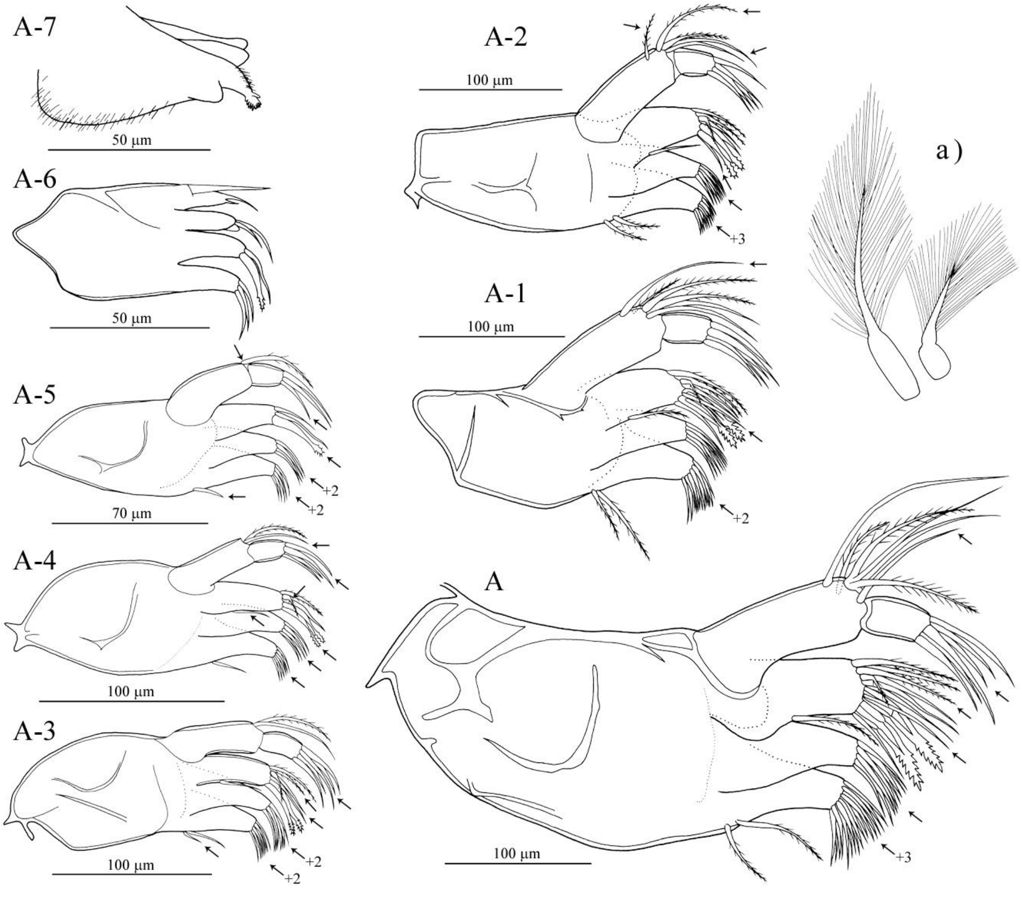

Fig. 11. Maxillula of juvenile and adult stages of *H. bosniaca* in lateral view (branchial plates and pre-Anlagen of instar A-8 can be seen on Fig. 6), (a) detail of hirsute setae on third endite.

In the next instar (A-5), the endopod is two-segmented; the first podomere is elongated and subrectangular with one long, hirsute seta on its outer apical edge; the second podomere is smaller and bears three medium-long stout setae. The previously rounded prolongation of the third endite is now transformed into a well-developed *Zahnborste*; the second endite has four apical setae in total, while the *Zahnborste* present in the previous instar has disappeared; the first endite has two new apical setae, and one short seta has appeared on the ventral edge near the base of the endite. The branchial plate has ten setae in total (Fig. 6).

In instar A-4 a new seta has appeared on both the first and the second segments of the maxillular palp (endopod). The third endite has a new *Zahnborste*, a new curved, plumose seta, and a new subapical seta, and its short seta is now hirsute. The first and second endites have six and five distal setae respectively (Fig. 11). The branchial plate has 11 setae along the posterior and ventral edges and two recurved setae on the anterior edge (11 + 2 setae) (Fig. 6).

The maxillular endopod of instar A-3 has an additional seta at the apex of the final podomere. The third endite has two new apical setae, one smooth and one hirsute; the second endite has seven apical setae; the first endite has eight apical setae and two basal setae. The respiratory plate has 14 + 3 setae.

In instar A-2 two new setae have appeared on the dorsoapical edge of the first endopodal podomere, in addition to one new hirsute subapical seta. The third endite has a new apical seta. The second endite has a new seta; the first endite has 11 apical setae and the two setae on the base are now hirsute. The branchial plate has 17 + 5 setae (Fig. 6).

In the palp of instar A-1 (Fig. 11), a new subapical simple seta has appeared on the first podomere. The third endite has a new apical seta and the basal seta is now hirsute; the second endite is unchanged; the first endite has two new apical setae. The branchial plate is unchanged from the previous instar.

In adults, the first and second endopodal segments both have a new apical seta. The third endite has two additional apical setae, one simple and one hirsute; the second endite has nine apical setae in total; the first endite has 16 apical setae. The branchial plate has 17 + 6 setae (Fig. 6).

Fifth Limb (Figs. 6, 12)

This appendage can be seen for the first time in primordial stage behind the maxillula in instar A-6 (Fig. 6). In the next instar it becomes more evident and takes the form of an elongated, curved podomere, pointed posteriorly and without any evidence of segmentation (Fig. 12).

In A-4 the L5 develops into an elongated appendage consisting of four podomeres. The first podomere has irregular shape, is weakly sclerotized and has a

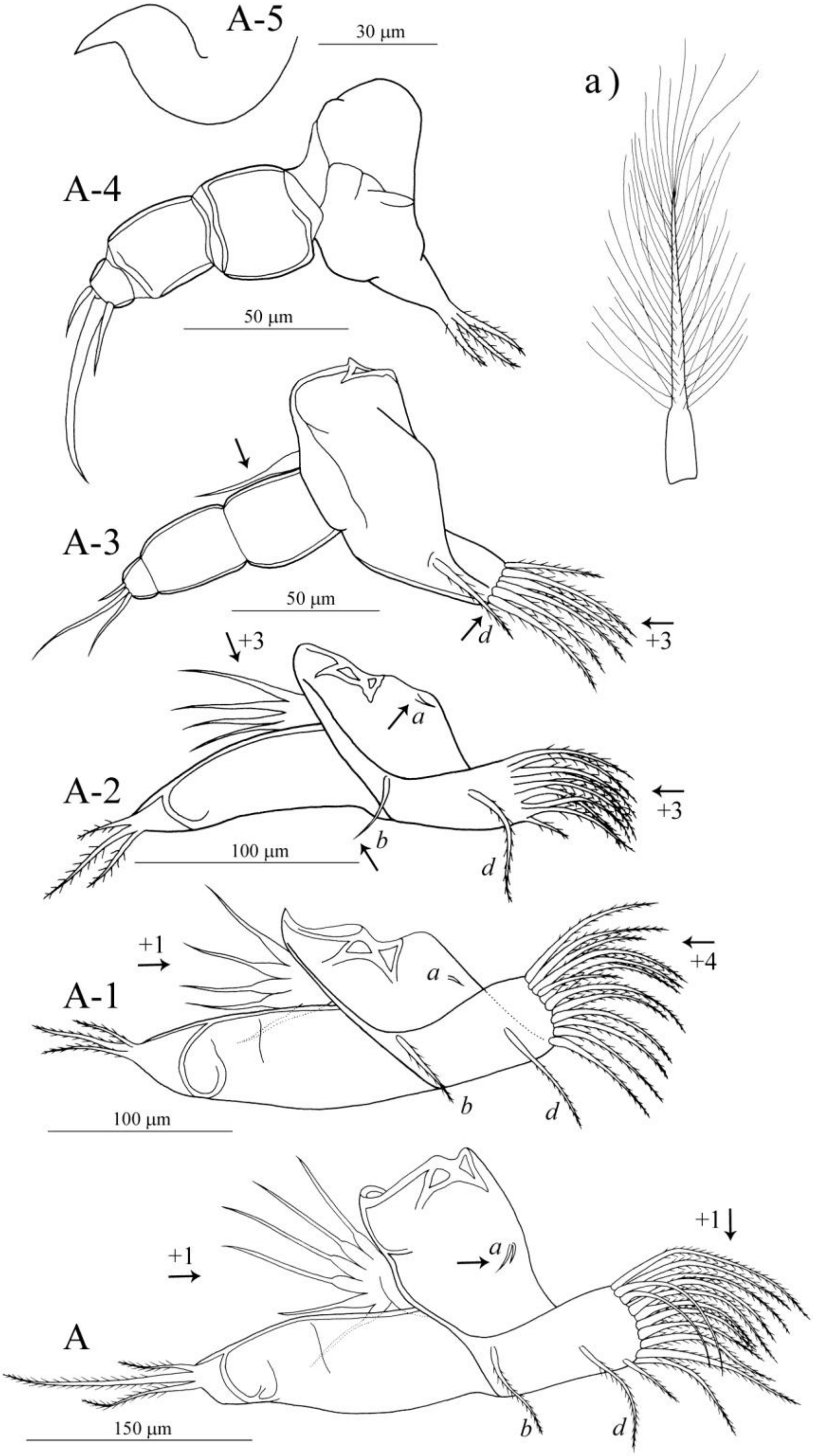

Fig. 12. Fifth limb of juvenile and adult stages of *H. bosniaca* in lateral view (pre-Anlagen of instar A-6 can be seen in Fig. 3), (a) detail of a hirsute setae on endite.

small projecting base (endite), which bears three medium length setae pointing anteriorly. The endopod is pointed posteriorly and is composed of three sclerotized podomeres. The first podomere of the endopod is quadrate and the second is subrectangular; the terminal podomere is trapezium-shaped and has a long claw and two short setae.

In instar A-3 the morphology of the L5 appendage has changed considerably from its previous form, and is now recognizable as a maxilliped. The endopod is similar to the previous instar but less sclerotized and with the claw turned now into a seta. The endite is transformed into a feeding process, and bears six plumed setae on its apical edge. The protopod has the plumose *d*-seta, plus a simple respiratory plate consisting of just one seta.

In instar A-2 the endopod segmentation becomes weakly expressed conforming two apparently fused segments with the apical setae now plumose. The endite has three additional setae (one of them short), forming a group of nine. The protopod has a small *a*-seta and a medium length *b*-seta; the branchial plate has now four setae (Fig. 12).

In instar A-1 the *b*-seta is now hirsute; the endite has four extra setae (13 in total) and the branchial plate has five setae.

In the adult stage the L5 appendage has two *a*-setae, one new short seta on the endite and one new seta on the branchial plate.

Sixth Limb (Fig. 13)

The Anlage of L6 appears for the first time in instar A-4. It is similar in shape to the Anlage of L5 in instar A-5.

In instar A-3 the L6 is transformed into a walking leg and has four podomeres; the first one is subtriangular forming a knee-like joint; the second podomere is subquadrate; the third segment is subrectangular and has a seta on its ventroapical corner; the last podomere is small and has two short apical setae and in between it holds one large and curved claw (Fig. 13).

In instar A-2 the L6 is composed of five podomeres, after the split of the third one into two new subquadrate podomeres; the second and third podomeres have a plumed seta on the ventro-apical corner; the setae of the fourth and the last podomeres are now hirsute and a new simple small seta has appeared in the fourth segment near the hirsute seta; a hirsute *d1*-seta is now present on the protopod. This structure is mostly unchanged until the adult stage but then all the setae will become hirsute.

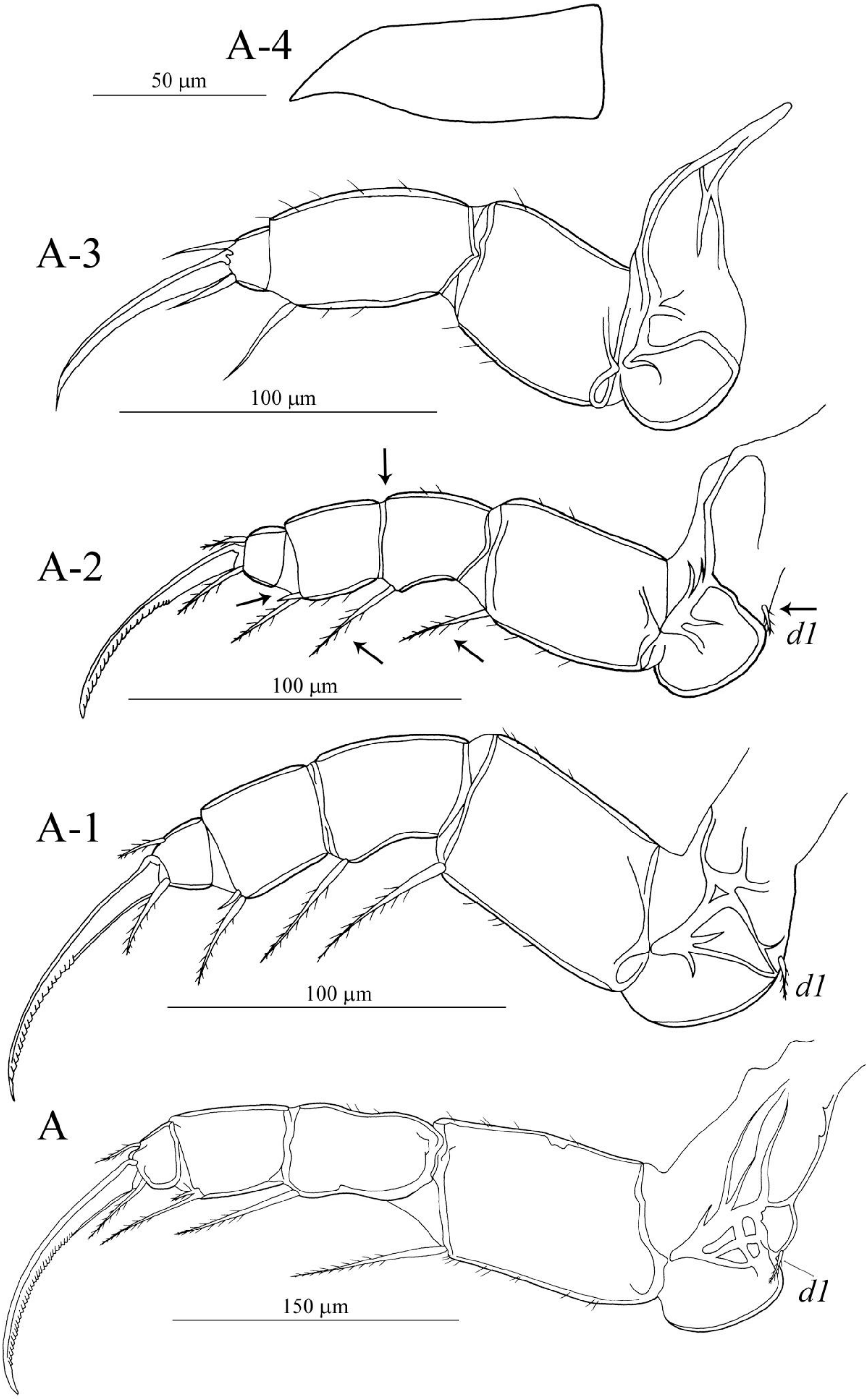

Fig. 13. Walking leg (L6) of juvenile and adult stages of *H. bosniaca* in lateral view.

Seventh Limb (Fig. 14)

This limb appears firstly as an Anlage in instar A-3 and its morphology is very similar to L6 in instar A-4 and L5 in instar A-5.

In the next instar (A-2) it has developed into a cleaning limb made up of three elongated podomeres; the first one has one plumed seta on the apical region and the terminal podomere holds distally a fully developed cleaning pincer similar to the final adult one (Fig. 14).

In instar A-1 the L7 has four new plumed setae: two ventrally situated on the first segment, one ventro-apical seta in the second podomere and one medial seta in the third podomere. This general structure does not change in the adult stage.

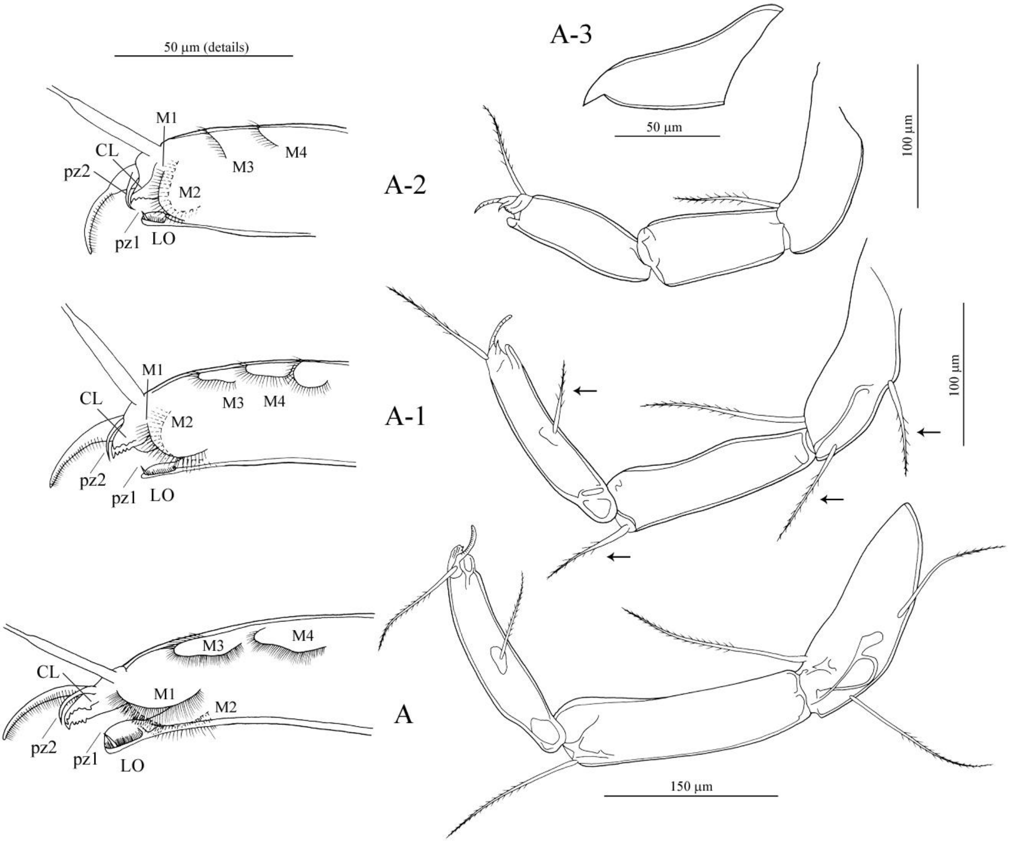

Fig. 14. Cleaning limb (L7) of juvenile and adult stages of *H. bosniaca* in lateral view.

Caudal Ramus (Figs. 6, 15)

It can be seen for the first time in the posterior region of instar A-7 and then it consists of a basal segment with a long, forward incurved, terminal claw (Fig. 15). This basic morphology is maintained without changes up to instar A-5.

In instar A-4 its previous morphology is altered and now consists of an elongated segment bearing apically the medium length claw *Gp* and subapically the thickened backwards curved seta s_p.

In instar A-3, this appendix is more sclerotized and elongated. The attachment of each Cr can be seen for the first time in this instar (Fig. 6). The s_p-seta becomes thinner and the short apical claw *Ga* has now appeared.

instar A-2 the s_p-seta is further reduced, the *Ga* becomes longer than the *Gp* claw and the short apical seta s_a is now present.

In the next instar (A-1) the basal segment and the s_a-seta are larger.

In the adult the only change observed is that the s_a-seta reaches the maximum length.

Discussion and conclusion

Individuals of *H. bosniaca* analysed in this study show some slight differences compared to the drawings of the original description by Petkowski *et al*. (2000). The chitinous attachment of the Cr looks different in that we observed only a bifurcation towards its proximal end (Fig. 6) and Petkowski *et al*. (2000) described it with two branching points and also with a T-junction at its edge, which we could not find in our specimens. We also noted that there are fewer setae in the drawings of Petkowski *et al*. (2000), but these differences may be just artefacts due to the fact that the original drawings show only the most important parts of the limbs, and to the absence of drawings for some appendages. Although there are slight differences in the morphology of our specimens compared to the original descriptions we have concluded that the ostracodes discussed here belong to *H. bosniaca* because of its general shell form and size, lack of tubercles and pustules on the right valves and An2 natatory setae arriving just to the tip of the claws.

During ontogenetic development, members of Cyprididae usually pass through eight juvenile stages that moult consecutively (from A-8 to A-1) before the final moult to reach adulthood (Cohen & Morin, 1990). Exceptionally, Roessler (1982a, 1982c, 1983) found that some species of Cyprididae have an earlier stage, and we observed the same in *H. bosniaca*. In summary, the A-9 stage develops inside the egg and begins the moulting process leading to the formation of instar A-8, therefore apolysis (the initial separation of the older A-9 exoskeleton from the newly formed A-

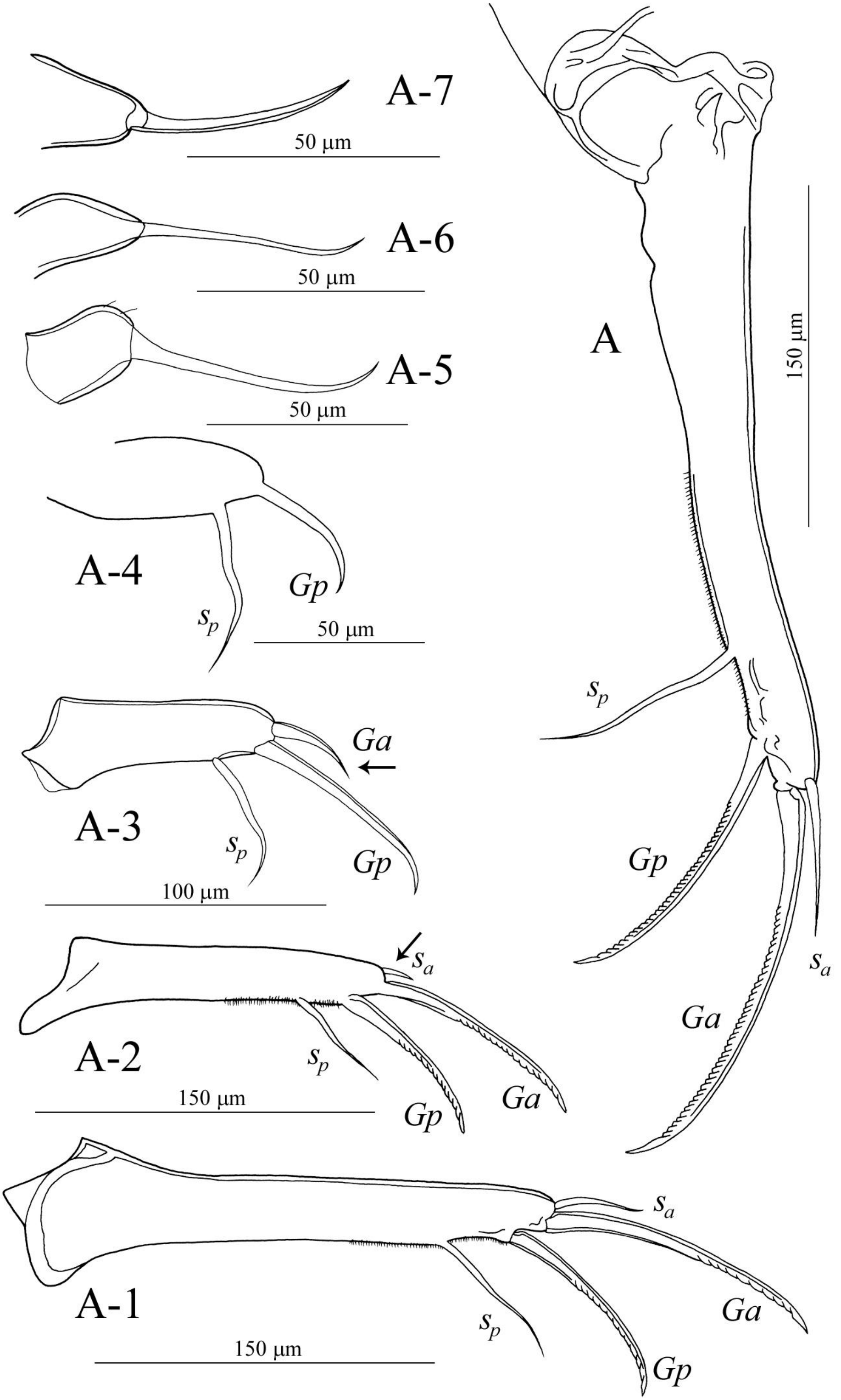

Fig. 15. Caudal ramus of juvenile and adult stages of *H. bosniaca* in lateral view.

8 cuticle) has already occurred inside the egg (Fig. 1). Later on, a crevice on the eggshell appears and the hatching stage (the exoskeleton of A-9 stage and instar A-8 inside its anterior chamber) emerges helped by the swelling process of the RPB (Fig. 2), in contrast to other crustaceans whose hatching is partially due to the movement of the nauplii (Lewis, 1975). This transitional stage remains immobile outside the egg for less than two minutes, whereupon ecdysis (opening of the old exoskeleton to facilitate the exit of the inner stage) occurs and instar A-8 emerges swimming from within the old A-9 exuvia, thus ending the moulting process. Consequently, in some ostracode species, the first stage appearing outside the egg is not a free A-8 instar, but one encapsulated inside the cuticle of the A-9 stage. The RPB of the A-9 stage seems to be needed for the hatching process, therefore the A-9 exoskeleton is not merely a protective cover for the A-8 instar, but it also appears to be functionally essential in this process. This type of hatching process in which individuals are enveloped in the prenaupliar membrane are not unique in ostracodes, but is present in other crustaceans. The anostracans have a similar type of hatching (Rosowski *et al*., 1997) but, unlike these, the prenauplii of ostracodes have limbs and a specialized bursa (RPB) used for hatching.

In the A-9 stage, Roessler (1998) observed three pairs of appendages (An1, An2 and Md) in *H. bogotensis*. However, by studying the exuviae of *H. bosniaca*, we interpret that there are only two pairs of limbs, because the structure that Roessler identified as a Md actually belongs to the second pair of limbs in our A-9 individuals. If we look at the hatching stage, it is possible to check the homology by formation, since structures of instar A-8 can be seen through the cuticle of the A-9 stage. This allows us to confirm that the first pair of limbs in this stage is homologous to the An1, but the second pair does not correspond (only) to An2, in fact they include both the An2 and the Md, and therefore we suggest this limb might be called the “antedible”. To our knowledge, this is the first time that this type of fusion is observed in crustaceans and may have important implications for phylogenetic reconstructions. Further research on the transition from the final embryonic to the first larval stage is needed in order to find out if this is an ancestral trait in crustaceans or conversely it is a derived, apomorphic character in (some) lineages of Ostracoda. However, the only functional exoskeletical structure of stage A-9 is the RPB but the appendages have very reduced mobility and do not seem to have any particular function; therefore the fusion could be probably considered a derived trait tending towards a reduction in the number and size of limbs in a phase of development encapsulated inside the egg shell.

The ostracode instar A-8 has been considered as an orthonauplius (Dahms *et al*., 2006) because it has three pairs of segmented appendages (An1, An2 and Md). However, it should be noticed that both *H. bogotensis* and *H. bosniaca* present at this stage already the pre-Anlagen of the Mx, what lead Roessler (1983) to consider it as a metanauplius, *i.e.*, a naupliar stage instar with more than three pairs of limbs, although one of them was still unsegmented. Roessler (1998) defined the stage A-9 as a prenauplius because their limbs were not segmented, and interpreted that it could be considered the lost orthonauplius because he observed three pairs of limbs, thus integrating again the ostracodes in the general theory that crustaceans basically start the development as orthonauplius (Dahms, 2000). However, according to our findings, we cannot consider that the A-9 stage is the lost orthonauplius since it has only two pairs of appendages, so we consider it as a prenauplius. The next stage, instar A-8 could be considered an orthonauplius because of the presence of three well-developed pairs of appendages, although a new appendage pre-Anlage can already be observed.

According to the detailed embryological study by Weygoldt (1960) on *Cyprideis torosa* (Jones, 1850), this cytheroidean does not have any stage similar to the A-9 prenauplius. Three pairs of appendages, An1, An2, and Md develop separately inside the egg, and no clear sign of a RPB was observed that could help during the hatching process in this species. For most ostracode taxa, the A-9 stage has never been described in the literature (with the exception of the above-mentioned works by Roessler), perhaps because those species do not have such transitional stage (like in *C. torosa*) or, most probably, because no just-hatched individuals were available to other authors. The short duration of the A-9 stage outside the egg, its lack of mobility, and the fragility of its shed cuticle (easily damaged through trampling by other individuals) might be the main reasons why this stage has passed unnoticed by most ostracodologists, as seems to be also the case of the first stage of other crustaceans, *i.e.*, in Anomopoda (see Kotov, 1997). It is therefore essential that more ostracode species be carefully observed just after hatching to acquire a better knowledge on the number of postembryonic stages in the group.

From instar A-8 onwards, the timing of the first appearance of each pair of appendages and their subsequent development were similar to those of other Cyprididae (Tab. 1). Some of the features observed during the development of the species compared with other ontogenetic studies on cypridid ostracodes are summarized on Table 2 (but keep in mind that some of these small differences may be due to observational errors; see appendix of Smith & Tsukagoshi, 2005), and the most important differences are discussed below. In some cases, we do not mention comparison with particular studies because of lack of clarity in the available drawings or descriptions.

- Central muscle scars appear first on instar A-7 in *H. bosniaca* and can be seen well defined from instar A-6 onwards but do not have the same pattern as the adults until instar A-1. In *Eucypris virens* the muscle scars appear for the first

Tab. 1. Summary tables of the ontogeny of five species of Cyprididae. ◦, pre-Anlagen; ○, Anlagen; ⊙, form resembling that of adults but not complete; ●, definitive form.

INSTAR	An1	An2	Md	Mx	L5	L6	L7	Cr	Cs
Heterocypris bosniaca (this work)									
0 (A-9)	○	○							
1 (A-8)	⊙	⊙	○	◦					●
2 (A-7)	⊙	⊙	⊙	○				○	●
3 (A-6)	⊙	⊙	⊙	⊙	◦			○	●
4 (A-5)	⊙	⊙	⊙	⊙	○			○	●
5 (A-4)	⊙	⊙	⊙	⊙	⊙	○		⊙	●
6 (A-3)	⊙	⊙	⊙	⊙	⊙	⊙	○	⊙	●
7 (A-2)	⊙	⊙	⊙	⊙	⊙	●	⊙	●	●
8 (A-1)	●	⊙	⊙	⊙	⊙	●	●	●	●
9 (A)	●	●	●	●	●	●	●	●	●
Heterocypris incongruens (after Schreiber, 1922)									
0 (A-9)	Not recovered								
1 (A-8)	⊙	⊙	○						
2 (A-7)	⊙	⊙	⊙	○				○	●
3 (A-6)	⊙	⊙	⊙	⊙	◦			○	●
4 (A-5)	⊙	⊙	⊙	⊙	○			○	●
5 (A-4)	⊙	⊙	⊙	⊙	⊙	○		⊙	
6 (A-3)	⊙	⊙	⊙	⊙	⊙	⊙	○	⊙	
7 (A-2)	⊙	⊙	⊙	⊙	⊙	●	⊙	●	
8 (A-1)	●	⊙?	⊙?	⊙	⊙?	●	●	●	
9 (A)	●	●	●	●	●	●	●	●	
Heterocypris salina (after Kubanç et al., 2007)									
0 (A-9)	Not recovered								
1 (A-8)	⊙	⊙	○						?
2 (A-7)	⊙	⊙	⊙	○				○	?
3 (A-6)	⊙	⊙	⊙	⊙				○	?
4 (A-5)	⊙	⊙	⊙	⊙	○			○	?
5 (A-4)	⊙	⊙	⊙	⊙	⊙	○		⊙	?
6 (A-3)	⊙	⊙	⊙	⊙	⊙	⊙	○	⊙	?
7 (A-2)	⊙	⊙	⊙	⊙	⊙	⊙	⊙	●	?
8 (A-1)	⊙	⊙	⊙	⊙	⊙	●	●	●	?
9 (A)	●	●	●	●	●	●	●	●	?
Heterocypris bogotensis (after Roessler, 1982, 1983)									
0 (A-9)	○	○	◦						
1 (A-8)	⊙	⊙	○	◦				◦	●
2 (A-7)	⊙	⊙	⊙	○				○	●
3 (A-6)	⊙	⊙	⊙	⊙	◦			○	●
4 (A-5)	⊙	⊙	⊙	⊙	○	◦		○	●
5 (A-4)	⊙	⊙	⊙	⊙	⊙	○		⊙	●
6 (A-3)	⊙	⊙	⊙	⊙	⊙	⊙	○	⊙	●
7 (A-2)	⊙	⊙	⊙	⊙	⊙	●	⊙	●	●
8 (A-1)	⊙	⊙	⊙	⊙	⊙	●	●	●	
9 (A)	●	●	●	●	●	●	●	●	
Eucypris virens (after Smith & Martens, 2000)									
0 (A-9)	Not recovered								
1 (A-8)	⊙	⊙	○						?
2 (A-7)	⊙	⊙	⊙	○				○	?
3 (A-6)	⊙	⊙	⊙	⊙				○	?
4 (A-5)	⊙	⊙	⊙	⊙	○			○	?
5 (A-4)	⊙	⊙	⊙	⊙	⊙	○		⊙	?
6 (A-3)	⊙	⊙	⊙	⊙	⊙	⊙	○	⊙	?
7 (A-2)	⊙	⊙	⊙	⊙	⊙	⊙	⊙	●	?
8 (A-1)	●	⊙	⊙	⊙	⊙	●	●	●	?
9 (A)	●	●	●	●	●	●	●	●	?

time in instar A-5 and are well defined in instar A-3 with a pattern similar to adulthood (Smith & Martens, 2000).

- The Rome organ appears for the first time in instar A-5, but the Wouters organ could not be found in any case in the present survey. This is in agreement with the absence of this organ in other members of the genus *Heterocypris* (Smith & Matzke-Karasz, 2008).
- Although there are observed differences in the Y aesthetasc segmentation appearing on the An2 of the species of *Heterocypris* studied so far, the study of its internal structure (Kaji & Tsukagoshi, 2008) shows that it must present segmentation from instar A-7 onwards, as seen in the present work.
- In instar A-8, the Md is very similar for the congeneric species *H. bosniaca*, *H. incongruens*, and *H. bogotensis* (Schreiber, 1922; Roessler, 1983). In these works and the present survey the mandible is drawn within the context of the whole body, not separating the individual limbs. In the description of the A-8 Md for other cypridid species such as *H. salina* or *E. virens* (Smith & Martens, 2000; Kubanç *et al.*, 2007) it seems that the authors only did drawings and descriptions of the distal end of the Md endopod, probably because the rest of the Md limb was lost during the dissection process, making difficult comparing between these species and our results.

Tab. 2. Summary tables of the most important differences in the development of selected appendages' (App.) traits of five species of Cyprididae. Quantity (0-4), size (L, long; M, medium; S, short) or time of appearance (instar A-8 to A) for some important setae, claws, aesthetascs and segmentation are indicated. Further abbreviations: s, segment; e, endite; N.A., not available. See text and Table 1 for further explanations. After information in: a (this work), b (Schreiber, 1922; Smith & Tsukagoshi, 2005; Meisch, 2000), c (Kubanç *et al.*, 2007), d (Roessler, 1982a, 1983), e (Smith & Martens, 2000; Smith & Tsukagoshi, 2005).

App.	Trait	*H. bosniaca*[a]	*H. incongruens*[b]	*H. salina*[c]	*H. bogotensis*[d]	*E. virens*[e]
An1	Setae on s1	0	0	0	0	1
	2nd seta on s2a	A-3	A-3	A-4	A-3	A-3
	2nd seta on s3	A-8	A-5	A-5	A-5	A-5
	Setae on s3b A-2	3	3	3	3	2
	Setae on s5	4	4	4+1	4	4
An2	Setae A-8 exopod	L, S, M	N. A.	L, S, S	L, L, L	M, M, M
	Aesthetasc y1	0	0	1	0	1
	Seta z3	A-1	N. A.	A	A-1	A-3
	Claw G1	A-8 (spine)	N. A.	A-8 (spine)	A-8 (spine)	A-7
Md	Seta α	A-7	N. A.	A-3	N. A.	A-3
	Seta β	A-7	N. A.	A-5	N. A.	A-5
	Seta γ	A-7	N. A.	A-7	N. A.	A-7
Mx	Palp segmentation	A-5	A-5	A-5	A-6	A-4
	*Zahnborsten*2nde	A-6 (lost A-5)	0	0	N. A.	0
	1st *Zahnborsten* 3rde	A-5	A-5	A-6	N. A.	A-5
	2nd *Zahnborsten* 3rde	A-4	A-5	A-5	N. A.	A-5
L5	Segmentation lost	Not completely from A-2	A-2	A	A-2	A-2
	Seta c	0	0	0	0	1
L6	Seta d2	0	0	0	0	1

- The Mx appears as a rudimentary Anlage in instar A-8, as with *H. bogotensis* (Roessler, 1983). In *H. bosniaca*, we could see it in the whole body preparations of A-8 individuals but not in preparations of the limbs separately, probably due to its fragility and small size. In *H. incongruens* and *E. virens* (Schreiber, 1922; Smith & Martens, 2000) apparently it was not found in this instar.
- In *H. incongruens* and *Eucypris virens* (Schreiber, 1922; Smith & Martens, 2000) the two *Zahnborsten* or tooth-like setae both appear on the third maxillular endite in instar A-5, while in *H. salina* (Kubanç *et al.*, 2007) one appears on this same endite in instar A-6, and the other in instar A-5. In contrast, in *H. bosniaca* the first *Zahnborste* appears in instar A-6, but on the second endite, not on the third. At the moult to instar A-5, this *Zahnborste* becomes transformed into a seta, and a rounded extension on the third endite becomes a new *Zahnborste*. This replacement of one *Zahnborste* by another was confirmed in *H. bosniaca* by the preparation of an A-6 individual just prior to moulting, *i.e.*, by checking "homology by formation" (Fig. 16). The second *Zahnborste* on the third endite appears later, in instar A-4.

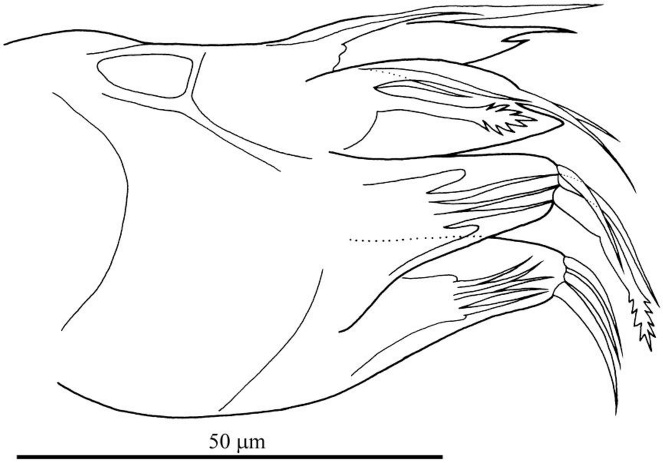

Fig. 16. Detail of the maxillula of an individual fixed just in the process of moulting from instar A-6 to A-5.

- The endopod of the L5 generally has three segments in instars A-4 and A-3 in the Cypridoidea. Individuals of *H. incongruens*, *H. bogotensis*, *E. virens*, *C. vidua*, and *Stenocypris* sp. lose this segmentation from instar A-2 onwards (Schreiber, 1922; Kesling, 1951; Ghetti, 1970; Roessler, 1983; Smith & Martens, 2000), while *H. salina* (Kubanç *et al*., 2007) displays two segments in this limb up to instar A-1. In none of these studies has segmentation been observed in the adult instar, but in *H. bosniaca* weak distal segmentation is still expressed from instar A-2 to adulthood.

- The development of the Cr is similar to that of most Cypridoidea except Cypridopsinae (*Cypridopsis vidua*; see Kesling, 1951) in which it is strongly reduced. In *H. salina* the Cr develops a shape similar to that of the adult in instar A-4, but with two claws (*Ga*, *Gp*) and one seta (s_p) (Kubanç *et al*., 2007). In *H. bosniaca*, it follows the general pattern of development, although we interpret instar A-4 as having the apical claw *Gp* and seta s_p (as suggested by its shape and subapical position) and not claws *Gp* and *Ga* as suggested for other species (Smith & Martens, 2000).
- The CS is present in all stages of *H. bosniaca* except A-9, while *H. bogotensis* (Roessler, 1983) did not show this seta in instars A-9, A-1 or adults, and in *H. incongruens* it was only possible to confirm CS in instars from A-7 to A-5 (Schreiber, 1922). In the Darwinuloidea, the CS is present from instar A-6 to A-2 (Smith & Kamiya, 2008). Little has been reported regarding CS in other studies.

Although some structures appear in *H. bosniaca* earlier than in other species of the genus (as for instance setae *α* and *β* on Md or seta *d1* on L6 when compared to *H. salina* (Kubanç *et al*., 2007)), others appear later (the second *Zahnborste* on Mx), or do not appear at all (aesthetasc y_1 on An2 or tubercles and pustules on the right valve margin). Similarly, *H. bosniaca* maintains throughout its development until adulthood some structures that would disappear in other congeneric species, including the CS (compared to *H. incongruens* and *H. bogotensis*) or the segmentation of the L5 (Tab. 2), which to our knowledge is the first time to be reported in adult Cyprinotinae. This segmentation is anyway present in adult individuals of other Cypridocopina, including *Propontocypris* sp. (see Fig. 2 in Kaji, 2010) and possibly also the cypridoidean *Pseudocandona eremita* (Vejdovský, 1882) (see Fig. 73 in Meisch, 2000).

The comparison of the appearance pattern of the limbs (Tab. 1), and some of their most important features (Tab. 2) during development MIGHT indicate that *H. bosniaca* is the more phylogenetically distant *Heterocypris* of the species analysed, whose adult shares some unique traits with *H. reptans* (Kaufmann, 1900) (reduction of marginal pustules on right valve and relatively short natatory setae; see Meisch, 1993) although *H. bosniaca* is a good swimmer compared to this species. Some of the ontogenetic development features of *H. bosniaca*, especially the maintenance of the segmentation of the L5 and the lack of tubercles and pustules (which are considered a diagnostic trait of the genus, but are also lacking in juveniles), together with a narrow calcified inner lamella on both valves (a typical juvenile trait in cypridoidean ostracodes) might be considered paedomorphic characters compared to the rest of *Heterocypris*. However, we would need to compare the time differences in somatic and reproductive growth with other species, to have some idea whether this paedomorphosis is originated by neotenic or progenetic processes (Ridley, 2004). Another feature to notice in this species is the change in position of *Zahnborste* between instars A-6 and A-5, which has not been described previously. This peculiarity could be an autapomorphy since it is not described in other ostracodes and provides valuable information that could be compared with future ontogenetic studies.

As we have shown, it is remarkable to notice that the stage A-9 has only been described in four ostracode species hitherto *H. bogotensis*, *Chlamydotheca incisa*, *C. unispinosa*, (Roessler, 1982a, 1982c, 1998), and now in *H. bosniaca*. Consequently, a detailed knowledge of the morphology of this early stage in other species could be very helpful to better understand the ontogeny of Cyprididae and the phylogenetic relationships among podocopid ostracodes and between ostracodes and other crustaceans (Roessler, 1998).

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Resumen global

Resumen ampliado

Los ostrácodos son un grupo de crustáceos con amplia distribución mundial. Su cuerpo está envuelto por dos valvas laterales y no suelen superar los 2 mm de longitud. *Heterocypris bosniaca* (Petkowski *et al.*, 2000) es una especie de aguas dulces temporales que se descubrió por primera vez en Bosnia el año 2000 y posteriormente también se encontraron poblaciones en Israel en el año 2002. Por tanto se conoce muy poco acerca de su biología, ontogenia y gran parte de su morfología. En el presente trabajo se cita por primera vez este ostrácodo en la Península Ibérica **(Capítulo I)**. En el Racó de Rius (Rafelguaraf, València, España) se pueden encontrar decenas de pequeñas pozas de roca (la mayoría con Ø < 1 m) sobre sustrato calizo, donde las únicas poblaciones Ibéricas conocidas de *Heterocypris bosniaca* fueron encontradas durante los muestreos realizados entre 2004 y 2006.

Debido a su pequeño tamaño, aislamiento e imprevisibilidad, las pequeñas pozas temporales presentan alto estrés ambiental y comunidades pobres en especies que se han adaptado a esas condiciones. Entre las adaptaciones especiales de los invertebrados que viven en estos hábitats se incluyen la tolerancia a la desecación y un desarrollo ontogenético rápido que les permite mantener poblaciones estables y hacer frente al elevado riesgo de extinción. En la zona de estudio se han encontrado 120 pozas excavadas en los afloramientos de roca caliza. Habitualmente se llenan con las lluvias otoñales, permanecen así durante el invierno y se secan en primavera. De todas las pozas muestreadas, 35 contienen poblaciones con elevadas densidades de *H. bosniaca*.

Tras conocer la distribución de la especie, se eligieron dos de esas pozas para hacer un seguimiento de sus poblaciones. Para ello se monitorearon semanalmente desde que se encontraron a principios de diciembre de 2004 hasta su desecación a finales de enero de 2005 **(Capítulo I)**. En cada visita se midieron una serie de variables ambientales y se tomaron muestras de agua y ostrácodos. Debido a la elevada densidad de *H. bosniaca* en las pozas seleccionadas, se recogieron los ostrácodos con un recipiente de plástico de 100 mL y 5 cm de diámetro el cual se arrastró por el sedimento aproximadamente 3-5 cm. Mediante este método se consiguieron grandes cantidades de individuos y se minimizó el impacto que las capturas pudieran tener en estos ambientes tan reducidos. Mediante el uso del estereomicroscopio, todos los ostrácodos recogidos fueron contados y asignados a estadíos de crecimiento para conocer los cambios en la población. Al mismo tiempo, algunos juveniles fueron criados aisladamente en el laboratorio para medir el crecimiento de los estadíos y conocer mejor la variación morfológica de las valvas a través del desarrollo larvario. Las medidas de longitud tomadas de estos animales permitió establecer los límites de tamaño entre los estadíos, lo cual resultó particularmente útil para clasificar los individuos que se recogieron del campo. Con este estudio se consiguió investigar las condiciones ambientales, la dinámica poblacional, el crecimiento de los estadíos y la historia vital de la especie. Durante el final de este ciclo hidrológico se encontró que la especie era tolerante a las condiciones de hipoxia. También se pudo observar que había individuos en el fango que parecían resistir eventos de desecación durante cierto tiempo.

Las pozas también fueron monitoreadas durante el principal hidroperiodo entre el otoño de 2005 y el verano de 2006 para comprobar la capacidad de las poblaciones partenogenéticas de *H. bosniaca* para hacer frente a los cambios ambientales a lo largo de un hidroperiodo impredecible **(Capítulo II)**. Aunque la metodología utilizada fue la misma que en el hidroperiodo anterior, teniendo en cuenta las observaciones previas sobre resistencia a la desecación, también se recogieron muestras de individuos enterrados en el substrato una vez se secaron las pozas. A lo largo de éste segundo ciclo hidrológico, se observaron varios periodos reproductivos, generalmente precedidos por una desecación y seguidos por una hidratación del substrato y dilución por el agua de lluvia. Los cambios demográficos observados indican que las poblaciones de *H. bosniaca* son capaces de persistir en ambientes intermitentemente inundados y producir varias generaciones por ciclo hidrológico anual. La velocidad de crecimiento de la especie varió a lo largo de los hidroperiodos, aparentemente por efecto de los cambios en la temperatura del agua. Además, los individuos adultos eran capaces de sobrevivir en el barro húmedo de las pozas secas durante más de cinco semanas.

Con el fin de evaluar la influencia de la humedad del sustrato en la supervivencia de los ostrácodos sometidos a un proceso de desecación **(Capítulo II)**, se llevaron a cabo cultivos de laboratorio con ostrácodos bajo dos tratamientos experimentales, uno con tasa de desecación rápida y otro con desecación lenta, además de un grupo control sin pérdida de agua. Los datos experimentales sugieren un menor tiempo medio de supervivencia cuando se exponen a procesos de desecación, y que la velocidad de pérdida de agua del substrato es un factor determinante para la tasa de supervivencia de los ostrácodos sometidos a eventos de desecación en las pozas temporales. Como muestran los estudios de los ostrácodos de ambientes acuáticos temporales sometidos a eventos impredecibles de desecación, una estrategia combinada de tolerancia de los adultos durante cortos periodos de escasez de agua y una rápida eclosión de los bancos de huevos puede ser ventajoso para la monopolización de pequeños hábitats efímeros.

La regla del tamaño-temperatura (en inglés *temperature-size rule:* TSR) es un fenómeno bien establecido que describe la respuesta del crecimiento de los ectotermos a la temperatura, por la cual los individuos que se mantienen a bajas temperaturas crecen más lentamente pero alcanzan mayor tamaño en la madurez. Aunque hay teorías adaptativas y no adaptativas sobre la plasticidad térmica del tamaño corporal, estas no se pueden aplicar a todos los ectotermos. Tras las variaciones en la velocidad de desarrollo observadas a lo largo de los hidroperiodos monitoreados anteriormente, se pretendió examinar las tasas de crecimiento y desarrollo del ostrácodo *H. bosniaca* a cuatro temperaturas diferentes (15ºC, 20ºC, 25ºC y 30ºC) controladas en el laboratorio durante las nueve fases de desarrollo **(Capítulo III)**. También se monitorearon hembras adultas partenogenéticas para evaluar los efectos de la temperatura y de la manipulación en la fecundidad. Los individuos se mantuvieron aislados en placas multipocillo y se revisaron diariamente para conocer su estado y buscar las exuvias que dejan tras la muda. Los resultados muestran que la tasa de desarrollo de la especie se acelera con un incremento de la temperatura, alcanzando el máximo valor a los 25-30ºC. El factor de crecimiento presenta una TSR inversa en estadíos juveniles, mientras que la típica TSR se establece en las dos últimas mudas. La fecundidad disminuye con la temperatura, y una mayor manipulación se traduce en una menor fecundidad y supervivencia de las hembras adultas. Estos resultados están de acuerdo con recientes investigaciones realizadas con crustáceos, las cuales muestran que hay un rango de temperatura óptimo para la producción de huevos y que la TSR puede variar durante la ontogenia, aparentemente en relación con el crecimiento somático frente la maduración de los órganos reproductores.

El creciente desarrollo de las sociedades en todo el mundo ha contribuido a un aumento en la contaminación global en términos de cantidad y variedad de contaminantes. Aunque las pequeñas pozas temporales no están tan directamente expuestas a la contaminación como otras masas de agua, debido a su reducido tamaño son muy vulnerables a los contaminantes porque pueden alcanzar concentraciones muy elevadas. Para realizar una evaluación completa de los efectos de los contaminantes en el medio ambiente se requiere el uso de un conjunto amplio de organismos de ensayo. Los crustáceos han sido ampliamente utilizados en ambientes acuáticos, pero dentro de este grupo, los ostrácodos han sido pasados por alto, a pesar de que son uno de los grupos de animales más comunes en aguas temporales. Dentro de este contexto, se realizaron bioensayos estáticos de toxicidad aguda con los metales pesados Cd y Pb, el tensioactivo SDS y esporas de la bacteria *Bacillus thuringiensis* var. *israelensis* usando hembras partenogenéticas adultas de *H. bosniaca* **(Capítulo IV)**. El experimento se realizó con 11 concentraciones diferentes de cada contaminante, a una temperatura constante de 20ºC y un fotoperiodo de 12:12 h. Cada cultivo se observó diariamente hasta la muerte de todos los individuos. En cada evento de observación se contó el número de individuos inmóviles y de muertos. Los resultados de las pruebas de toxicidad se expresan como LC_{50} y EC_{50} a 48 y 96 horas utilizando el análisis probit. Como se esperaba, con los metales pesados y con el SDS la especie respondió presentando efectos negativos en proporción a la concentración de contaminante en el cultivo. Aunque solo a largo plazo, la presencia de esporas de la bacteria siempre tuvo efectos negativos en la supervivencia, incluso en las menores concentraciones utilizadas. Este resultado es sorprendente porque la mayoría de estudios no muestran efectos negativos sobre organismos que no sean diana de las esporas. La sensibilidad de *H. bosniaca* a 48 h disminuyó en el siguiente orden Cd>Pb>SDS>*Bti*, mientras que el Pb y SDS intercambian sus posiciones a 96 h. La comparación de los valores de LC_{50} para esta especie con la información disponible en la literatura revela que en general es más resistente que otros crustáceos.

A pesar de ser la familia de ostrácodos más común y extendida en agua dulce, los Cyprididae son todavía poco conocidos con respecto a la ontogenia de las especies constituyentes. La serie completa de ocho estadíos juveniles ha sido descrita en detalle para sólo cinco de las aproximadamente 1000 especies actuales, y sólo en un caso se incluyó el primer estadío A-9. El objetivo fue llenar este vacío con un estudio morfológico del desarrollo postembrionario de *H. bosniaca* **(Capítulo V)**, especie con inusuales características morfológicas dentro del género, incluyendo caracteres putativamente pedomórficos tales como valvas sin tubérculos y una lamela interna calcificada estrecha en ambas valvas. En el interior del huevo, la poco conocida etapa A-9 (prenauplio) se desarrolla e inicia el proceso de muda. Tras la rotura del huevo, el estadío juvenil A-8, todavía dentro del exoesqueleto de la etapa A-9, surge pasivamente del huevo y se termina el proceso de muda a un estadío A-8 libre en menos de dos minutos. La etapa prenaupliar A-9 tiene dos pares de extremidades; el primer par son las anténulas, mientras que el segundo se corresponde al grupo fusionado de antenas y mandíbulas, observado mediante una comprobación de homología por formación. Aparentemente esta es la primera vez que tal fusión del segundo y tercer par de apéndices se describe en los crustáceos. Los siguientes estadíos juveniles (del A-8 al A-1) y la etapa adulta de *H. bosniaca* siguen un patrón de crecimiento similar al del resto de los Cyprididae, y particularmente cercano al de los otros miembros del género, pero notablemente diferentes en el desarrollo de los *Zahnborsten* maxilulares y la segmentación del endopodio de la quinta extremidad.

Discusiones y perspectivas

El estudio de pequeñas masas de agua temporales ha ido aumentando a lo largo de los últimos años debido a que los científicos se han percatado de la importancia que tienen estos ambientes (Jocque *et al*., 2010). Son focos de biodiversidad y resultan muy útiles para entender el funcionamiento de los ecosistemas acuáticos debido a su relativa sencillez comparados con otros sistemas de mayor tamaño en el que las interacciones entre organismos se complican mucho más (Brendonck *et al*., 2010). En general, el mayor problema que presenta su estudio es el reducido tamaño, lo cual conlleva que no suelan aparecer en los mapas topográficos, así que resulta difícil conocer su existencia y ubicación en una zona a no ser que se realicen salidas en el momento que puedan contener agua y por tanto es difícil estimar su verdadera abundancia. En la zona de estudio, tras varias expediciones exploratorias, se han llegado a encontrar y catalogar hasta 120 pequeñas pozas temporales (ver Capítulo II) distribuidas en una superficie de 65 ha, aunque se intuye que podrían existir muchas más en los alrededores.

La amplia presencia de *H. bosniaca* y el tiempo transcurrido desde su primera observación, nos indican que la especie está bien establecida en la zona. La ausencia de machos nos podría indicar que se trata de un área de reciente expansión de la especie en términos evolutivos o geológicos a partir de poblaciones más orientales (Petkowski *et al*., 2000; Martens *et al*., 2002). Aunque debido a la escasez de estudios disponibles relacionados con estos ambientes, la especie podría tener una expansión mucho mayor de lo que se conoce hasta el momento, e incluso podrían existir poblaciones sexuales cercanas a la zona de estudio pero que todavía permanecen desconocidas. Este hecho queda patente puesto que recientemente se han conseguido muestras de pozas temporales de Carcaixent y Alfarb (Valencia) y en Eivissa (Baleares) en las que se han encontrado individuos de *H. bosniaca*, aunque de momento todas hembras. Por lo tanto son necesarios nuevos muestreos en ambientes similares de otras zonas para conocer la expansión real de la especie y sería interesante realizar estudios genéticos con las diferentes poblaciones para conocer mejor el origen geográfico y su estructura genética. La dispersión a larga distancia podría ser un evento azaroso y relativamente poco frecuente en esta especie quizás comparada con ostrácodos de ambientes más extensos en humedales (Figuerola & Green, 2002; Green & Figuerola, 2005), pero parece tener buenas habilidades dispersoras a nivel local, especialmente si tenemos en cuenta la capacidad que individuos adultos y subadultos tienen para resistir cortos eventos de desecación que les pueden servir para ir de una poza a otra ayudados por vertebrados, como podrían ser los jabalíes (Vanschoenwinkel *et al*., 2008).

Aunque las actividades antrópicas pueden acarrear graves efectos negativos en el medio ambiente, en la zona de estudio se han podido observar las consecuencias de las actuaciones de cazadores que permiten la continuidad de estos sistemas. Las pozas temporales son abrevaderos naturales para muchas especies sujetas a la cacería, así que los cazadores evitan su colmatación vaciándolas de sedimento y asegurando su continuación. El caso más claro observado es el de la poza X1, la cual, fue vaciada durante el hidroperiodo de 2006-2007, momento en el que el sedimento ocupaba un gran volumen y solamente cabían entre 4-6 cm de agua. Tras el vaciado, el volumen de agua libre fue mucho mayor, lo cual propició la eclosión y supervivencia de anostráceos, nunca antes observados en esta poza durante los muestreos realizados para estudiar las dinámicas poblacionales de *H. bosniaca*. Por otra parte, estas actividades humanas de caza han podido propiciar la contaminación (por ejemplo con munición de plomo) de algunas de las charcas. Podría plantearse en un futuro realizar análisis de este u otros contaminantes que pudieran afectar a las poblaciones naturales de invertebrados en estos sistemas tan vulnerables.

En los estudios de la dinámica poblacional de este tipo de ambientes tan reducidos resulta complicado obtener suficientes organismos en las muestras como para que sean representativos del ambiente en general sin destruir el hábitat. Gracias a las elevadas densidades a las que se encuentra *H. bosniaca* en las pozas y al método de muestreo utilizado, se consiguieron muestras bastante representativas sin ocasionar un daño aparente en las charcas. Durante el estudio de la dinámica poblacional en las pozas, se observó que la única especie dominante fue *H. bosniaca* y no hubo sucesión de especies tal y como se muestra en muchos otros trabajaos (McLay, 1978; Lahr *et al*., 1999). Parece ser que las adaptaciones de la especie a estos ambientes impredecibles junto con la capacidad de utilización de los recursos y la elevada densidad de individuos provocan que no haya espacio ecológico para que se establezcan otras especies competidoras, y mucho menos que logren desplazarla. Seria pues interesante llevar a cabo prospecciones en charcas cercanas con mayor tamaño o más recursos para ver si hay posibilidad de coexistencia con otras especies (como ocurre en charcas mayores; Martins *et al*., 2009), así como experimentos de competencia con grados variados de densidad de varias especies de ambientes temporales para ver si se produce exclusión competitiva a partir de cierto tamaño o nivel de recursos del hábitat.

Algunas de las nuevas incógnitas que han surgido a lo largo del trabajo se han conseguido investigar y se han obtenido resultados esclarecedores. Por ejemplo, durante el primer estudio de la dinámica poblacional se observaron individuos que podían resistir eventos de desecación, así que se realizó el estudio de la segunda dinámica poblacional teniéndolo en cuenta y realizando un experimento en el laboratorio para comparar con los resultados de campo. Con este estudio posterior quedó clara la posibilidad de aguante de individuos adultos y juveniles en el substrato húmedo pero sin agua libre durante varias semanas, lo que sugiere que esta estrategia puede ser más habitual de lo que se pensaba en ostrácodos y otros organismos, aunque algunos estudios ya apuntaban esta posibilidad (Delorme & Donald, 1969; Horne, 1993). En la segunda dinámica poblacional se observaron diferentes velocidades de crecimiento a lo largo del hidroperiodo y por tanto se realizaron cultivos en el laboratorio a temperaturas controladas para saber si la temperatura era la causante de la diferente velocidad de desarrollo y también conocer los efectos sobre el tamaño y fecundidad. En éstos cultivos se observaron algunas eclosiones y en los cultivos de huevos se vieron unas membranas transparentes y se quiso indagar la cuestión. En la bibliografía se encontró que se podía tratar de un estadío bastante desconocido (el prenauplio o A-9; Roessler, 1982a, 1982b), así que se realizaron cultivos y disecciones de huevos para conocer su morfología. Queda por ver si este estadío está realmente presente en todos los miembros de la familia Cyprididae o al menos del género *Heterocypris*, a lo que apuntan nuevos datos inéditos, y las observaciones iniciales de Roessler (1998).

Respecto a los estudios ecotoxicológicos, cabe destacar la elevada resistencia a ciertos contaminantes, aunque quizás lo más sorprendente es que las esporas de la bacteria *Bti* siempre afectan la supervivencia de la especie a largo plazo, incluso a muy bajas concentraciones. Este hecho puede tener graves implicaciones en aquellos lugares en los que se use este tipo de tratamiento para controlar las plagas de mosquitos, puesto que se podría ver afectada la comunidad de ostrácodos e incluso de otros crustáceos, aunque hasta el momento no se han descrito efectos adversos en otros organismos no diana del *Bti* (Duchet *et al.*, 2010; Caquet *et al.*, 2011). Serían necesarios nuevos estudios con comunidades completas y en poblaciones naturales para poder entender mejor los efectos sobre el medio (Nayar *et al.*, 1999).

Una característica sorprendente de la especie, observada al estudiar su ontogenia, fue la presencia de un *Zahnborste* en el segundo endito maxilular únicamente durante el estadío A-6, el cual desaparece y surge uno nuevo en el tercer endito tras la muda al siguiente estadío. Hasta el momento no había ningún trabajo en el que se describiera esta característica y en un primer momento llevó a pensar que se había cometido algún tipo de error en algún punto del procesado de los individuos. Gracias a la preparación de un espécimen en fase de muda se pudo corroborar el proceso mediante chequeo de homología por formación, es decir, se observaron las nuevas estructuras formadas en el interior de la cutícula del estadío anterior y de esa forma no hubo dudas de que el cambio de posición era real. Este hecho fue sorprendente, y también muestra la efectividad de los estudios de ontogenia cuando se pueda usar individuos en el proceso de muda que faciliten la observación del origen de ciertos rasgos característicos. Pero una de las partes más importantes de este estudio fue el redescubrimiento de la fase eclosionadora en los ostrácodos. Al principio se realizaron muchos intentos de diseccionar un huevo mediante una gran variedad de técnicas, aunque en ningún caso se obtuvieron buenos resultados. Posteriormente, tras conocer con mayor detalle la técnica utilizada para decapsular quistes de anostráceos (Sorgeloos *et al*., 1977), se decidió probarla con los huevos de ostrácodo puesto que también están cubiertos por una doble membrana, la más externa calcificada. A lo largo del tratamiento con hipoclorito la capa más externa del huevo se volvía transparente y se podía observar la membrana interna separada de la externa por una capa de líquido. Al final del tratamiento, la membrana externa se desprendía y en los casos de huevos en avanzado estado de desarrollo, el hinchamiento de la RPB provocaba la rotura de la membrana interna y la expulsión de la fase eclosionadora. El estudio de esta fase del desarrollo resulta muy interesante no solo en el contexto de la ostracodología, sino también en el estudio de los crustáceos en general debido a las implicaciones filogenéticas y evolutivas que podrían tener.

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Conclusiones generales

1. Las pozas de roca caliza encontradas en la zona del Racó de Rius (Rafelguaraf) albergan de forma frecuente densas poblaciones partenogenéticas del ostrácodo *Heterocypris bosniaca* (Petkowski *et al*., 2000). Estas pozas son muy similares en cuanto a sus condiciones ambientales, siendo la variabilidad en el hidroperiodo la mayor diferencia observada. Las fluctuaciones en la conductividad y composición iónica pueden explicarse por procesos de desecación (aumento de los valores) y dilución por las lluvias.

2. La especie tiene poblaciones estables en estas pozas y es capaz de producir tanto huevos de resistencia (puesto que se conoce su presencia en las pozas desde 1998 y tiene que soportar las fuertes desecaciones estivales) como subitáneos (porque a lo largo del hidroperiodo aparecen nuevas cohortes de individuos sin desecación previa de las pozas y en los cultivos individualizados se produjeron eclosiones de huevos recientes sin desecación de los pocillos).

3. A lo largo de los principales hidroperiodos la especie muestra un patrón de historia vital bivoltino o multivoltino y pueden llegar a estar desarrollándose simultáneamente varias cohortes. La velocidad de crecimiento de cada cohorte puede ser muy diferente en épocas distintas, probablemente por la influencia principal de la temperatura sobre el crecimiento individual. Se observan fuertes patrones de eclosión tras la desaparición de la lámina de agua libre en épocas con pocas precipitaciones y el posterior rellenado por lluvias.

4. Tanto el contenido total como la velocidad de pérdida de agua en el substrato son factores determinantes para la supervivencia de los ostrácodos enterrados en el sedimento. La capacidad de resistir ciertos periodos de desecación por parte de individuos desarrollados los convierte en posibles dispersores gracias a vectores como las aves o mamíferos. Esta característica podría explicar el porqué *H. bosniaca* parece tener buena capacidad dispersora, al menos a pequeña escala local, puesto que se encuentra ampliamente distribuido en la zona.

5. La temperatura tiene un efecto directo sobre la velocidad de crecimiento de *H. bosniaca* en cultivos de laboratorio. El desarrollo se acelera con el aumento de la temperatura, aunque esta aceleración también disminuye con la temperatura, dando lugar a que a los 25-30ºC se alcance la máxima velocidad de desarrollo.

6. El incremento de tamaño entre mudas no sigue el mismo patrón a lo largo del desarrollo en respuesta a la temperatura, con lo que la longitud de los individuos adultos no se corresponde con los resultados previstos por la regla del tamaño-temperatura (TSR). En consecuencia no hay una respuesta monotónica del tamaño frente a la temperatura.

7. La fecundidad de *H. bosniaca* responde a la temperatura (disminuye con el aumento de la temperatura) mientras que no muestra relación lineal con el tamaño de la hembra adulta.

8. La manipulación durante las observaciones de laboratorio tiene efectos negativos significativos sobre la supervivencia y fecundidad de las hembras cultivadas de forma aislada.

9. Con los dos metales pesados (Cd, Pb) testados y el SDS *H. bosniaca* responde presentando efectos negativos de mortalidad e inmovilidad en proporción a la concentración del contaminante en los cultivos. A largo plazo, la presencia de *Bti* tiene efectos negativos incluso a bajas concentraciones. Por lo tanto, al contrario de lo que generalmente se considera, las esporas de la bacteria tienen un impacto negativo en este crustáceo.

10. *Heterocypris bosniaca* es generalmente más resistente que otros invertebrados acuáticos frente a los contaminantes testados (Cd, Pb, SDS), con una tolerancia al menos un orden de magnitud mayor que su congénere *H. incongruens*. Esta mayor resistencia puede ofrecerle ventajas frente a otros invertebrados en la colonización de nuevos ambientes.

11. Aplicando el protocolo para decapsular quistes de anostráceos en huevos de *H. bosniaca* se ha conseguido observar su interior y obtener eclosiones artificiales que han permitido el estudio en detalle de aspectos morfológicos durante el proceso de eclosión. También se ha corroborado la función esencial que tiene el exoesqueleto del prenauplio (A-9) en dicho proceso.

12. Mediante comprobaciones de homología por formación realizados en la fase del desarrollo necesaria para la eclosión (estadío A-8 formado en el interior de la exuvia del A-9), se ha podido observar que el prenauplio (A-9) presenta las antenas y mandíbulas fusionadas en un único par de apéndices.

13. Se considera que la primera fase del desarrollo es un prenauplio porque solo presenta dos pares de extremidades no funcionales. La siguiente fase de desarrollo se debe considerar ortonauplio porque posee tres pares de apéndices bien desarrollados, aunque presente los *pre-Anlagen* de un nuevo par de extremidades.

14. *Heterocypris bosniaca* presenta caracteres pedomórficos (mantenimiento de una débil segmentación en la quinta pata, falta de tubérculos y pústulas en las valvas y una lamela interna calcificada estrecha en ambas valvas) y autapomórficos ("cambio de posición" del *Zahnborste* maxilular entre los estadíos A-6 y A-5) que junto al resto de características morfológicas durante el desarrollo indican que se trata del cipridido filogenéticamente más distante dentro del género *Heterocypris* de los que se dispone su ontogenia.

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Anexos

En formato de anexo electrónico se proporcionan dos programas ejecutables en sistema operativo Windows (con formato .exe) que sirven como apoyo visual a ciertas explicaciones de la Tesis. Tienen un tamaño no redimensionable de 1000 x 650 píxeles. Para acceder al contenido hay que hacer doble clic sobre el icono correspondiente mientras que con la tecla escape podemos salir en cualquier momento. En la página de inicio de cada aplicación se proporciona información sobre su utilización. También se proporciona el vídeo (con formato .avi) obtenido en la grabación de una de las eclosiones que se comentan en el capítulo V.

Anexo 1: Zona de estudio

La aplicación es un mapa interactivo de la zona de estudio en la que podemos ver la posición de las pozas en el área donde se ha estudiado la ecología de la especie *Heterocypris bosniaca* y algunas características del terreno. También dispone de imágenes de todas las pozas encontradas con algunas de sus características.

Anexo 2: Morfología de *Heterocypris bosniaca*.

En la aplicación se puede navegar de forma interactiva entre las imágenes y dibujos de la morfología de *H. bosniaca* mostradas en el capítulo V. Las imágenes se muestran en un gran tamaño y sirven como ayuda durante la lectura de las descripciones.

Anexo 3: Eclosión de un huevo de *Heterocypris bosniaca*.

Vídeo de 10 minutos y 27 segundos en el que se pueden ver nadando varios individuos pertenecientes al estadío A-8 y la eclosión de uno de los huevos que se pueden ver al fondo. El primer indicio de rotura del huevo ocurre en el minuto tres aproximadamente, mientras que la fase eclosionadora no ha salido completamente del huevo hasta pasado el minuto nueve. Aproximadamente en el minuto 10 y 19 segundos es cuando el individuo rompe la cutícula de la fase A-9 y sale nadando libremente el estadío A-8.

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Esta Tesis empezó su andadura hace tanto tiempo que ya casi ni recuerdo sus inicios. Parecía que nunca llegaría el final, pero aquí estoy, escribiendo el último apartado. Ahora es momento de echar la vista atrás y recordar a todos aquellos que han hecho posible este trabajo. No soy una persona muy dada a este tipo de discursos, y en cada capítulo aparece una sección de agradecimientos con aquellas personas e instituciones que más han aportado, intelectual y económicamente, a los trabajos concretos. De todos modos, siempre hay mucha gente que no se puede nombrar por falta de espacio en las revistas y ésta es mi oportunidad, aunque seguro que alguien se me queda en el tintero y espero que no se moleste.

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