Ecologia d'ostracodes simbionts (Entocytheridae) de carrancs invasors a Europa

Tesi doctoral

2014

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Ecology of symbiotic ostracods (Entocytheridae)

inhabiting invasive crayfish in Europe

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Ecologia d'ostracodes simbionts (Entocytheridae) de carrancs invasors a Europa

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La imatge de la portada està composada a partir de la foto d'un carranc de riu americà *Pacifastacus leniusculus* i una foto al microscopi electrònic (feta per Burkhard Scharf) d'una còpula d'ostracodes entocitèrids pertanyents a l'espècie *Uncinocythere occidentalis*, la qual s'ha trobat associada a poblacions exòtiques europees de *P. leniusculus* en aquest treball.

Tesi presentada per **Alexandre Mestre Pérez** per optar al grau de Doctor en Biologia per la Universitat de València.

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Tesi dirigida pels doctors

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Firmat: Francesc Mesquita Joanes   Firmat: Juan S. Monrós González

Aquest treball ha estat finançat per un projecte del Ministeri de Ciència i Innovació (ECOINVADER, CGL2008-01296/BOS) i una beca predoctoral ("Cinc Segles") de la Universitat de València.

A ma mare,
a mon pare
i al meu germà

Agraïments

Em considere molt afortunat i estic molt agraït d'haver gaudit, durant el llarg camí d'aprenentatge que representa la tesi, d'unes condicions excel·lents per poder desenvolupar aquest treball. En primer lloc, he tingut dos directors, Francesc Mesquita i Juan Monrós, que han sabut conduir-me, baix el meu punt de vista, d'una manera molt encertada, marcant-me molt bé el camí a seguir i, al mateix temps, donant-me marge de llibertat per desenvolupar idees pròpies. He d'agrair als dos la confiança que han tingut en mi, sobretot Juan que, des de que el conec, sempre m'ha recolzat en la investigació, i em va motivar per iniciar la tesi. També vull agrair els coneixements que m'han aportat els dos sobre totes les fases de la investigació i el seu esforç per donar a aquest treball la millor qualitat possible. En referència al darrer aspecte, he de destacar a Francesc Mesquita que, amb molta paciència, m'ha ajudat a resoldre infinitats de dubtes i m'ha ensenyat a ser millor investigador. I què dir dels companys del departament? Laia Zamora, Olivier Schmit, Josep Antoni Aguilar, Andreu Escrivà, Andreu Castillo, Lluis Valls, Carla Olmo, Maria Antón, Juan Rueda, Jose Antonio Gil-Delgado, Antonio Sanz, Javi Armengol, Romina Liberto i Susana Romo; tots ells m'han ajudat d'alguna o altra manera, en tasques de mostreig o de laboratori, consells tècnics, proveïment d'espai de treball o material, recolzament anímic o bé, simplement, m'han fet la vida laboral en el departament més plaent amb la seua companyia, gràcies a tot(e)s!. Per la seua implicació més directa, vull agrair especialment a Josep Antoni Aguilar, Jose Antonio Gil-Delgado, Juan Rueda i Andreu Castillo la seua contribució a la tesi. Ma tia, Alexandra Pérez, que és una artista, em va ajudar amb el disseny de la portada.

Vull donar les gràcies als meus dos tutors d'estada a la LSU i a la Universitat de Sheffield, per haver-me acollit i haver-me ajudat en tot el que vaig necessitar: Bill Kelso and Roger Butlin, thank you very much! També agraesc als tècnics del TAB LAB de Sheffield, Andy Kruppa i Gavin Horsburgh, la seua gran ajuda i assessorament, d'ells vaig aprendre moltíssim. He de fer menció a tota la gent que va contribuir a la meua adaptació a la vida en Sheffield: Juan Galindo, Maria

José Rivas, Yung Wa Sin, Camille Jan, Nicolas Santkin, Ricardo Romero, Daniel Leocadio, Pawel Lee, Julián Merino, Virginia Loza, Mauricio Carter, Paz Durán, Steeves Buckland, Charilaos Pilides, Emma Morley i Laura Brettell. I el mateix he de dir de la gent de Baton Rouge: Robert Romaire, Mike Kaller, Will Sheftal i Allison McKiernan, Chris Bonvillain, Kevin kyle, Charlie Brown, Noel Novelo, Kouassi Da Costa, Rafael Cuevas, Mauricio Toscano, Washington da Silva (and the rest of members of my soccer team)... moltes gràcies a tot(e)s vosaltres.

Una cosa que m'ha sorprès molt gratament de la tesi ha sigut la predisposició que ha tingut molta gent d'altres institucions a col·laborar en la nostra investigació: David Baldry, Husamettin Balkis, Joan Carles Casanova, Adam Ellis, Karsten Grabow, Göran Klobučar, Antonín Kouba, Sergi López, Ivana Maguire, Andreas Martens, Aysegül Mülayim, Alex Ribas, Burkhard Scharf, Menno Soes. Moltes gràcies a tot(e)s vosaltres. Agraesc, també, el suport dels governs de les comunitats autònomes de Andalucía, Extremadura, Castilla La Mancha, Castilla León, Navarra, Aragó, Illes Balears, Catalunya i el Pais Valencià, a l'hora de proporcionar-nos els permisos necessaris per mostrejar, al ministeri de Ciència i Innovació, per haver contribuït al finançament d'aquest treball, a través del Projecte ECOINVADER (CGL2008-01296/BOS), i a la Universitat de València, per la beca predoctoral que em va concedir.

Finalment, vull concloure aquest apartat, com no, destacant el suport de la meua família, que m'han demostrat sempre estar al meu costat.

Resum

Els entocitèrids són una família d'ostracodes (Crustacea), majoritàriament d'origen americà, ectosimbionts (bàsicament comensals) d'altres crustacis, principalment decàpodes d'aigua dolça. Les darreres dècades, els carrancs natius europeus s'han vist perjudicats per la invasió de carrancs exòtics, degut a fenòmens d'exclusió competitiva i transmissió de simbionts parasitaris. En la present tesi, hem avaluat la presència d'entocitèrids associats a carrancs de riu (natius i exòtics) a Europa. Hem contribuït a sentar unes bases bibliogràfiques i metodològiques per a l'estudi dels entocitèrids. S'ha analitzat l'especificitat d'hoste d'Entocytheridae i el patró latitudinal de riquesa de les espècies americanes del grup. També hem estudiat els factors abiòtics i biòtics que afecten la distribució i els paràmetres poblacionals d'un entocitèrid exòtic. Finalment, hem testat la transmissió d'entocitèrids exòtics a carrancs de riu europeus sota condicions experimentals. Per primera vegada, hem registrat la presència a Europa de dues espècies d'entocitèrids exòtics (*Ankylocythere sinuosa* i *Uncinocythere occidentalis*) associats a carrancs de riu invasors d'origen americà (*Procambarus clarkii* i *Pacifastacus leniusculus*). Hem aportat una base de dades georeferenciada d'escala global d'accés lliure i gratuït per a la comunitat científica, els llistats actualitzats de les espècies i la bibliografia del grup, i informació pràctica per l'establiment de protocols de mostreig d'entocitèrids. Hem pogut constatar que l'especialització en una única espècie d'hoste no és majoritària en els entocitèrids. D'altra banda, els entocitèrids americans mostren un patró inhabitual de distribució de riquesa d'espècies amb la latitud, probablement, relacionat amb el vincle evolutiu amb els seus hostes. El nostre model de simbiont, *A. sinuosa*, suposadament no parasitari, s'ajustà a tres patrons generals en ecologia de poblacions de macroparàsits, suggerint que algunes regles poblacionals establertes en paràsits podrien ser regles més generals dels simbionts. Les poblacions exòtiques d'*A. sinuosa* es veieren marcadament afectades per les condicions climàtiques i limnològiques, mostrant que alguns grups particulars de simbionts poden ser especialment sensibles a la variabilitat ambiental, per exemple els ectosimbionts d'aigua dolça no parasitaris, com els entocitèrids. D'acord amb els nostres resultats, l'expansió d'un simbiont exòtic com *A. sinuosa* podria veure's afectada per filtres espacials restrictius deguts a limitacions climàtiques del simbiont, els quals implicarien la pèrdua del simbiont en aquelles àrees envaïdes per l'hoste afectades pels filtres. Finalment, hem confirmat la transmissió de l'entocitèrid exòtic, *A. sinuosa*, a un carranc de riu natiu europeu, sota condicions experimentals. Per tant, seria convenient avaluar experimentalment el grau d'amenaça real per a la conservació del carranc europeu que suposaria l'esdeveniment en cas d'ocórrer en la natura.

Resumen

Los entocitéridos son una familia de ostrácodos (Crustacea), mayoritariamente de origen americano, ectosimbiontes (básicamente comensales) de otros crustáceos, principalmente decápodos de agua dulce. Durante las últimas décadas, los cangrejos de río nativos europeos se han visto perjudicados por la invasión de cangrejos exóticos, debido a fenómenos de exclusión competitiva y transmisión de simbiontes parasitarios. En la presente tesis, hemos evaluado la presencia de entocitéridos asociados a cangrejos de río (nativos y exóticos) en Europa. Hemos contribuido a sentar unas bases bibliográficas y metodológicas para el estudio de los entocitéridos. Se han analizado la especificidad de hospedador de los Entocytheridae y el patrón latitudinal de riqueza del grupo. También hemos estudiado los factores abióticos y bióticos que afectan a la distribución y los parámetros poblacionales de un entocitérido exótico. Finalmente, hemos testado la transmisión de entocitéridos exóticos a cangrejos de río europeos bajo condiciones experimentales. Por primera vez, hemos registrado la presencia en Europa de dos especies de entocitéridos exóticos (*Ankylocythere sinuosa* y *Uncinocythere occidentalis*) associados a cangrejos de río invasores de origen americano (*Procambarus clarkii* y *Pacifastacus leniusculus*). Hemos aportado una base de datos georeferenciada de escala global, de acceso libre y gratuito para la comunidad científica, los listados actualizados de las especies y la bibliografía del grupo, e información práctica para el establecimiento de protocolos de muestreo de entocitéridos. Hemos podido constatar que la especialización en una única especie de hospedador no es mayoritaria en los entocitéridos. Por otro lado, los entocitéridos americanos muestran un patrón inusual de distribución de riqueza de especies con la latitud, probablemente, relacionado con el vínculo evolutivo con sus hospedadores. Nuestro modelo de simbionte, *A. sinuosa*, supuestamente no parasitario, se ajustó a tres patrones generales en ecología de poblaciones de macroparásitos, sugiriendo que algunas reglas poblacionales establecidas en parásitos podrían ser reglas más generales de los simbiontes. Las poblaciones exóticas de *A. sinuosa* se vieron marcadamente afectadas por las condiciones climáticas y limnológicas, mostrando que algunos grupos particulares de simbiontes pueden ser especialmente sensibles a la variabilidad ambiental, como por ejemplo los ectosimbiontes de agua dulce no parasitarios (e.g. los entocitéridos). De acuerdo con nuestros resultados, la expansión de un simbionte como *A. sinuosa* podría verse afectada por filtros espaciales restrictivos debidos a limitaciones climáticas del simbionte, los cuales implicarían la pérdida del simbionte en aquellas áreas invadidas por el hospedador afectadas por los filtros. Finalmente, hemos confirmado la transmisión del entocitérido exótico, *A. sinuosa*, a un cangrejo de río nativo europeo, bajo condiciones experimentales. Por tanto, sería conveniente evaluar experimentalmente el grado de amenaza real para la conservación del cangrejo europeo que supondría el suceso en caso de ocurrir en la naturaleza.

Abstract

Entocytherids are an ostracod family (Crustacea), mainly native to America, ectosymbionts (basically commensal) on other crustaceans, chiefly freshwater decapods. During the last decades, native European crayfish have been threatened by the invasion of exotic crayfish, due to competitive exclusion and transmission of pathogenic symbionts. In this work, we checked the presence of entocytherids associated with crayfish (native and exotic) in Europe. We contributed to establish bibliographic an methodological bases for research focused on entocytherids. We analysed host specificity in Entocytheridae and the latitudinal pattern of species richness in American entocytherids. We also assessed abiotic and biotic factors affecting the distribution and population parameters in an exotic entocytherid. Finally, we tested the transmission of an exotic entocytherid to a European native crayfish under experimental conditions. For the first time, we recorded the presence of two exotic entocytherid species (*Ankylocythere sinuosa* and *Uncinocythere occidentalis*) associated with American invasive crayfish (*Procambarus clarkii* and *Pacifastacus leniusculus*). We provide a global-scale georeferenced database of Entocytheridae, freely available to the scientific community, the updated species and bibliographic checklists of the group, and practical information for the establishment of standardised protocols for entocytherid sampling. We showed that the specialization in a unique host is not the rule in Entocytheridae. On the other hand, American entocytherids had an unusual latitudinal pattern of species richness, probably related with the tight evolutionary link with their hosts. Our symbiont model, *A. sinuosa*, supposedly non-parasitic, fitted three general patterns in population ecology of macroparasites, suggesting that some population rules established for parasites could actually be more general rules of symbionts. The exotic populations of *A. sinuosa* were markedly affected by climatic and limnological conditions, showing that some particular symbiont groups can be specially sensitive to environmental variability, for example freshwater non-parasitic ectosymbionts, such as the entocytherids. According to our results, the geographical expansion of an exotic symbiont as *A. sinuosa* could be affected by restrictive spatial filters as a consequence of climatic limitations of the symbiont, which would imply the symbiont loss in those areas invaded by the host and affected by the filters. Finally, we confirmed the transmission success of an exotic entocytherid, *A. sinuosa*, to a European native crayfish, under experimental conditions. Therefore, we consider convenient to experimentally assess the actual degree of threat for crayfish conservation that would suppose the event in case of occurrence in nature.

Índex

Introducció

La present tesi neix a raó de la detecció d'un nou grup d'organismes exòtics a Europa per part d'un grup d'investigadors liderat pel Departament de Microbiologia i Ecologia de la Universitat de València. Els organismes al·ludits havien romàs probablement durant dècades desapercebuts per la comunitat científica europea degut a la seues peculiar forma de vida i mida diminuta. Pertanyen a una família (Entocytheridae) d'ostracodes ectosimbionts[1] d'altres crustacis, és a dir, viuen en estreta associació amb l'exoesquelet de crustacis de major mida que exerceixen d'hostes[2].

Ecologia de simbionts en les invasions biològiques

Els simbionts, és a dir organismes "que viuen junts" amb altres (els seus hostes) sense implicacions de cost o benefici (Sapp, 1994), representen un estil de vida reeixit, donat el seu ample rang taxonòmic i la seua ubiqüitat. De fet, la vida associada a un hoste aporta avantatges importants als simbionts, com és protecció front als depredadors, estabilitat ambiental i de disponibilitat de recursos o bé un augment de la capacitat dispersiva (Athias-Binche & Morand, 1993; Hairston Jr & Bohonak, 1998; Poulin, 2007b; Fernandez-Leborans, 2010). La seua importància en ecologia és evident, ja que poden promoure canvis en l'hàbitat i distribució de les espècies (Poulin & FitzGerald, 1989; Zamora-Vilchis et al., 2012), regulen les dinàmiques poblacionals dels seus hostes (Combes, 2001) i exerceixen influència sobre l'estructura de les comunitats i els processos dels ecosistemes (Hay et al., 2004). També juguen un paper destacat en biologia de les invasions (Torchin et al., 2003; Prenter et al., 2004; Roy & Handley, 2012) i han sigut responsables de seriosos problemes de conservació (Strauss et al., 2012).

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[1] Simbionts que habiten les regions externes de l'hoste

[2] Organismes capaços de sustentar el desenvolupament i reproducció d'un simbiont

Ecologia de poblacions de metazous simbionts: patrons i factors

Degut a la importància dels simbionts metazous en ecologia i conservació, s'ha fet una intensa recerca en aquest camp, a fi de trobar models de poblacions i dinàmiques de transmissió de simbionts (Anderson & May, 1978; May & Anderson, 1978; McCallum et al., 2001; Torgerson et al., 2003). També s'ha investigat a fons amb l'objectiu de reconèixer patrons generals (Shaw & Dobson, 1995; Poulin, 2007a; Morand & Krasnov, 2008) i factors (Whiteman & Parker, 2004; Readel et al., 2008) que afecten els seus paràmetres poblacionals. No obstant, la gran majoria d'esforç s'ha enfocat en simbionts parasitaris, principalment d'hostes vertebrats (Poulin, 2007b). En canvi, malgrat que existeixen estudis sobre simbionts no parasitaris (Damborenea, 1998; Pennuto et al., 2002; Galván & Sanz, 2006; Fernandez-Leborans, 2010; DeWitt et al., 2013), aquests han sigut generalment poc tractats.

Els estudis d'ecologia de simbionts han evidenciat l'existència de patrons en els paràmetres poblacionals que apareixen de forma molt freqüent en diferents grups. El patró més general és l'agregació de les abundàncies[1], és a dir, la gran majoria d'hostes d'una població tenen molts pocs simbionts i sols uns pocs hostes acumulen grans quantitats de simbionts (Poulin, 2013). Els orígens dels patrons agregats poden ser molt variats, i estar relacionats amb diferències entre hostes en la susceptibilitat de rebre o mantenir els simbionts, preferències dels propis simbionts per uns tipus d'hostes determinats, heterogeneïtat espacial en la distribució dels estadis infectius dels simbionts, etc. (Poulin, 2007b). Un altre patró observat, relacionat amb aquest, és l'estreta relació entre l'abundància de simbionts i la seua variància, expressats en forma de logaritmes (Shaw & Dobson, 1995). Per explicar aquest darrer patró, els parasitòlegs es basen en l'existència de mecanismes reguladors característics de les infrapoblacions[2] de paràsits, com és la mortalitat de l'hoste induïda per un excés de simbionts (Shaw & Dobson, 1995). També s'ha observat una correlació positiva entre la taxa d'ocupació d'hostes[3] i l'abundància de simbionts, en altres paraules, els simbionts més abundants aconsegueixen ocupar una major proporció d'hostes de la població (Morand & Guégan, 2000). El darrer patró és una versió particular de l'amplament documentada relació entre l'abundància local i la distribució geogràfica dels organismes de vida lliure (Gaston, 1996; Gaston et al., 1997).

En referència als factors que afecten els paràmetres poblacionals dels simbionts, aquests poden ser agrupats en: (1) característiques de l'hoste i (2) condicions

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[1] L'abundància és el nombre mitjà de simbionts per hoste en una població de simbionts

[2] Nombre total d'individus d'una espècie de simbiont que viuen en un hoste

[3] Percentatge d'hostes amb simbionts respecte al total d'hostes de la població

ambientals. En general, hi han dos característiques de l'hoste que solen ser importants: la grandària del cos i la densitat poblacional. Hostes més grans solen proveir de més recursos espacials i alimentaris a la infrapoblació de simbionts que mantenen (Poulin, 2013). A més, poblacions d'hostes més denses faciliten la transmissió de simbionts degut a la major proximitat entre individus (Anderson & May, 1978). Respecte al segon cas, existeixen excepcions quan el simbiont presenta taxes reproductives o de transmissió més baixes que les reproductives i de dispersió de l'hoste. En aquests casos, un percentatge dels nous hostes nascuts o dispersants poden no arribar a ser colonitzats pel simbiont (Stanko et al., 2006), o bé es pot produir un "efecte de dilució" de les abundàncies dels simbionts com a resultat de les colonitzacions d'una nova cohort d'hostes (Krasnov et al., 2007). Pel que fa a les condicions ambientals, poden afectar la fecunditat del simbiont o la seua supervivència indirectament a través de l'efecte sobre l'hoste (Kadlec et al., 2003) o directament (DeWitt et al., 2013). A una escala temporal intraanual, l'estacionalitat de les abundàncies de simbionts és freqüent, especialment en sistemes aquàtics, i pot estar lligada a fluctuacions en les condicions ambientals, com ara la temperatura i la salinitat (Tucker et al., 2001), encara que algunes vegades és resultat d'ajusts del simbiont a la dinàmica poblacional de l'hoste (Blanco & Frías, 2001). Alguns treballs suggereixen que les condicions ambientals externes a l'hoste tenen un paper minoritari comparades amb les característiques de l'hoste o del propi simbiont (Krasnov et al., 2005; Poulin, 2006). Aquesta afirmació casa bé amb la hipòtesi de l'estabilitat ambiental i de disponibilitat de recursos com a benefici de la vida simbiont.

La gran majoria de dades que suporten els patrons anteriorment descrits i el paper minoritari de les condicions ambientals a l'hora de modular les poblacions de simbionts corresponen a simbionts parasitaris d'hostes vertebrats. L'ús de models alternatius de simbiont ajudarien a comprovar fins a quin punt es poden generalitzar aquests fenòmens a tots els simbionts i a comprendre millor els processos implicats.

Simbionts invasors

Les interaccions biòtiques són fonamentals per entendre els fenòmens de les invasions (Peterson et al., 2011). Les distribucions de les espècies estan determinades per tres grups de factors: abiòtics, biòtics i dispersius (Soberón & Peterson, 2005). En aquest context, els models de nínxol ecològic (MNEs) són ferramentes estadístiques que ens permeten predir les distribucions de les espècies (Thuiller et al., 2005). Generalment estan basats en l'ús de factors abiòtics, usualment condicions climàtiques, com a predictors, de manera que el que s'aconsegueix com a resultat és un mapa geogràfic de les àrees de disponibilitat climàtica de l'espècie analitzada. Els MNEs han sigut aplicats a la biologia de les invasions per tractar de predir les àrees de risc d'invasió per part d'una espècie exòtica invasora (e.g. Reshetnikov & Ficetola, 2011). Un dels grans problemes que presenta l'aplicació de MNEs a l'anàlisi dels processos d'invasió és que, en la immensa majoria de casos, es desestimen els factors biòtics i s'obtenen prediccions a partir de models basats en la relació entre la distribució de l'espècie i les condicions climàtiques en el seu rang d'origen. Però hem de tenir en compte que les interaccions amb altres espècies com la depredació, competència, mutualisme, o relacions simbiont-hoste també influeixen sobre la distribució d'una espècie. I aquestes interaccions poden sofrir modificacions entre el rang natiu, d'on s'obtenen les dades, i el rang invasor, objecte de les prediccions (Elith & Leathwick, 2009). El rang invasor pot estar lliure d'espècies competidores, depredadors o simbionts que controlaven la distribució de l'espècie invasora en el seu rang natiu, de manera que la seua expansió en el nou rang es veu afavorida. L'anomenada hipòtesi de "l'alliberament d'enemics" (Keane & Crawley, 2002) atribueix l'èxit invasor d'una nova espècie introduïda a la pèrdua d'enemics naturals del seu rang natiu durant les etapes inicials de la invasió.

Els simbionts juguen un paper important en la biologia de les invasions, fins el punt que alguns autors recorren a la hipòtesi de l'alliberament d'enemics aplicada als simbionts per explicar l'èxit de les espècies invasores. Torchin et al. (2003) van demostrar, per a un ample rang taxonòmic d'espècies animals, que les poblacions natives mantenen major riquesa d'espècies i majors abundàncies de paràsits que les poblacions exòtiques. La pèrdua pot ser deguda a la baixa probabilitat que els pocs espècimens introduïts continguen tota la riquesa de paràsits natius de l'espècie, o a l'extinció primerenca d'algunes espècies de paràsits després de l'establiment de l'hoste. Com que els paràsits poden produir reduccions de grandària de cos i de densitat poblacional dels seus hostes, la pèrdua de paràsits té un efecte d'alliberament demogràfic de l'espècie invasora, la qual esdevé plaga. Els efectes dels simbionts sobre els processos d'invasió poden arribar a ser molt complexes i tenir amples implicacions per l'estructura de la comunitat envaïda, interferint sobre les interaccions de l'espècie exòtica amb les natives de l'àrea receptora, com ara la competència o la predació (Prenter et al., 2004). Un dels perills potencials de les espècies invasores es troba en la biota simbiont associada als seus cossos que viatja amb elles i pot interactuar amb espècies natives de la regió envaïda afavorint, en alguns casos, l'èxit invasor a través de l'efecte nociu del simbiont exòtic sobre espècies natives possibles competidores de l'hoste invasor. La transmissió de simbionts exòtics a noves espècies d'hoste natives de l'àrea envaïda és una amenaça potencial per a la conservació (Strauss et al., 2012). Els hostes novells, els quals no estan evolutivament adaptats a tindre el nou tipus de simbiont, poden veure's seriosament perjudicats per la nova associació (Mastitsky et al., 2010). Un exemple molt ben documentat d'efecte d'un simbiont exòtic sobre la conservació de la biodiversitat el tenim en la plaga del carranc de riu natiu europeu (Alderman, 1996).

Europa acull sis espècies autòctones de carranc de riu. Algunes d'elles han sofert severes reduccions de les seues poblacions en les darreres dècades. Una de les principals causes ha sigut la introducció, establiment i expansió de carrancs exòtics a Europa durant els darrers 50 anys (Holdich, 2002). Els carrancs invasors han perjudicat al natiu europeu a través de fenòmens de competència interespecífica i transmissió de simbionts patògens. Particularment nociva ha sigut la plaga del carranc de riu, també anomenada afanomicosi, produïda per un Oomycota parasitari (*Aphanomyces astaci*) natiu de Nord Amèrica i transportat a Europa per mitjà de carrancs exòtics americans, els quals són portadors i el toleren. Aquest paràsit produeix la mort del carranc natiu europeu i representa una greu amenaça per a la seua conservació. Actualment, almenys cinc espècies de carrancs exòtics gaudeixen de poblacions estables a Europa (Holdich, 2003). Com a conseqüència de l'impacte dels carranc exòtics, junt amb la contaminació i la pèrdua d'hàbitat, algunes de les espècies de carranc autòcton europeu es troben actualment molt amenaçades, fins al punt que apareixen en el llistat d'espècies en perill d'extinció de la UICN[1] i estan emparades per la legislació europea. Concretament, apareixen citades com a protegides en l'Apèndix III de la Convenció de Berna del 1979 i en els Apèndix II i V de la Directiva d'Hàbitats de la Unió Europea (Taylor, 2002). D'acord amb la Directiva d'Hàbitats, alguns països europeus s'han implicat en programes de seguiment poblacional i reintroducció per recuperar les espècies amenaçades. Així s'han pogut restablir noves poblacions de carrancs natius amb èxit en àrees on prèviament existien i havien sigut extints (Füreder et al., 2010).

Ostracodes entocitèrids com a models

El grup Ostracoda és un taxó ample de crustacis amb unes 13,000 espècies existents en l'actualitat, que inclou formes marines i d'aigua dolça. La particularitat més evident que els caracteritza és la presència d'una closca bivalva que protegeix els seu cossos i els assembla a cloïsses diminutes. Els ostracodes d'aigua dolça, composats per unes 2000 espècies i 200 gèneres, representen un dels grups d'invertebrats més abundants i ubics dels cossos d'aigua continentals, i formen part de la microfauna habitual de qualsevol bassal, sèquia o, inclús toll temporal format per les darreres pluges amb què ens puguem creuar. Malgrat això, no

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[1] Unió Internacional per a la Conservació de la Natura

resulten familiars a la major part de gent no especialitzada degut a que tot just arriben als pocs mil·límetres de longitud i, per tant, la seua observació detallada requereix l'ús d'estereomicroscopis.

Entocytheridae

La gran majoria d'ostracodes són organismes de vida lliure, amb excepció de la família Entocytheridae, íntegrament constituïda per ectosimbionts d'altres crustacis, a la qual ens hem referit a l'inici de la introducció. Els entocitèrids presenten una sèrie d'adaptacions a la vida sobre un hoste, com és una reducció de la seua grandària (no solen superar els 500 µm de longitud), la forma de les valves sol ser poc bombada per facilitar la mobilitat entre les fissures de l'exoesquelet de l'hoste i tenen estructures especialitzades de fixació a l'hoste en els extrems dels apèndixs locomotors. Respecte al seu efecte sobre l'hoste, la hipòtesi més acceptada és el comensalisme, és a dir, els entocitèrids no tenen ningun efecte (ni nociu ni beneficiós) sobre l'hoste (Hart & Hart Jr, 1974). Encara que existeixen alguns grups minoritaris d'entocitèrids que viuen sobre isòpodes i amfípodes, el 95 % de les espècies són ectosimbionts de carrancs de riu, i pertanyen a dues subfamílies: Entocytherinae i Notocytherinae. La família Entocytherinae és originària d'Amèrica del Nord on viu associada a carrancs de riu americans de la superfamília Astacoidea (natius de l'Hemisferi Nord). Per la seua banda, la família Notocytherinae habita els carrancs de riu Parastacoidea (Hemisferi Sud) d'Oceania. Els carrancs natius de l'Hemisferi Nord (Astacoidea) estan constituïts per dues famílies: Astacidae i Cambaridae. Les dues famílies presenten una distribució disjunta (Scholtz, 2002). La distribució nativa de Cambaridae comprèn Nord Amèrica i Àsia de l'Est, mentre que Astacidae presenta espècies natives en l'Oest de Nord Amèrica i Europa. Curiosament, no s'han descrit entocitèrids propis dels carrancs Astacidae natius europeus.

Informació existent sobre els entocitèrids

La creació de bases de dades de biodiversitat lliures per a la comunitat científica és una feina valuosa per estimular la investigació en ecologia i biogeografia. Entre altres repositoris, la Infraestructura Mundial d'Informació en Biodiversitat (GBIF) es un destacat recurs lliure i gratuït que ofereix accés a dades de biodiversitat de grups taxonòmics molt diversos d'ecosistemes terrestres i marins, incloent els ostracodes. També existeixen bases de dades georeferenciades específiques d'ostracodes (e.g. NODE[1], OMEGA[2]; Horne et al., 1998, 2011), algunes d'elles

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[1] Nonmarine Ostracod Distribution in Europe

[2] Ostracod Metadatabase of Environmental and Geographical Attributes

d'accés lliure, altres no. Tanmateix, els entocitèrids, els quals representen la tercera família d'ostracodes no marins (d'un total de 14) en nombre d'espècies, han sigut notablement subrepresentats en les bases de dades disponibles. Malgrat que podem trobar en la bibliografia especialitzada un gran nombre de publicacions que inclouen cites d'entocitèrids (e.g. Hobbs Jr & Walton, 1975, 1976; Hobbs Jr & Peters, 1977; Andolshek & Hobbs Jr, 1986), aquestes cites no estan incloses en la majoria de bases de dades de diversitat generals i específiques d'ostracodes, amb la sola excepció de NODE. A més, la citada base de dades està restringida a cites europees d'ostracodes mentre que hem de tenir en compte que les dues subfamílies principals d'entocitèrids (i.e. Entocytherinae i Notocytherinae) són natives d'Amèrica del Nord i Central i d'Australàsia (Hart & Hart Jr, 1974). D'altra banda, el GBIF només contenia, abans del nostre estudi, 44 registres d'entocitèrids, els quals corresponen principalment a localitats de mostreig dels espècimens conservats en col·leccions de museus (e.g. Smithsonian Museum of Natural History, Australian Museum). Un altre exemple de la reduïda atenció dedicada als entocitèrids per la majoria d'ostracodòlegs són les recents revisions d'ostracodes no marins del món, les quals només consideren els ostracodes de vida lliure (Martens et al., 2008; Martens & Savatenalinton, 2011), desestimant als entocitèrids, o bé tractant-los d'una manera molt superficial (Karanovic, 2012).

Justificació de l'ús dels entocitèrids com a models

Els entocitèrids han estat molt poc tractats pels biòlegs actuals. Malgrat que hi ha un volum important de literatura científica sobre aquesta família d'ostracodes, el seu període d'esplendor investigadora foren els anys 70, durant els quals alguns biòlegs feren una intensa labor de recerca d'entocitèrids a través de la revisió de mostres de carrancs de museus i de grans campanyes de mostreig de carrancs, sobretot en Nord Amèrica. El resultat fou la descripció d'un gran nombre de noves espècies d'entocitèrids i la recol·lecció d'un gran volum d'informació sobre la seua distribució. Tota aquesta informació repartida en forma de publicacions disperses fou concentrada en un treball de revisió de la família Entocytheridae per Hart & Hart Jr (1974). Després d'aquesta revisió, la recerca en forma de publicacions disperses continuà, però ja ningú ha tornat a produïr un treball integrador del grup i, poc a poc, la taxa d'aparició de publicacions sobre entocitèrids ha anat caient fins l'actualitat. El fet de ser una família atípica d'ostracodes, per ser l'única integrada totalment per espècies amb un estil de vida simbiont, que requereixen de mostrejos específics no del medi sinó dels seus hostes, probablement ha sigut la raó per la qual els entocitèrids no són considerats per la gran majoria d'ostracodòlegs. Tanmateix, la condició de simbionts els fa particularment atractius des de la perspectiva de l'estudi de la biologia de les interaccions ecològiques. Els entocitèrids són ectosimbionts no parasitaris de crustacis d'aigua dolça, un model molt poc tractat en ecologia de simbionts metazous, on predominen els estudis basats en simbionts parasitaris de vertebrats. A més, la presència d'entocitèrids exòtics a Europa associats a carrancs invasors, alguns dels quals representen els macroinvertebrats amb major èxit invasor del planeta (com és el cas de *Procambarus clarkii*), aporta un nou interès cap als entocitèrids des del punt de vista de la biologia de les invasions. Finalment, l'ús dels entocitèrids com a model de simbiont invasor adquireix un plus d'importància en el context de la conservació de la biodiversitat per raons biogeogràfiques, ja que els carrancs natius europeus, objectes de greus problemes de conservació, en part relacionats amb processos d'invasió, han estat històricament lliures d'entocitèrids. Aquest fet els podria fer vulnerables a un hipotètica recepció dels entocitèrids exòtics, un fenomen del qual no tenim dades sobre la seua probabilitat de produir-se ni de les seues possibles conseqüències en cas de succeir.

Objectius

Com hem dit, recentment s'ha registrat la presència d'entocitèrids exòtics a Europa. Els objectius principals de la present tesi són:

1. Avaluar la presència d'entocitèrids en diferents espècies de carrancs de riu (exòtics i natius) a escala europea.
2. Contribuir a establir unes bases metodològiques i de disponibilitat de recursos d'informació per a l'estudi dels entocitèrids.
3. Analitzar l'especificitat d'hoste d'Entocytheridae i el patró latitudinal de riquesa de les espècies americanes del grup.
4. Comprovar si els paràmetres poblacionals d'un ectosimbiont comensal de crustacis d'aigua dolça (i.e. els entocitèrids) segueixen els patrons generals observats en altres grups de simbionts (i.e. paràsits de vertebrats).
5. Analitzar, a nivell espacial i temporal, els factors abiòtics (clima i química de l'aigua) i biòtics (relacionats amb característiques de l'hoste) que afecten els paràmetres poblacionals d'un entocitèrid exòtic, i avaluar la seua importància relativa a l'hora de regular l'expansió del simbiont en el procés d'invasió.
6. Comprovar si és possible la transmissió d'entocitèrids exòtics des d'una espècie de carranc americà a un carranc de riu europeu sota condicions experimentals.

Breu descripció dels capítols

La tesi està presentada en forma de compendi d'articles prèviament publicats o acceptats. Únicament dos treballs estan en fase de revisió, pendents d'acceptació (in review) o de ser enviats a una revista (in prep.). Aquest és el llistat d'articles que conformen la tesi:

Capítol I. Aguilar-Alberola, J. A., Mesquita-Joanes, F., López, S., Mestre, A., Casanova, J. C., Rueda, J., & Ribas, A. (2012). An invaded invader: high prevalence of entocytherid ostracods on the red swamp crayfish *Procambarus clarkii* (Girard, 1852) in the Eastern Iberian Peninsula. *Hydrobiologia*, *688*, 63–73.

Capítol II. Mestre, A., Monrós, J. S., & Mesquita-Joanes, F. (2014). A review of the Entocytheridae (Ostracoda) of the world: updated bibliographic and species checklists and global georeferenced database, with insights into host specificity and latitudinal patterns of species richness. *Crustaceana*. In press.

Capítol III. Mestre, A., Monrós, J. S., & Mesquita-Joanes, F. (2011). Comparison of two chemicals for removing an entocytherid (Ostracoda: Crustacea) species from its host crayfish (Cambaridae: Crustacea). *International Review of Hydrobiology*, *96*, 347–355.

Capítol IV. Castillo-Escrivà, A., Mestre, A., Monrós, J. S., & Mesquita-Joanes, F. (2013). Population dynamics of an epibiont Ostracoda on the invasive red swamp crayfish *Procambarus clarkii* in a western Mediterranean wetland. *Hydrobiologia*, *714*, 217–228.

Capítol V. Mestre, A., Monrós, J. S., & Mesquita-Joanes, F. (in review). Factors affecting abundance and host occupancy of a non-parasitic freshwater ectosymbiont (Ostracoda, Entocytheridae) inhabiting the invasive crayfish *Procambarus clarkii*. *Freshwater Biology*.

Capítol VI. Mestre, A., Aguilar-Alberola, J. A., Baldry, D., Balkis, H., Ellis, A., Gil-Delgado, J. A., Grabow, K., Klobučar, G., Kouba, A., Maguire, I., Martens, A., Mülayim, A., Rueda, J., Scharf, B., Soes, M., Monrós, J., & Mesquita-Joanes, F. (2013). Invasion biology in non-free-living species: interactions between abiotic (climatic) and biotic (host availability) factors in geographical space in crayfish commensals (Ostracoda, Entocytheridae). *Ecology and Evolution*, *3*, 5237–5253.

Capítol VII. Mestre, A., Castillo-Escrivà, A., Rueda, J., Monrós, J. S., & Mesquita-Joanes F. (in prep). Experimental spillover into a novel endangered crayfish host.

L'establiment de l'ordre dels articles, que representen els capítols de la tesi, ha estat basat en criteris de contingut i no pas de caire cronològic, per tal de proporcionar una estructura lògica al conjunt del treball.

El capítol I mostra els resultats del primer mostreig d'entocitèrids simbionts de carrancs a escala europea. L'estudi se centra en el carranc roig americà *Procambarus clarkii* (Girard, 1852), introduït el 1973 al Sudoest de la Península Ibèrica per a la seua explotació amb fins alimentaris. Actualment, l'espècie està amplament estesa per gran part d'Europa, fent gala del seu gran potencial invasor. De fet, és l'espècie de carranc de riu invasor més reeixida, i ha arribat a envair moltes regions del món gràcies a tenir amples rangs de tolerància a condicions abiòtiques, un període per adquirir la maduresa sexual molt curt i gran capacitat dispersiva (Siesa et al., 2011). En aquest treball s'examinen més de 200 carrancs de riu *P. clarkii* procedents de 12 localitats distribuïdes per l'Est i Sud de la Península Ibèrica. Per primera vegada en Europa, s'utilitzen mètodes específics de mostreig d'entocitèrids per a la seua detecció, evidenciant la ampla presència anteriorment desconeguda d'una espècie d'entocitèrid exòtic americà associat a poblacions invasores ibèriques de *P. clarkii*.

El capítol II és un estudi bibliogràfic amb l'objectiu de cobrir algunes de les carències del coneixement actual de la família Entocytheridae. Amb aquesta finalitat, presentem una base de dades georeferenciada d'accés lliure i gratuït sobre presències de les diferents espècies d'entocitèrids a nivell mundial, publicada en GBIF. També revisem el coneixement actual del grup mitjançant una actualització del llistat d'espècies conegudes d'entocitèrids i de la seua bibliografia. A més, analitzem l'especificitat d'hoste del grup i el patró de riquesa d'espècies en Amèrica del Nord i Central.

El capítol III consisteix en un estudi experimental per testar dos mètodes per desprendre els entocitèrids dels seus hostes prèviament usats: clorobutanol (Young, 1971) i aigua en gas (capítol I). L'estudi avalua l'efectivitat del clorobutanol i l'aigua amb gas carbònic per extraure l'entocitèrid *Ankylocythere sinuosa* del carranc de riu *Procambarus clarkii*. L'espècie d'entocitèrid de l'experiment pertany a algunes de les poblacions exòtiques descobertes en l'estudi del capítol I. Vam avaluar l'efecte del tipus d'anestèsic emprat i el temps d'exposició al tractament sobre l'efectivitat en l'extracció d'entocitèrids, la supervivència del carranc i la dels entocitèrids alliberats. Amb això, aportem informació de caire metodològic útil per l'elecció de protocols estandarditzats d'extracció d'entocitèrids adaptats a les necessitats requerides pel tipus concret d'estudi a realitzar. La nostra pretensió és facilitar la investigació sobre aquest grup d'ectosimbionts molt poc estudiat.

Els dos capítols següents tracten l'ecologia de poblacions dels entocitèrids. En el capítol IV, fem una descripció de la dinàmica poblacional anual de l'entocitèrid *A. sinuosa* en una població de *P. clarkii* del Parc Natural de la Marjal Pego-Oliva (Est de la Península Ibèrica), a partir de dades obtingudes d'una campanya de mostrejos mensuals al llarg d'un any. Analitzem les principals variables que modulen els patrons demogràfics del simbiont. D'entre les variables considerades, contemplem condicions ambientals (e.g. temperatura i conductivitat de l'aigua) i característiques de l'hoste (i.e. grandària del cos, estat de muda i densitat poblacional). Prèviament només s'havia tractat la dinàmica poblacional anual d'aquestos entocitèrids una vegada (Young, 1971). A diferència de Young (1971), nosaltres realitzem un estudi molt més detallat i enfocat en aquest tema i, a més, ens basem en poblacions exòtiques del simbiont.

El capítol V aborda l'ecologia de poblacions dels entocitèrids des d'un punt de vista espacial. En aquest estudi, en primer lloc, veiem si *A. sinuosa* segueix els tres patrons observats en paràsits anteriorment esmentats. En segon lloc, avaluem la relativa importància de característiques de l'hoste (sexe, mida del cos, densitat i muda) front a condicions ambientals (clima i química de l'aigua) a l'hora d'influir sobre l'abundància i la taxa d'ocupació d'hostes d'*A. sinuosa*. L'estudi està basat en les poblacions exòtiques associades a *P. clarkii* de la Península Ibèrica. Amb això tractem temes d'interès general en ecologia de simbionts metazous des d'un punt de vista poc explorat, basant-nos en un model de simbiont atípic en la majoria d'estudis previs: un ectosimbiont de crustacis d'aigua dolça no parasitari.

En el capítol VI exposem una nova aproximació metodològica a l'anàlisi espacial d'un procés d'invasió, on incorporem factors biòtics, fent ús d'un model de simbiont invasor. En la nostra aproximació combinem un marc teòric basat en la teoria de conjunts junt amb l'ús dels MNEs per avaluar les interaccions entre factors abiòtics (i.e. clima) i biòtics (disponibilitat d'hoste) en l'espai geogràfic d'un simbiont exòtic. En primer lloc, realitzem el primer mostreig extensiu per avaluar la distribució d'entocitèrids en carrancs de riu a Europa. Mostregem un total de 94 localitats europees i 12 espècies de carranc de riu (incloent natius i exòtics). Amb les dades recollides del mostreig, junt amb aquelles provinents de la base de dades presentada en el capítol II, apliquem la nostra metodologia emprant als entocitèrids exòtics a Europa com a organismes model. En suma, mostrem una manera d'avaluar gràficament si l'expansió d'un simbiont podria veure's principalment restringida per les seues pròpies limitacions fisiològiques a les condicions climàtiques o, pel contrari, la seua capacitat invasora es veu frenada tan sols per la disponibilitat d'hoste. De les dues hipòtesis oposades amb un gradient de possibilitats intermèdies, en el primer cas, l'hoste invasor seria capaç d'arribar a envair àrees no adequades per al simbiont amb la conseqüent pèrdua d'aquest, mentre que, en la segona hipòtesi, el simbiont arribaria fins on ho fa l'hoste invasor al qual acompanya. Fent ús dels entocitèrids, discutim les possibles implicacions de les diferents hipòtesis en el procés d'invasió i la conservació de la biodiversitat.

Finalment, el capítol VII és un experiment on provem la transmissió d'un entocitèrid exòtic a un carranc natiu europeu amenaçat. El principal objectiu d'aquest darrer estudi és la comprovació de la transmissió de l'entocitèrid *A. sinuosa* del carranc invasor *P. clarkii* al natiu *A. italicus*, sota condicions experimentals. Els resultats de l'experiment ofereixen informació valuosa per saber si cap la possibilitat que es produïra una transmissió dels entocitèrids exòtics a poblacions salvatges de carranc natiu europeu. Pensem que és important tenir informació sobre aquesta qüestió per decidir si s'hauria de tenir en compte o no la possible transferència, ja que el procés duu associats riscos potencials per a la conservació del carranc de riu europeu. A més, proporcionem nova informació sobre característiques del mecanisme de transmissió dels entocitèrids.

En resum, la tesi mostra la presentació de la nova troballa d'entocitèrids exòtics a Europa com a punt de partida d'una investigació que inclou temes diversos relacionats amb metodologies específiques de mostreig d'ectosimbionts de crustacis, bases de dades de biodiversitat, ecologia de poblacions de simbionts metazous i biologia de les invasions i conservació d'espècies, mantenint un fil conductor clarament establert a través dels entocitèrids, amb un enfoc especial en aquelles espècies exòtiques recentment trobades en Europa.

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CAPÍTOL I

An invaded invader: high prevalence of entocytherid ostracods on the red swamp crayfish *Procambarus clarkii* (Girard, 1852) in the Eastern Iberian Peninsula

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Hydrobiologia, *688*, 63-73

Hydrobiologia (2012) 688:63–73
DOI 10.1007/s10750-011-0660-1

OSTRACODA – BIOSTRATIGRAPHY AND APPLIED ECOLOGY

An invaded invader: high prevalence of entocytherid ostracods on the red swamp crayfish *Procambarus clarkii* (Girard, 1852) in the Eastern Iberian Peninsula

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Abstract The American red swamp crayfish *Procambarus clarkii* (Girard, 1852) was introduced in 1973 into the Iberian Peninsula for commercial purposes. As a result of both the expansion from the Iberian Peninsula and, probably, further introductions in other European countries, now it is widely distributed throughout much of Europe. The ecological impacts of this invading crayfish have received increasing attention, but nothing is known about its symbiotic entocytherid ostracods outside the American continent. The present survey has examined more than 200 crayfishes from 12 localities distributed over a wide area of Eastern Spain. Entocytherid ostracods were extracted from individual crayfishes and they were identified, counted, assigned to developmental instars and sexed. In all the study locations but one, we found at least one crayfish individual infected by entocytherid ostracods and the species determined was the same in all cases: *Ankylocythere sinuosa* (Rioja, 1942). The number of ostracods on individual *P. clarkii* varied notably in relation to crayfish size and also differed significantly among sampling sites. The crayfish size effects on ostracod densities might be related to the amount of resources and to the crayfish age and moulting frequency affecting ostracod distribution and population structure. In addition, the spatial variation in ostracod densities could also be related to site-specific habitat traits and the variability of crayfish population dynamics. Our study represents the first citation of an alien entocytherid species in Europe and demonstrates its wide distribution in the Iberian Peninsula. Further research is needed to know the potential effects of this ostracod species on the ecology of *P. clarkii* and of native species, with implications on the management of this aquatic invader.

Keywords *Ankylocythere* · Ostracoda · Entocytheridae · Commensalism · Alien species · Biological invasion

Introduction

The red swamp crayfish, *Procambarus clarkii* (Girard, 1852), native to north-east Mexico and to

Guest Editors: D. A. Do Carmo, R. L. Pinto & K. Martens / Ostracoda – Biostratigraphy and Applied Ecology

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south-central United States, has a great invasion capacity that has enabled it to become the most cosmopolitan crayfish in the world. Its current distribution includes all the continents, except Australia and Antarctica (Huner, 1977, 2002; Gherardi, 2006). Its expansion has been favoured by humans, mainly because of its culinary value. In 1973, it was introduced into the southern Iberian Peninsula for aquaculturing purposes (Geiger et al., 2005). Later, the escapes of individuals from aquaculture plants and intentional releases allowed the red swamp crayfish to rapidly spread throughout the Iberian Peninsula (Gherardi, 2006). Both this expansion from the Iberian feral populations, probably added to multiple introduction events in other European regions (as in Italy; Gherardi, 2000), resulted in the current wide European distribution of *P. clarkii*.

The expansion of the red swamp crayfish has caused serious ecological problems in the invaded territories, including short-term reduction of biodiversity (Rodríguez et al., 2003; Gherardi & Acquistapace, 2007). Consequently, the red swamp crayfish has been the centre of numerous eradication research studies (Blake, 1995; Peay & Hiley, 2001; Stebbing et al., 2003; Peay et al., 2006; Aquiloni et al., 2009). Besides predation, competition and habitat modification, the negative impact of the red swamp crayfish on native biodiversity sometimes comes about through symbiotic biota associated with crayfish. The most striking case is the oomycete *Aphanomyces astaci*, infecting alien crayfishes (*Pacifastacus leniusculus*, *Orconectes sapidus*, *P. clarkii*) with minor effects on their populations, but which causes the death of the European native crayfishes through aphanomycosis disease (Gil-Sánchez & Alba-Tercedor, 2002).

Crayfishes are organisms with a wide variety of associated biota, including viruses, bacteria, fungi, protozoan and metazoan groups (Edgerton et al., 2002). The introduction of crayfishes into new environments could also facilitate the introduction of parasites and epibionts in those cases where associated organisms withstand the introduction process (Torchin et al., 2003). Despite some crayfish pathogen and symbiotic groups being subjected to intense research, including oomicetes such as *A. astaci* (e.g. Cerenius et al. 2003; Oidtmann et al., 2008; Dunn et al., 2009; Hochwimmer et al., 2009; Kozubikova et al., 2009; Vrålstad et al., 2009), or protists such as *Psorospermium haeckeli* (e.g. Rug & Vogt, 1994; Gydemo, 1996; Vogt, 1998; Vogt & Rug, 1999; Bangyeekhun et al., 2001), there are other more poorly studied groups. This is the case of Ostracoda belonging to the family Entocytheridae.

Entocytheridae are ostracods symbiotic (i.e. living together with) of other crustaceans (Decapoda, Amphipoda and Isopoda). There are around 180 species, 31 genera and 5 subfamilies known (Hart & Hart, 1974). Generally, they have low host specificity (Hart & Hart, 1974), and in the few cases of species with only one host known, the isolation of the host habitat (burrowing crayfish species) is seemingly remarkable (Young, 1971). Very little knowledge about these ostracods' feeding and symbiotic relationship with hosts is available, although some authors have suggested that they are commensals or, at least, facultative commensals (Hoff, 1942; Baker, 1969).

Among entocytherids, Entocytherinae are the largest subfamily, and the majority of species are symbionts of crayfishes belonging to the superfamily Astacoidea (Hart & Hart, 1974). Ostracods live in the setiferous areas and ventral crevices of the crayfish exoskeleton, and can invade the branchial chambers (Hobbs & Peters, 1977). Although entocytherids can survive for long periods (weeks and even months) outside the host, it seems that they require the host for reproduction and development (Baker, 1969; Young, 1971). Transfer between hosts does not require direct contact between them (Young, 1971).

There are some cases of alien entocytherid colonisation of new areas which were documented prior to the present survey, which is probably the case of the entocytherids in Hawaii (Hart & Hart, 1971) or the well-documented case of *Uncinocythere occidentalis* in Japan, introduced with *Pacifastacus leniusculus trowbridgii* from the United States between 1926 and 1930 (Smith & Kamiya, 2001). Prior to our study, however, the existence of alien entocytherid fauna in Europe was unknown and, besides, no Entocytherinae members have been cited in Europe. Studies on the ecological population traits of entocytherids are scarce and, in any case, the few there are focus on native populations (Young, 1971; Hart et al., 1985). With this work, we aimed to know whether the American crayfish *Procambarus clarkii*, a successful invader of European water bodies, carries alien entocytherids in its European range and, whether or not their populations have similar ecological patterns to those described by Young (1971) and Hart et al.

(1985) for native entocytherid populations in the United States. In this way, we aimed at extending the knowledge of this group of alien organisms whose ecological role remains unknown in European aquatic ecosystems.

Materials and methods

During the fieldwork, we collected 203 individual red swamp crayfishes from 12 localities widely distributed over the Iberian Peninsula, from Doñana (SW Spain) to Catalonia (NE Spain), between 2003 and 2009. Crayfishes were captured using bait traps and hand nets, and were kept in water to be transferred from the sampling site to the laboratory, where they immediately underwent the process to extract entocytherid fauna. To do that the protocol consisted in immersing each individual crayfish separately in a bath of carbonated water for 15 min. Then crayfishes were removed from the bath, measured (total length and weight), sexed and preserved in 70% ethanol. The carbonated water resulting from each individual crayfish bath was filtered through a small 30-μm mesh-sized net. Next the filtered residue was submerged in a vial with 96% ethanol and was stored in the laboratory until observation under a stereomicroscope. The entocyhterids found were determined at the species level by using mainly the monography of Hart & Hart (1974) and the paper of Peters & Pugh (1999). All ostracod individuals were counted and classified as belonging to one of these groups: adult and subadult males, adult and subadult females and remaining juveniles. We measured the carapace length and height of a sample of 30 individuals for each developmental instar and sex (stages were differentiated based on the presence-absence of different appendages).

In order to check the effectiveness of the carbonated extraction method, after more than 1 week of applying the filtering process explained above, we removed the individual crayfishes from the ethanol bottles where they were stored, and we filtered the ethanol from each bottle with the same net type as before. Finally, we counted and preserved the remaining entocytherids as above.

A linear regression analysis was done to test whether the total number of ostracods (the sum of the ostracods collected using the filtered carbonated water protocol plus the remaining ostracods obtained weeks later from the bottle where crayfishes were stored) could be calculated from the number of ostracods collected after following the initial extraction protocol. Both numbers of ostracod individuals where $\log_{10}$-transformed. In order to check for differences in the number of entocytherid ostracods per crayfish between populations, we carried out an analysis of covariance (ANCOVA) using the logarithm of crayfish weight as a covariate. For this analysis, we discarded the localities whose number of infected crayfishes was lower than 15, and we excluded crayfishes without ostracods. Consequently, only four samples were used for this analysis. Furthermore, we analysed the linear correlation between the number of adult entocytherids and the total number (adults plus juveniles) of entocytherids per crayfish. A paired *t* test was done to compare the number of ostracod males with the number of females per crayfish individual to test for deviations from a 1:1 male:female sex ratio. All the analyses were performed with the SPSSwin software, v.17.

Results

At all the localities sampled (Fig. 1; Table 1), with the exception of Marjal dels Moros and Cubillas, we collected more than one crayfish individual, with a maximum of 52 individuals in Pinedo. The only entocytherid species found in this study was *Ankylocythere sinuosa* (Rioja, 1942) after having inspected 203 crayfish individuals, of which 147 contained ostracods. The morphological aspect of the peniferous part of the hemipenis (Figs. 2, 3) resembled more that of *Ankylocythere tiphophila* (Crawford, 1959), considered a different species from *A. sinuosa* by Hart & Hart (1974). However, we followed the more recent evaluation of the taxonomic status of these two species by Peters & Pugh (1999), who considered *A. tiphophila* to be a junior synonym of *A. sinuosa*. Some individuals presented small flat rounded structures which were usually attached to the external surface of the valves (Fig. 2). These structures correspond to peritrichid ciliates belonging to Lagenophryidae, a group with species that are epibionts on crustaceans (Fernandez-Leborans & Tato-Porto, 2000; Fernandez-Leborans, 2009). Furthermore, bacteria-like structures were also observed over the external penis surface (Fig. 2). *A. sinuosa* individuals were present in all the

Fig. 1 Sites where *Procambarus clarkii* specimens have been collected in the Eastern Iberian Peninsula to be analysed for the presence of entocytherid ostracods. The numbers refer to the codes in Table 1

studied localities, except the Laguna de los Ojos de Villaverde site in Albacete. Furthermore, we found a 100% prevalence of entocytherids in crayfishes for all the sites with ostracods, except two: Pinedo (with nearly half the infected crayfishes) and Torreblanca where we could find only one infected crayfish. Taking into account crayfish size, the highest ostracod densities were found in Granada (Padul) and Oliva with more than 10 individuals per gram of crayfish fresh weight (Table 1).

A size-class (carapace length and height) inspection of *A. sinuosa* instars (Fig. 4), together with an examination of the soft parts of selected individuals (in agreement with Smith & Kamiya, 2005), allowed us to discriminate between seven juvenile instars of *A. sinuosa.* We found no sexual differences in the morphology of the five earliest juvenile instars, but sexual differences were obvious from the A-2 to the adult instar, as was the presence of more or less developed penises in males. When we compared the size of the three oldest instars between males and females (Fig. 4), we saw that the females' size increased in each moult at a higher rate than that for males. Consequently, the size of adult males (360 ± 4 µm long and 200 ± 6 µm high) was similar to that of the A-1 females (361 ± 6 µm long and 201 ± 7 µm high). Adult females were larger in size (421 ± 21 µm long and 239 ± 13 µm high). In most localities with entocytherids, we observed male–female ostracod associations that could represent either copulating pairs or males guarding immature females (Danielopol, 1977). In all sites but one, coupled (copulating or guarded) females were immature and belonged to instar A-1 ("biunguis females" according to Hobbs, 1971). The only exception was site Oliva, where we found both A-1 and A-2 coupled females (with an ~1.7:1 A-1:A-2 coupled females ratio), and only an exceptional case of one adult female coupled to a male ("triunguis females").

The total number of ostracods obtained from a crayfish related to the number of ostracods extracted from the first filtering process using carbonated water. A linear regression analysis showed a significant relationship as follows: $\mathrm{Log}_{10}(\text{Total Ostr}) = 0.19 + 0.944 * \log_{10}(\text{filtered ostracods})$; $R^2 = 0.975$, $F = 4937$, $P < 0.01$. Figure 5 shows the relationship between crayfish weight and the number of ostracods collected after filtering the carbonated water used for extracting entocytherids. Only the four samples containing more than 15 crayfishes with ostracods are shown, that is, the same samples as used for the ANCOVA analysis. Although the regression lines for these populations present different slopes (ranging from 0.48 for Pinedo to 1.3 for Zaragoza), the results of the ANCOVA test do not show significant differences among slopes (interaction term site × log crayfish weight, $F = 1.64$, $P = 0.18$). This allowed comparisons to be made of the (log-transformed) mean number

Table 1 Summary table of the main results of the study

Code	Locality	Habitat	Date	NC	C Weight (g)	C Length (cm)	Prevalence	NE/C	NE/g C	NE/cm C	% Extraction	sex ratio C	sex ratio E
1	Riera d'en Xunclà	River	Jul-08	12	10.1 ± 6.1	7.0 ± 1.2	100	11.5 ± 10.1	1.4 ± 1.2	1.7 ± 1.4	60	0.7	0.4
2	Riu Congost	River	Jun-08	11	15.9 ± 6.1	7.9 ± 1.1	100	109.7 ± 129.9	6.4 ± 6.5	13.5 ± 15.5	90	0.8	0.7
3	Riells del Fai	River	Jul-08	8	11.0 ± 4.3	7.4 ± 0.6	100	6.8 ± 4.5	0.7 ± 0.5	0.9 ± 0.6	65	1.0	1.3
4	Cartuja	Oxbow	Jan-08	11	11.2 ± 9.4	6.7 ± 2.8	100	71.5 ± 103.1	5.7 ± 3.5	8.3 ± 10.8	83	0.2	0.8
5	Cascarro	Pond	Jan-08	3	27.6 ± 18.0	9.5 ± 1.6	100	141.3 ± 160.6	4.1 ± 2.5	13.6 ± 14.5	68	F	1.0
5	Cascarro	Pond	Jan-09	19	14.1 ± 8.5	7.8 ± 1.3	100	190.1 ± 131.8	13.1 ± 6.5	22.5 ± 13.4	NA	0.4	0.9
6	Torreblanca	Wetland	Apr-08	29	29.9 ± 14.8	9.7 ± 1.8	3	0.2 ± 0.9	0.0 ± 0.1	0.0 ± 0.1	100	1.2	0.3
7	Marjal dels moros	Wetland	Jun-08	1	26 9	9.0	100	83.0	3.1	9.2	75	M	1.0
8	Pinedo	River	Dec-07	52	6.0 ± 5.4	5.5 ± 1.9	65	4.6 ± 7.4	0.5 ± 0.7	0.7 ± 1.0	49	0.8	1.1
9	Ojos de Villaverde	Lake	Dec-07	6	22.6 ± 4.5	8.7 ± 0.8	0	–	–	–	–	5.0	–
10	Cubillas	River	Jun-08	1	14.1	7.8	100	216.0	15.3	27.9	91	M	0.6
11	Padul	Wetland	Jun-08	33	18.0 ± 9.8	8.1 ± 1.4	100	331.3 ± 321.8	18.5 ± 12.4	38.1 ± 32.3	92	1.5	0.6
12	Doñana	Wetland	Jul-03	31	20.6 ± 8.0	7.7 ± 0.9	100	3.6 ± 20	0.2 ± 0.1	0.5 ± 0.3	NA	1.2	0.3
13	Oliva	Wetland	Mar-09	17	14.4 ± 1.7	8.3 ± 1.7	100	192.9 ± 153.5	14.3 ± 9.1	22 2 ± 15.1	NA	1.4	0.9

Codes (first column) refer to the numbers in Fig. 1. *N* number, *C* crayfish, *E* Entocytherid ostracods. Measurements and ratios are averages ± standard deviations. % Extraction refers to the proportion of ostracods extracted from the crayfishes by the carbonated water method. *NA* not available (in these cases, no total amount of ostracods is known because remaining individuals after applying the carbonated water method were lost). Sex ratios are the number of males / the number of females. – no data because of the absence of entocytherids on the crayfishes. In sex ratio C, *F* only females; *M* only males

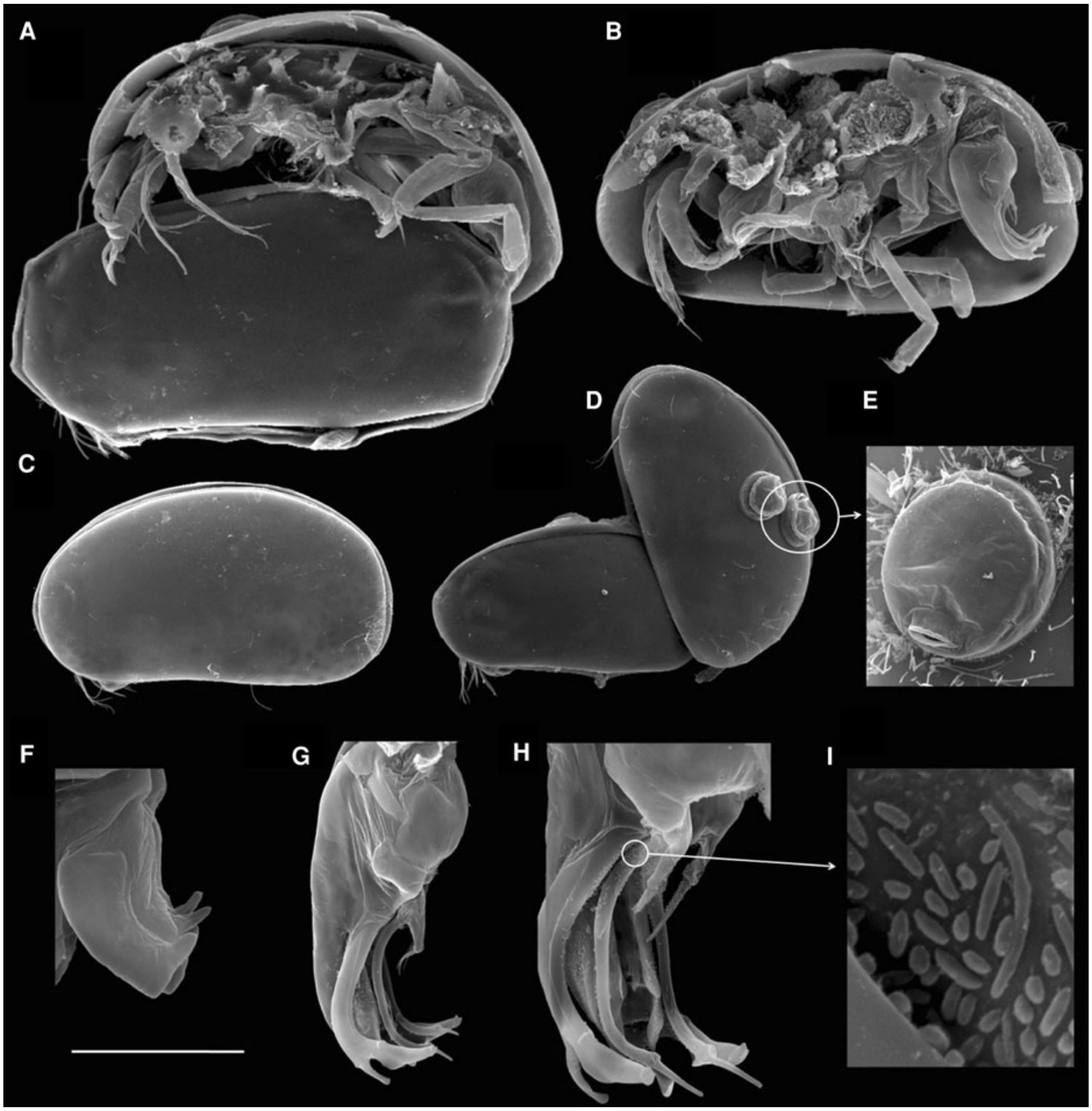
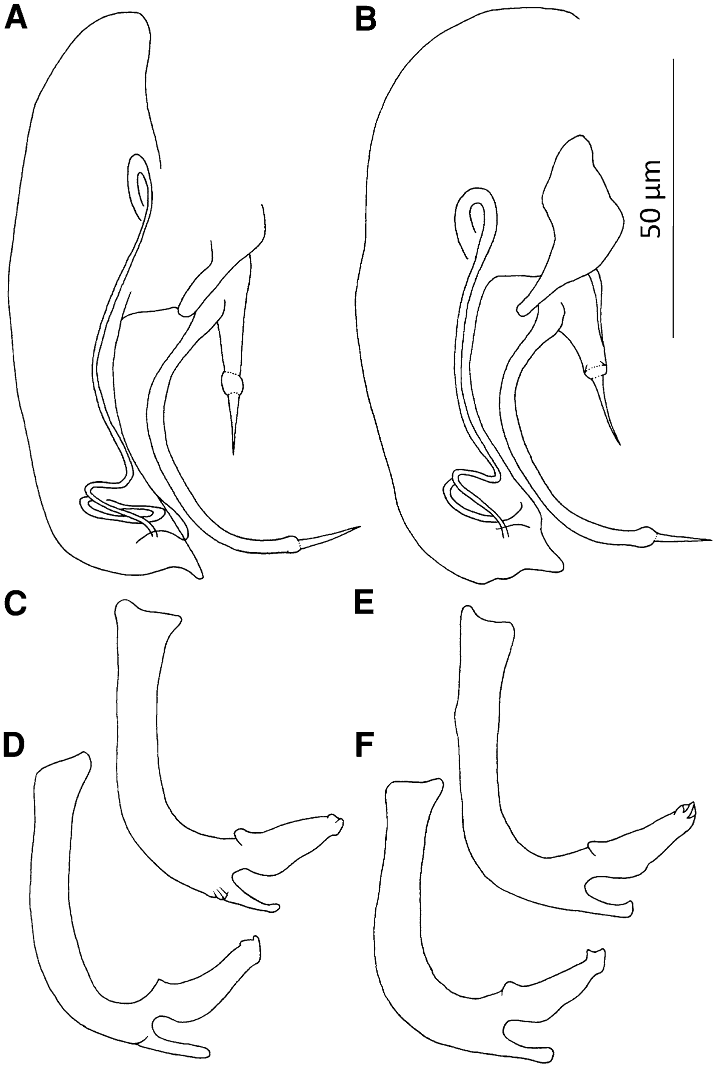


Fig. 2 SEM photographs of *Ankylocythere sinuosa* specimens: **A**, **D** two coupled pairs in different positions (the **A** couple shows a male with the left valve removed to observe attachment to the female); **B** adult male with the left valve removed; **C** adult female; **E** detail of a peritrichid ciliate structure appearing on the valves of *A. sinuosa*; F, copulatory complex from a subadult (A-1) male; **G**, **H** copulatory complex from adult males; lateral (**G**) and sublateral (**H**) views; **I** detail of bacteria-like structures. *Scale bars*: **A**, **B** = 129 µm; **C**, **D** = 200 µm; **E** = 20 µm; **F** and **G** = 59 µm; **H** = 35 µm; **I** = 6 µm

of ostracods per crayfish, corrected by $\log_{10}$(weight), among the sites, which resulted in significant differences ($F = 119.4, P < 0.05$). Bonferroni tests for pair-wise comparisons identified Pinedo to be the only site with significant differences when compared with the other three sites as it contained less ostracods per gram of crayfish than the other localities. The number of male *A. sinuosa* per crayfish individual was, on average, lower ($15.5 \pm 21.5, N = 147$) than the number of females (21.0 ± 28.1, $N = 147$), and showed

Fig. 3 Variation in male copulatory complex (**A** and **B**), with the clasping apparatus drawn separately (**C–F**), in lateral view of *Ankylocythere sinuosa* males from different localities (**A**, **C**, **D** from Riu Congost, locality code 2; **B**, **E** Padul, code 11; **F** Cascarro, code 5)



statistically significant differences according to the paired *t* test done ($t = -6.19$, $P < 0.01$). Furthermore, the proportion of adult entocytherids in a crayfish poorly, but significantly and negatively, correlated with the logarithm of the total number of ostracods (after Pearson's correlation, $r = -0.416$, $P < 0.01$).

Discussion

In our survey conducted in the Iberian Peninsula, we found only one entocytherid species associated with *Procambarus clarkii* crayfishes with a high frequency of occurrence and high prevalence (all the populations but one, and 100% of infected crayfishes in most cases). This does not coincide with the data from the area of origin (North America), where apparently one host crayfish species can harbour a varied number of entocytherid species (Hart & Hart, 1974). The impoverished ostracod entocytherid fauna found in Iberian *Procambarus clarkii* populations is possibly a result of the jump dispersal and the fast colonisation process noted in this area. Alien invaders are known to contain a reduced subsample of potential symbionts and parasites as a result of a chance selection of the individuals transported, together with certain negative effects on some hosted

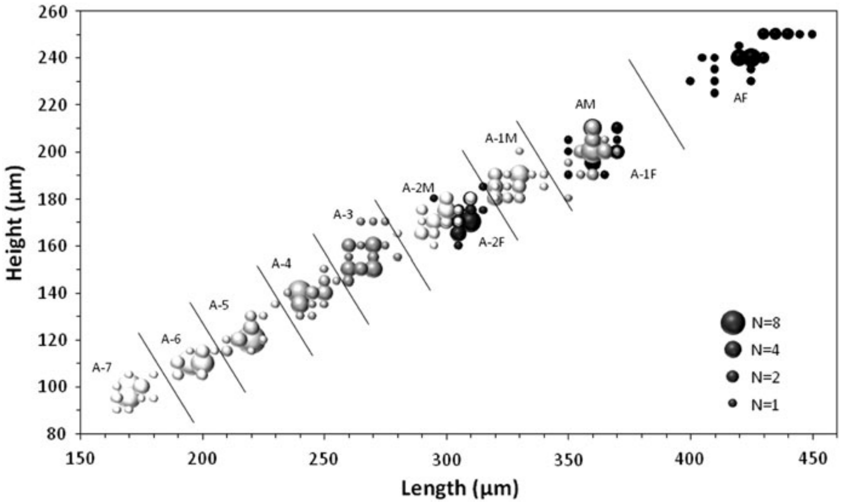


Fig. 4 Carapace length–height relationship for developmental instars of *Ankylocythere sinuosa.* Juvenile instars: A-7 to A-1; *AF*, *AM* adult individuals *F* female, *M* male. *Bubble size* corresponds to the number of individuals measured

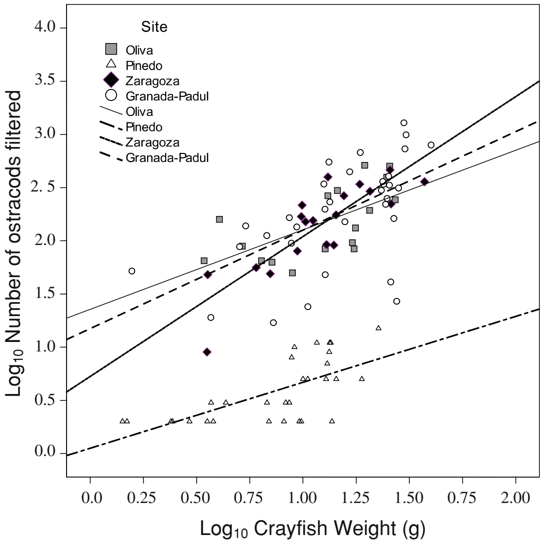


Fig. 5 Relationship between crayfish size ($\log_{10}$weight, grams) and the number of *Ankylocythere sinuosa* individuals extracted by the carbonated water method ($\log_{10}$number of ostracods filtered). *Regression lines* are shown separately for all four samples used for the analysis

species (Torchin et al., 2003). On the other hand, *A. sinuosa* has a large number of potential hosts, more than 40 crayfish species, mostly belonging to the genus *Procambarus* (Hart & Hart, 1974; Peters & Pugh, 1999). At present, it is completely unknown whether or not *A. sinuosa* lives in association with other crayfish species (either native or alien) in Europe. The original distribution of *A. sinuosa* is remarkably wide, and includes the southern United States, Mexico, and Cuba (Hart & Hart, 1974). Host non-specificity and wide distribution are features that could favour *A. sinuosa*'s invasive ability. Possibly one indication of the invasive character of *A. sinuosa* could lie in the fact that it is the only species of the 12 entocytherid species found in Mexico that extends beyond the state; the other 11 species are endemic to Mexico (Hobbs, 1971). However, an entocytherid's invading success is intrinsically linked to the host's success and, in this sense, the success of *A. sinuosa* could largely relate to its association with the world' most invasive crayfish, *Procambarus clarkii*. In addition, *A. sinuosa*'s high prevalence could facilitate the colonisation of new areas as the probability of translocated crayfishes carrying entocytherids is considerably great.

Generally, most podocopan ostracod species have 8 immature stages (Cohen & Morin, 1990; Meisch, 2000), but we could find only seven juvenile instars in *A. sinuosa*. This reduced number of juvenile instars is a characteristic phenomenon of entocytherids. Perhaps this reduction of juvenile free living instars is attained by the first instar moult occurring in the egg phase (Smith & Kamiya, 2005). The sexual dimorphism we observed for *A. sinuosa*, showing a larger body size in the adult females, is also found in other entocytherids such as *Uncinocythere occidentalis* (Smith & Kamiya, 2005), and could result from an evolutionary trend in the reduced body size of entocytherids as a response to a commensal life. In this evolutionary process of reducing size, females might be less advantaged than males because females need to produce eggs, and the number of spawned eggs usually increases with female size. Males of some other non-commensal ostracods are also smaller than females, such as species of the genus *Heterocypris* and *Eucypris* (Meisch, 2000). Regarding sexual size dimorphism, entocytherid females typically mate during the penultimate developmental instar (A-1 or "biunguis females"), and copulating "triunguis" (adult) females are rare exceptions (Hobbs, 1971). Oliva, one of the sites with the highest ostracod densities of this study, was the only place where we found A-2 females associated with adult males forming copulating or guarding pairs, a type of entocytherid association not described in the literature (Hobbs, 1971; Danielopol, 1977). Perhaps, in highly favourable reproduction situations, adult entocytherid males are less selective in the search for mates and form copulatory or guarding complexes also with A-2 females. Further research is needed to understand the reproductive process of entocytherids, and to know if the females are able to keep the sperm from A-1 (and perhaps also A-2) juvenile instars until the adult egg production stage or, really, the mating males are guarding these juvenile females for a future copulation in a mature female stage, as suggested by Danielopol (1977).

At the population level, our results agree with previous studies on entocytherids for several aspects. The positive correlation found between crayfish body size and the number of entocytherids harboured has already been described by Young (1971). This can be explained not only by the larger amount of resources offered by a larger host, but also the longer life time of larger (usually older) crayfishes that provides more time for the colonization and reproduction of the entocytherids (Kuris et al., 1980). Moreover we found that the number of entocytherids in a crayfish varied widely for each population and for a given crayfish length. Our results coincide with those obtained by Hobbs et al. (1967) and Young (1971). Young suggested that the crayfish moulting process could account for the variation found. In this sense, lower density values would belong to recently moulted crayfishes, which would have lost entocytherids when discarding the old exuvia. Therefore, higher entocytherid densities would correspond to the crayfishes that moulted a long time ago; consequently, entocytherids had enough time to reproduce without suffering losses. This is in agreement with the finding by Young (1971) which shows that the proportion of adult ostracods was higher (and ostracod densities lower) in young (small-sized) crayfishes as these moult more frequently than older (larger) crayfishes and, therefore, small decapods were colonised by adult entocytherids after each moult, while large ones (with low frequency moults) allowed entocytherids to reproduce on their bodies.

The differences we found in entocytherid densities between populations (by even controlling crayfish size) could result from ecological differences in the population or in environmental parameters (temperature, salinity, concentration of nutrients, host densities, etc.) Nevertheless, seasonal effects could be involved because different sites were sometimes sampled on different occasions. Seasons can affect entocytherid density, largely due to the crayfish reproductive cycle (Young, 1971). However, Young (1971) did not test statistically for significant differences, so these could appear by chance. Monitoring populations at different sites is necessary to evaluate the differential effects of time and space on ostracod population traits dynamics.

To our knowledge, this is the first citation of an alien entocytherid species in Europe. Mezquita et al. (2000) reported the presence of isolated entocytherid individuals, but did not assign them to a known species. The effects of entocytherids on their hosts and their indirect effects on the new ecosystems into which they were introduced are completely unknown. We know that ostracods can be intermediate hosts of some parasites (e.g. Grytner-Ziecina, 1996; Moravec, 2004), and entocytherids are not likely to be an exception. We found peritrich ciliates fixed on the valves of *A. sinuosa* individuals in our study, as well as undetermined bacteria on their hemipenises. Probably neither the ciliates nor the bacteria are much harmful to the ostracods or their hosts, but we did not find published information on the effects of such relationships. Understanding the functioning of entocytherid interspecific relationships, particularly in relation to the performance of their invasive host *Procambarus clarkii* and of the native European crayfishes has important management consequences. Although most papers on entocytherids consider that they act as commensals on crayfishes, some negative or positive effects may come about. Lee et al. (2009) showed how another group of crayfish epibionts, branchiobdellids, which was also considered to be mainly commensals, can act as mutualists in some circumstances by cleaning biofouling growth on crayfish individuals, which helps improve their living performance. Entocytherid-crayfish interactions may also show this pattern if ostracods are able to clean crayfish branchial plates or other exoskeleton parts. Nonetheless, new research works are required to verify this. Furthermore, Cuéllar et al. (2002) suggested that ostracods (unidentified, but probably entocytherids according to the picture shown in their paper) were the cause of pathogenic effects on crayfishes. If this is confirmed, the up to now widely accepted commensal role of entocytherids should be reviewed. With the present survey, we show the wide distribution and high prevalence of alien entocytherids living on one of the most important invading macroinvertebrates in European water bodies, which calls for a more intense research programme into the effects of this relationship.

Acknowledgements We thank J. Pretus (Univ. of Barcelona) for establishing contacts among the authors of this paper and for attracting our attention to the ostracods extracted from crayfishes. J. A. Gil-Delgado (Univ. Valencia) and CIAMA (La Alfranca) are acknowledged for organising the sampling and for providing permits in Zaragoza. We thank Ignacio Lacomba, Conselleria de Medio Ambiente, Agua, Urbanismo y Vivienda, Generalitat Valenciana for his help and support. We thank master students F. Peris, O. Mollá, F. J. Peris, and F. J. Vicent who helped in the field with the sample collections. J. M. Poquet (Univ. Granada) assisted FM in the fieldwork conducted in Granada and J. M. Gil-Sànchez provided information on adequate sampling sites. We thank A. Portheault for field assistance in the Doñana National Park. Yasushi Kusuoka and Gregorio Fernández-Leborans assisted us for the identification of the ciliates commensals on the ostracods. The personnel at the Electron Microscopy unit of the SCSIE (Univ. Valencia) helped us with sample preparation and the SEM pictures. This work was largely funded by the ECOINVADER (CGL2008-01296/BOS) project from the Spanish Ministry of Science and Innovation. We are also grateful to all the project members for their discussion and ideas. Finally, we are very grateful to Robin Smith and an anonymous referee for their suggestions to improve an earlier version of this paper.

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CAPÍTOL II

A review of the Entocytheridae (Ostracoda) of the world: updated bibliographic and species checklists and global georeferenced database, with insights into host specificity and latitudinal patterns of species richness

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Crustaceana. In press

A review of the Entocytheridae (Ostracoda) of the world: updated bibliographic and species checklists and global database, with insights into host specificity and latitudinal patterns of species richness

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April 17, 2014

Abstract

The creation of biodiversity datasets freely available for the scientific community is a valuable task to stimulate global research on biodiversity. Among others, the Global Biodiversity Information Facility (GBIF) is a remarkable resource providing free online access to biodiversity data on many diverse taxonomic groups (including Ostracoda) from both terrestrial and marine ecosystems. More specific databases for Ostracoda georeferenced data have been built (e.g. NACODe, OMEGA...), some with freely available data. However, the Entocytheridae, a family of ostracods living commensal on other crustaceans, with 220 living species, representing the third non-marine ostracod family in number of species, has been significantly under-represented in the currently available biodiversity databases. To cover this gap, we present here a free-access world database of Entocytheridae published in GBIF and review the current knowledge of the group by updating a bibliographic and species checklists of the Entocytheridae. We also analyse the host specificity of the group and the latitudinal species richness pattern in North and Central America (including 186 spp.). The current database includes 3509 georeferenced records from 220 species, in contrast to just 44 entocytherid georeferenced records previously published in GBIF. In addition, the updated species list accounts for 43 species and 2 genera that were not included in the previous compendium on Entocytheridae published by Hart & Hart Jr in 1974, so as 40 species not included in the 2013 Checklist provided by the Catalogue of Life. We show that the specialization in one unique host species is not the rule in Entocytheridae, and evidence an unusual latitudinal pattern of species richness in North and Central American entocytherids, most probably related to the biogeographic history of their hosts.

Introduction

The integration of biodiversity information datasets covering all the taxonomic groups to create a global biodiversity database freely available to all the scientific community is a valuable task to stimulate global research on biodiversity. To be able to attain such an aim, the existence of a consistent, reliable and updated list of the known biological species is a previous and essential step. With this aim, the Integrated Taxonomy Information System (ITIS) partnership (`http://itis.gov`) and the Species 2000 federation (`http:`

`//sp2000.org/`) planned the Catalogue of Life (`http://catalogueoflife.org/`), in order to build a list of all known species of organisms on Earth from multiple peer-reviewed taxonomic databases, periodically providing updated species checklist editions, such as the Annual Checklist (e.g. Roskov et al., 2013). Wikispecies (`http://en.wikipedia.org/wiki/Wikispecies`) is another project with similar aims to provide an open access and dynamic catalogue of all species directed at scientists, with high potential to store useful information related to each species. Another important step is the creation of standard formats to store biodiversity information, in order to facilitate its transference and sharing. Along this line, the Biodiversity Information Standards (TDWG, Berendsohn et al., 2011) created the Darwin Core (Darwin Core Task Group & Biodiversity Information Standards – TDWG, 2009), a body of standards to store information about taxa, their occurrence in nature as documented by observations, specimens, and samples, and related information.

Taxonomically supported by the Catalogue of Life and based on Darwin Core standard formats, the Global Biodiversity Information Facility (GBIF, `http://data.gbif.org`) is an international organisation to provide open free access to biodiversity data via internet through a global network of countries and organizations. The Ocean Biogeographic Information System (OBIS, `http://iobis.org`), World Register of Marine Species (WoRMS, Appeltans et al., 2012) and the Freshwater Animal Diversity Assessment (FADA, Balian et al., 2008) are similar projects involving marine and freshwater species databases.

More specific databases restricted to a given taxonomic group are more or less available to scientists. There are various examples of this type of database for Ostracoda. Indeed, the interest of ostracodologists in the construction of Ostracoda databases is remarkable. One of the first works on this area is the Kempf Database on Ostracoda (KDO, Kempf, 1980a,b,c,d, 1991, 1997a,b,c,d, 2002, 2006), a bibliographic compendium for the ostracodologists. In addition to this, a series of ostracod database projects rose with a biogeographic approach: the North American Combined Ostracode Database (NACODe, Curry & Smith, 2012), the Nonmarine Ostracod Distribution in Europe (NODE, Horne et al., 1998), the Ostracod Metadatabase of Environmental and Geographical Attributes (OMEGA, Horne et al., 2011) and the World Ostracoda Database (Brandão et al., 2013). The NACODe is a fusion of two previous databases, the Delorme Ostracode Database, that contains data from Canadian freshwater bodies, accessible via the Canadian Museum, and the North American Nonmarine Ostracode Database (NANODe), built from North American freshwater collections and with free internet access; NODE is a database restricted to freshwater European data mainly from the literature, but not freely accessible; OMEGA, also privately hosted, is a more recent database that includes the above-mentioned two databases with additional South African and Japanese records, and was designed to calibrate nonmarine ostracods for palaeoclimate and palaeoenvironmental applications; finally, the World Ostracoda Database incorporates at present a free-access world database of marine ostracods integrated into WoRMS.

The entocytherids are a peculiar family of ostracods because of their symbiont (i.e. non-free-living, including parasites, commensals and mutualists) life mode of all its component species. Their hosts are crustaceans belonging to Decapoda, Amphipoda and Isopoda. Commensalism is the most accepted hypothesis about the relationships between the entocytherids and their hosts (Hart & Hart Jr, 1974). The ostracod family Entocytheridae, represented by 178 species, 31 genera and 5 subfamilies according to Hart & Hart Jr (1974), the last review of this family hitherto, is the third non-marine Ostracoda family (out of 13 living families) with the highest number of species after the Cyprididae and Candonidae (Martens et al., 2008). However, despite we can find a high number of publications dealing with the presence of entocytherids (e.g. Hobbs Jr & Peters, 1977; Hobbs Jr & Walton, 1975, 1976; Andolshek & Hobbs Jr, 1986), they are not included in major ostracod biogeographic databases, with the exception of NODE. However, this resource is restricted to European records, while the two main entocytherid subfamilies (Entocytherinae and Notocytherinae), representing almost 95 % of the entocytherid species, are native to North and Central America (Entocytherinae) and Australasia (Notocytherinae) (Hart & Hart Jr, 1974). Moreover, the GBIF online resource only included 44 entocytherid occurrences hitherto, mainly corresponding to sampling localities of specimens preserved in Museum collections (e.g. National Museum of Natural History, Australian Museum...) Another example of the reduced attention paid to the entocytherids by most ostracodologists are recent reviews of the world non-marine ostracod fauna that only consider free-living ostracods (Martens et al., 2008; Martens & Savatenalinton, 2011), disregarding this commensal group, or describing it in a very succinct way (e.g. Karanovic, 2012).

Host specificity is an important parameter to understand the symbiont-host relationships, and it is closely related to the evolution of their association. Generally, parasites tend to be strongly host-specific (Poulin, 2007). In contrast, following the theory about the forces involved on host specificity proposed by Combes (1991), we expect lower host specificity in commensals than in parasites because the "compatibility filter" that the biont has to overcome to colonize a host should be less restrictive for commensals than parasites. Nevertheless, compared with studies carried out on parasites, the commensal-host relationship has been under-treated. Entocytheridae is a group that has been found in association with a wide variety of hosts composed by crustacean species from three different orders: Decapoda, Amphipoda and Isopoda. Due to the wide range of host species in the group and their commensal relationships with their host, the entocytherids are an interesting group to analyse issues on host specificity in symbiotic interactions.

Georeferenced databases with large-scale geographic ranges are essential in testing one of the most recognised patterns in biogeography: the latitudinal gradient of species richness, which decrease from the tropics toward the poles (Hillebrand, 2004). This is a very general pattern, but there are some exceptions (e.g. Rivadeneira et al., 2011). Non-free-living species represent special systems that may be strongly cued to particular factors such as their host specificity, dispersal abilities and coevolution (Poulin, 2007). In this sense, the entocytherids could be useful for checking this type of patterns in non-free-living species.

The main aim of this work was to build a free-access biogeographic database of the family Entocytheridae with all the published world occurrences of the group hitherto. We also aimed at compiling updated bibliographic and species checklists of this ostracod family, not updated since the review of Hart & Hart Jr (1974). Additionally, we analyse the host specificity of the group and the latitudinal species richness pattern of the North and Central American species.

Material and methods

In order to build our biogeographic database, we previously carried out an intensive bibliographic search to obtain all the publications containing in the text body (excluding the references section) a direct mention to the family Entocytheridae or any member of the group. Afterwards, we checked these publications by looking for all the entocytherid occurrences. Then, we created an entocytherid database using the Database Management System PostgreSQL v. 9.1 (The PostgreSQL Global Development Group, 2012) where we transferred the occurrence data obtained from the bibliographic search. The database's variables followed the standard Darwin Core. When finished, the "Entocytheridae (Ostracoda) World Database" (`http://www.gbif.es/gbif/ic_colecciones.php?ID_Coleccion=10114`) was converted to a Darwin Core archive by using the Integrated Publishing Toolkit (IPT) v. 2.0.5 (`http://www.gbif.es:8080/ipt/`) in GBIF, where a copy of the free-access database (Mestre & Mesquita-Joanes, 2013) has been hosted.

To analyse the host specificity of Entocytheridae species, we used the information obtained from the literature regarding to the host species cited in association with the entocytherids in the localities where they were found together. We only considered those citations where the host species was identified. So, we removed from the analysis those records without identified hosts, leaving a total of 203 entocytherid species for the analysis. With this information, we did a histogram for the distribution of entocytherid species according to the number of associated host species. To analyse the latitudinal changes in species richness for the North and Central American entocytherids we represented the entocytherid species richness per 5° latitudinal interval on the American continent between 15-55° of latitude. The species richness of each latitudinal interval was estimated using the Margalef's diversity index: $D_{MG} = (S-1)/\ln N$, where $S =$ Number of species, and $N =$ Number of occurrences (Magurran, 2004), in order to minimize the effects of sampling bias between latitude intervals. All the analyses were made using PostgreSQL v. 9.1 and R-cran v. 3.0.1 (R Core Team, 2013) through the RPostgreSQL package v. 0.3 (Conway et al., 2013) connecting both softwares.

Results

As a result of our bibliographic search, we found a total of 215 publications containing direct citations to entocytherids (Annex 1). The temporal variation of publications on entocytherids (Figure 1) shows that the main effort was made during the sixties and early seventies with a maximum of 35 publications in 5 years (this is, 7 publications per year on average). During the late seventies and in the eighties there was a reduction in the number of publications until it reached a mean number below one publication per year until present, with an slight increasing trend during the last few years.

The present database contains entocytherid records for 220 species, 35 genera and 5 subfamilies (included in the species checklist in Annex 2) with a total of 3509 occurrences. The main body of the data, with 3429 occurrences, comes from North and Central America, dominated by members of the subfamily Entocytherinae (with 183 species), together with some records of three Sphaeromicolinae species of the genus *Hobbsiella*. Oceania, represented by the subfamily Notocytherinae, contains 37 records. The European entocytherid records, with also 37 occurrences, correspond to the subfamilies Sphaeromicolinae and Hartiellinae, with the exception of some exotic American entocytherids (Entocytherinae), mainly located in the Iberian Peninsula. Finally, there are isolated records of Entocytherinae in Hawaii (1 occurrence) and Japan (2 occurrences), and Microsyssitrinae in southern India (2 occurrences) and South Africa (1 occurrence) (Figure 2).

Regarding the analysis of host specificity, the number of identified host species found in association with the entocytherids were 248 (234 Decapoda, 13 Isopoda and 1 Amphipoda). The species of the main host group, Decapoda, were composed by 217 Cambaridae, 12 Parastacidae, 4 Astacidae and 1 Pseudothelphusidae.

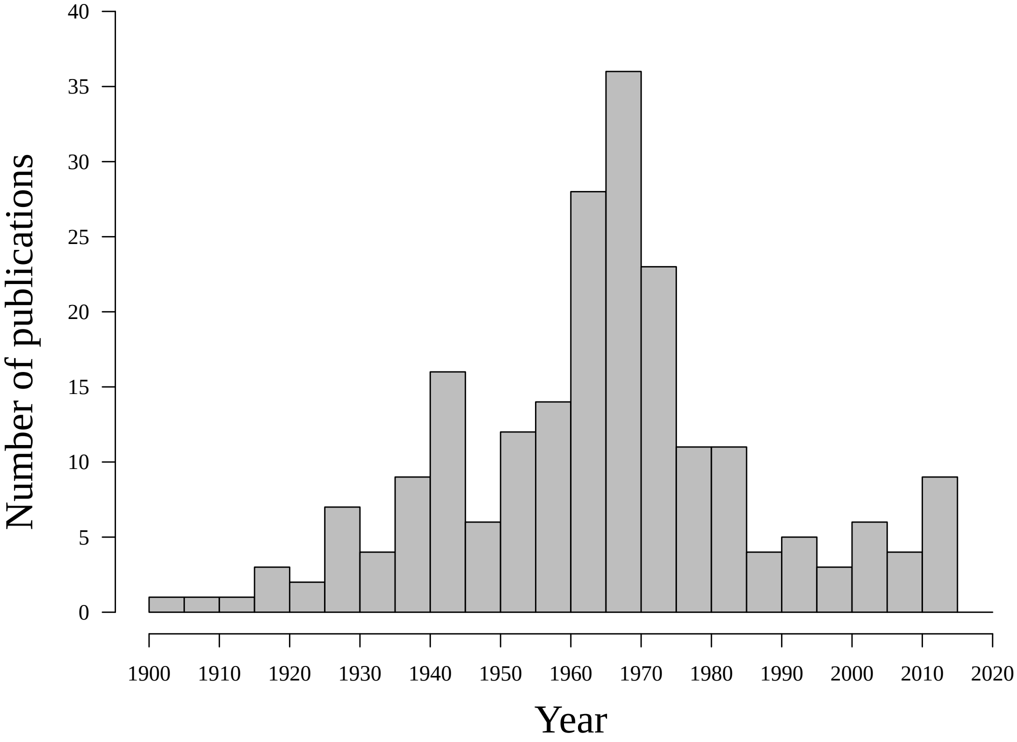

Figure 1. Temporal evolution of the number of publications on entocytherids. Publications that have a direct mention of the family Entocytheridae or any component of the group in their text body (excluding the references section) are included (Annex 1), summing up a total of 215 publications.

The distribution of the entocytherid species according to the number of hosts (Figure 3) shows a truncated pattern over lower numbers of host species, with 104 species (approx. 50 % of the 203 species analysed) having less than three hosts. However, only 73 entocytherid species had only one associated host species. Moreover, 32 entocytherids were cited in association with more than 20 host species, with some cases exceeding 40 host species (i.e. 47 for *Ankylocythere sinuosa*, 48 for *A. ancyla*, 50 for *Uncinocythere simondsi* and 64 for *Entocythere elliptica*).

The data selected for the analysis of latitudinal variation in species richness of North and Central American entocytherids included 186 entocytherid species, recorded in a total of 2410 American locations between 15 and 55° of latitude. Our results show a non linear latitudinal pattern of entocytherid species richness characterized by a maximum peak at intermediate latitudes (around 30-40°) with a strong decrease towards higher and lower latitudes (Figure 4).

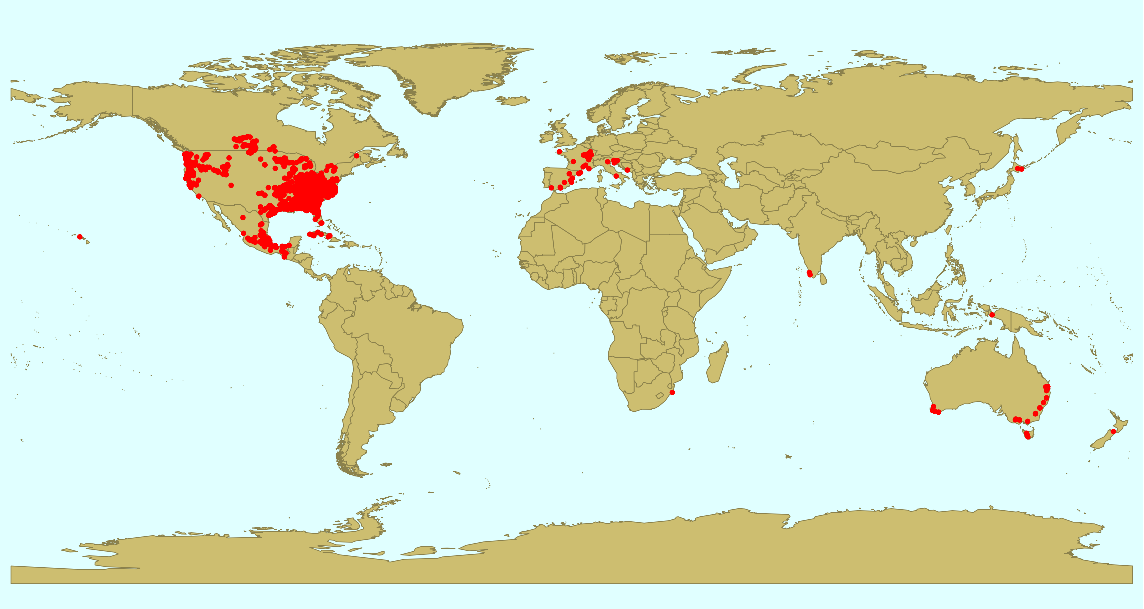

Figure 2. World distribution of the ostracod family Entocytheridae based on the free-access Entocytheridae (Ostracoda) World Database published in GBIF (Mestre & Mesquita-Joanes, 2013) and presented in this study, composed by 3509 occurrences and 220 species.

Discussion

Research on the Entocytheridae, with a pronounced peak in productivity around the sixties and the first half of the seventies, that culminated in the review by Hart & Hart Jr (1974), suffered a negative trend in number of publications since the last seventies until present, probably due to the decrease of scientific activity of some authors that have considerably contributed to the study of entocytherids (e.g. H. H. Hobbs Jr. and C. W. Hart Jr.). The slight increase of publications in recent years are mainly related to the discovering of well-established exotic entocytherid populations in Europe and Japan, associated with the invasive American crayfishes *Procambarus clarkii* (Girard, 1852) and *Pacifastacus leniusculus* (Dana, 1852) (Smith & Kamiya, 2001; Grabow & Martens, 2009; Grabow et al., 2009; Mestre et al., 2011, 2012, 2013; Aguilar-Alberola et al., 2012; Castillo-Escrivà et al., 2012, 2013). In this sense, the Entocytheridae could represent a new model of exotic ostracod dispersal, more related to the invasive success of their hosts and in which the entocytherid-host relationships have an important role, unlike other ostracod dispersal processes such as the passive dispersal by human activities (McKenzie & Moroni, 1986; Escrivà et al., 2012) or bird dispersal (Figuerola & Green, 2002; Frisch et al., 2007), where other different types of factors are involved, such as geographic parthenogenesis and desiccation-resistant eggs (McKenzie & Moroni, 1986).

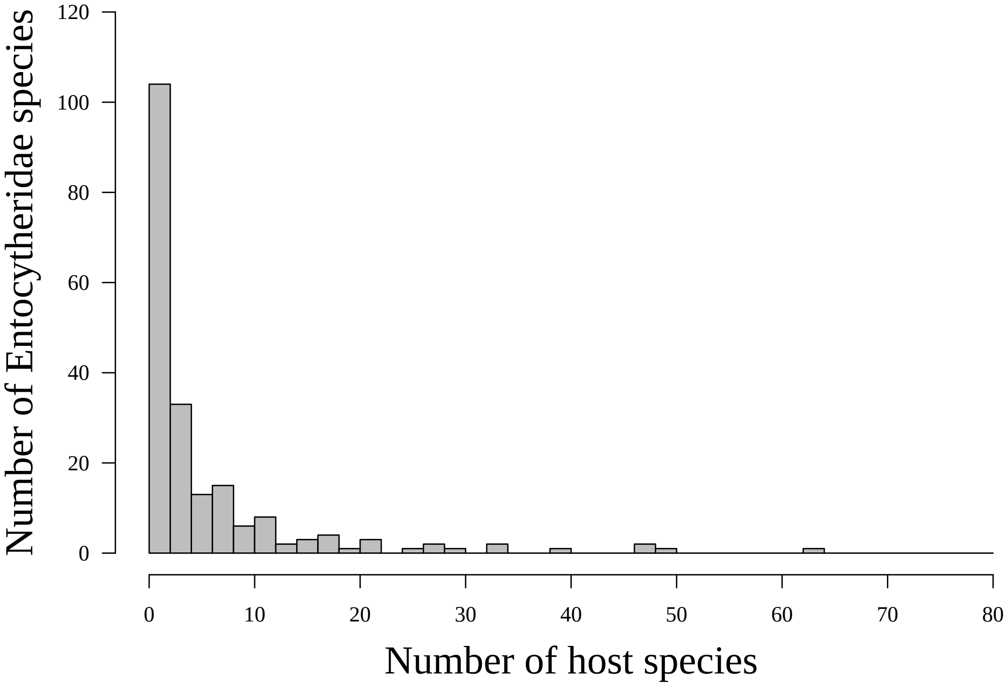

Figure 3. Frequency distribution of Entocytheridae species in relation to the number of associated host species. The data were obtained from the literature about the group (Annex 1), and only considering the citations of identified host species found in association with entocytherids, including 203 entocytherid species and 248 host species.

The Entocytheridae (Ostracoda) World Database (Mestre & Mesquita-Joanes, 2013) increases the number of georeferenced entocytherid sites in GBIF with 3509 new records, compared to the previous number of just 44 records. The database and species checklist also integrate 43 species and 2 genera that were not included in the last review of the family published by Hart & Hart Jr (1974), because they were described later. The two new genera are *Psittocythere*, erected to name a new species discovered by Hobbs Jr & Walton (1975), and *Hobbsiella*, a name proposed by Danielopol & Hart Jr (1985) to group three American species belonging to Sphaeromicolinae: *Hobbsiella cirolanae* (Rioja, 1951), *Hobbsiella coahuiltecae* (Hobbs & Hobbs, 1973) and *Hobbsiella moria* (Hart, 1978). Moreover, the updated species checklist (Annex 2) contains 40 species not present in the 2013 Annual Checklist of the Catalogue of Life (Roskov et al., 2013): *Ankylocythere barbouri*, *Ankylocythere spargosis*, *Dactylocythere scotos*, *Entocythere prisma*, *Plectocythere odelli* and all the species belonging to Notocytherinae (except *Laccocythere aotearoa*), Sphaeromi-

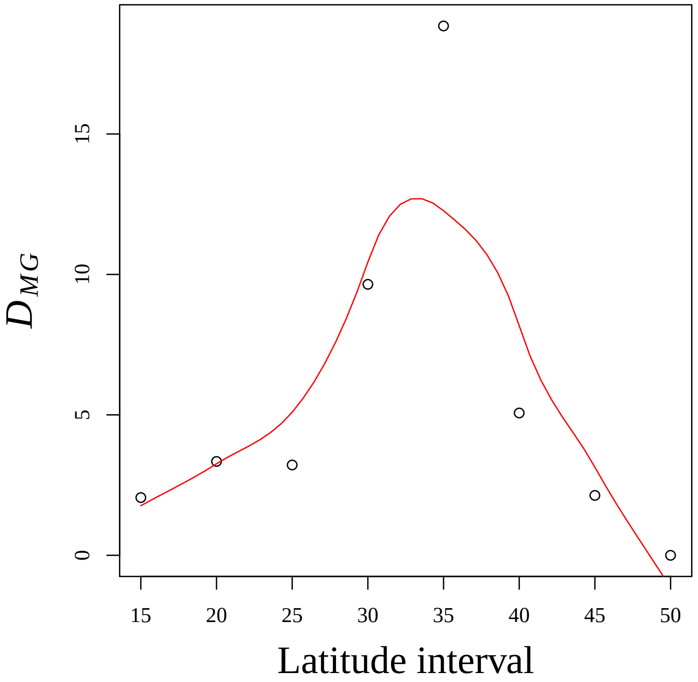

Figure 4. Species richness of North and Central American Entocytheridae (186 spp.) per 5° latitudinal interval on the American region between 15-55° of latitude. The species richness was estimated using the Margalef's diversity index ($D_{MG} = (S-1)/\ln N$ where S = Number of species, and N = Number of occurrences). The fitted curve corresponds to a LOESS model of the Gaussian family with a smoothing parameter $\alpha = 2/3$.

colinae (except *Hobbsiella moria*), Hartiellinae and Microsyssitrinae. Besides the new taxa, we incorporated some other posterior taxonomic changes proposed for the group. These are the cases of the synonymy of *Ankylocythere sinuosa* and *Ankylocythere tiphophila* (Crawford, 1959) in a unique species, *Ankylocythere sinuosa*, as established by Peters & Pugh (1999), and the substitution of the generic name *Aphelocythere* by *Waltoncythere*, as proposed by Hobbs Jr & Peters (1978).

Our results confirm that low host specificity is well established in the family Entocytheridae (approx. The 64 % of the 203 species analysed have been found associated with more than one host species), as suggested Hart & Hart Jr (1974). The lower restrictions in host compatibility related to the host defences compared with parasites might facilitate this establishment. Nevertheless, probably other factors involved on the evolution of the group's host specificity, such as interspecific competition, promoted the specialization of some entocytherid species in a unique host, as evidence the 73 entocytherid species with only one associate host. In this sense, there are some evidences of spatial segregation between entocytherid species within the host to avoid the interspecific competition and be able to share the same host (Hobbs Jr et al., 1967).

The peculiar latitudinal pattern in species richness followed by the American entocytherids, with a peak around 30-40° N may be related to the similar observed pattern in the American crayfishes, their main host group, by France (1992). This pattern could be explained by the radiation in diversity of the American crayfishes in the Southeast of North America that occurred from the last Cretaceous and through the Cenozoic (Hobbs Jr, 1988), favoured by the great diversity of semi-aquatic habitats in the region. The decrease of species richness at higher latitudes is probably due to the effect of the recent glaciation events that preclude a long history of colonisation and evolution. The absence of crayfishes and entocytherids in lower latitudes is thought to be a result of competitive exclusion by the freshwater crabs inhabiting these regions (Scholtz, 2002).

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Annex 2

Taxonomic list of the 220 extant species of the family Entocytheridae.

Class Ostracoda Latreille, 1806
  Subclass Podocopa G.W. Müller, 1894
    Order Podocopida G.O. Sars, 1866
      Suborder Cytherocopina Gründel, 1967
        Superfamily Cytheroidea Baird, 1850

- **Family Entocytheridae Hoff, 1942**
  - **Subfamily Entocytherinae Hoff, 1942**
    - *Ankylocythere ancyla* Crawford, 1965
    - *Ankylocythere barbouri* Villalobos & Hobbs, 1974
    - *Ankylocythere bidentata* (Rioja, 1949)
    - *Ankylocythere burkeorum* Hobbs III, 1971
    - *Ankylocythere carpenteri* Hobbs & McClure, 1983
    - *Ankylocythere chipola* Hobbs III, 1978
    - *Ankylocythere copiosa* (Hoff, 1942)
    - *Ankylocythere cubensis* (Rioja, 1955)
    - *Ankylocythere ephydra* Hart & Hart, 1971
    - *Ankylocythere freyi* Hobbs III, 1978
    - *Ankylocythere hamata* (Hobbs, 1957)
    - *Ankylocythere harmani* Hobbs, 1966
    - *Ankylocythere heterodonta* (Rioja, 1940)
    - *Ankylocythere hobbsi* (Hoff, 1944)
    - *Ankylocythere hyba* Hobbs & Walton, 1963
    - *Ankylocythere krantzi* Hobbs III, 1978
    - *Ankylocythere maya* Hobbs, 1971
    - *Ankylocythere prolata* Hobbs & Peters, 1991
    - *Ankylocythere sinuosa* (Rioja, 1942)
    - *Ankylocythere spargosis* Andolshek & Hobbs, 1986
    - *Ankylocythere tallapoosa* Hart & Hart, 1971
    - *Ankylocythere talulus* (Hoff, 1944)
    - *Ankylocythere telmoecea* (Crawford, 1959)
    - *Ankylocythere toltecae* Hobbs, 1971

Ankylocythere tridentata Hart, 1964
Ankylocythere villalobosi Hobbs, 1971
Ascetocythere asceta (Hobbs & Walton, 1962)
Ascetocythere batchi Hobbs & Walton, 1968
Ascetocythere bouchardi Hobbs & Walton, 1975
Ascetocythere coryphodes Hobbs & Hart, 1966
Ascetocythere cosmeta Hobbs & Hart, 1966
Ascetocythere didactylata Hobbs & Hart, 1966
Ascetocythere hoffmani Hobbs & Hart, 1966
Ascetocythere holti Hobbs & Walton, 1970
Ascetocythere hyperoche Hobbs & Hart, 1966
Ascetocythere jezerinaci Hobbs & McClure, 1983
Ascetocythere lita Hobbs & Hobbs, 1970
Ascetocythere myxoides Hobbs & Hart, 1966
Ascetocythere ozalea Hobbs & Hart, 1966
Ascetocythere pseudolita Hobbs & Walton, 1975
Ascetocythere riopeli Hobbs & Walton, 1976
Ascetocythere sclera Hobbs & Hart, 1966
Ascetocythere stockeri Hobbs & Peters, 1989
Ascetocythere triangulata Hobbs & Walton, 1975
Ascetocythere veruta Hobbs & Walton, 1975
Cymocythere clavata Crawford, 1965
Cymocythere cyma (Hobbs & Walton, 1960)
Cymocythere gonia Hobbs & Hart, 1966
Dactylocythere amicula Hart & Hart, 1966
Dactylocythere amphiakis Hart & Hart, 1966
Dactylocythere apheles Hobbs & Walton, 1976
Dactylocythere arcuata (Hart & Hobbs, 1961)
Dactylocythere astraphes Hobbs & Walton, 1977
Dactylocythere banana Hart & Hart, 1971
Dactylocythere brachydactylus Hobbs & Walton, 1976
Dactylocythere brachystrix Hobbs & Walton, 1966
Dactylocythere chalaza (Hobbs & Walton, 1962)
Dactylocythere charadra Hobbs III, 1971
Dactylocythere chelomata (Crawford, 1961)
Dactylocythere coloholca Hobbs & Hobbs, 1970
Dactylocythere cooperorum Hobbs & Walton, 1968

Dactylocythere corvus Hobbs & Walton, 1977
Dactylocythere crawfordi Hart, 1965
Dactylocythere crena Hobbs & Walton, 1975
Dactylocythere cryptoteresis Hobbs & Peters, 1993
Dactylocythere daphnioides (Hobbs, 1955)
Dactylocythere demissa Hobbs & Walton, 1976
Dactylocythere enoploholca Hobbs & Walton, 1970
Dactylocythere exoura Hart & Hart, 1966
Dactylocythere falcata (Hobbs & Walton, 1961)
Dactylocythere guyandottae Hobbs & Peters, 1991
Dactylocythere isabelae Hobbs & Peters, 1977
Dactylocythere jeanae Hobbs, 1967
Dactylocythere koloura Hart & Hart, 1971
Dactylocythere lepta Hobbs & Peters, 1991
Dactylocythere leptophylax (Crawford, 1961)
Dactylocythere macroholca Hobbs & Hobbs, 1970
Dactylocythere mecoscapha (Hobbs & Walton, 1960)
Dactylocythere megadactylus Hart & Hart, 1971
Dactylocythere myura Hobbs & Walton, 1970
Dactylocythere pachysphyrata Hobbs & Walton, 1966
Dactylocythere peedeensis Hobbs & Peters, 1977
Dactylocythere phoxa Hobbs, 1967
Dactylocythere prinsi Hobbs & Walton, 1968
Dactylocythere prionata (Hart & Hobbs, 1961)
Dactylocythere prominula Hobbs & Walton, 1977
Dactylocythere pughae (Hobbs & Hobbs, 1970)
Dactylocythere pygidion Hobbs & Peters, 1991
Dactylocythere runki (Hobbs, 1955)
Dactylocythere sandbergi (Hart & Hart, 1971)
Dactylocythere scissura Hobbs & Walton, 1975
Dactylocythere scotos Norden & Norden, 1985
Dactylocythere speira (Hart & Hart, 1971)
Dactylocythere spinata (Hobbs & Walton, 1970)
Dactylocythere spinescens Hobbs & Walton, 1977
Dactylocythere steevesi (Hart & Hobbs, 1961)
Dactylocythere striophylax (Crawford, 1959)
Dactylocythere susanae (Hobbs III, 1971)

Dactylocythere suteri (Crawford, 1959)
Dactylocythere ungulata (Hart & Hobbs, 1961)
Dactylocythere xystroides (Hobbs & Walton, 1963)
Donnaldsoncythere ardis (Hobbs & Walton, 1963)
Donnaldsoncythere cayugaensis (Hobbs & Walton, 1966)
Donnaldsoncythere donnaldsonensis (Klie, 1931)
Donnaldsoncythere hiwasseensis (Hobbs & Walton, 1971)
Donnaldsoncythere humesi (Hoff, 1943)
Donnaldsoncythere ileata Hobbs & Walton, 1963
Donnaldsoncythere leptodrylus Hobbs & Peters, 1977
Donnaldsoncythere pennsylvanica (Hart, 1960)
Donnaldsoncythere scalis Hobbs & Walton, 1963
Donnaldsoncythere truncata Hobbs & Walton, 1963
Donnaldsoncythere tuberosa (Hart & Hobbs, 1961)
Entocythere cambaria Marshall, 1903
Entocythere claytonhoffi Rioja, 1942
Entocythere costata Hobbs & Peters, 1977
Entocythere dentata Crawford, 1965
Entocythere dorsorotunda Hoff, 1944
Entocythere elliptica Hoff, 1944
Entocythere harrisi Peters, 1975
Entocythere illinoisensis Hoff, 1942
Entocythere internotalus Crawford, 1959
Entocythere kanawhaensis Hobbs & Walton, 1966
Entocythere lepta Hart & Hart, 1971
Entocythere mexicana Rioja, 1943
Entocythere prisma Andolshek & Hobbs, 1986
Entocythere reddelli Hobbs & Walton, 1968
Entocythere ruibali Rioja, 1955
Entocythere tyttha Hobbs & Hobbs, 1970
Geocythere acuta Hart & Hart, 1971
Geocythere geophila (Hart, 1959)
Geocythere gyralea Hart, 1965
Geocythere nessoides Hobbs & Hobbs, 1970
Harpagocythere baileyi Hobbs & Peters, 1977
Harpagocythere georgiae Hobbs III, 1965
Harpagocythere tertius Hobbs & Walton, 1968

Hartocythere torreya (Hart, 1959)
Litocythere lucileae Hobbs & Walton, 1968
Lordocythere petersi Hobbs & Hobbs, 1970
Okriocythere cheia Hart, 1964
Ornithocythere aetodes Hobbs III, 1970
Ornithocythere gypodes Hobbs III, 1969
Ornithocythere popi Hobbs III, 1970
Ornithocythere rhea Hobbs III, 1970
Ornithocythere waltonae Hobbs, 1967
Ornitocythere thomai Hobbs & McClure, 1983
Phymocythere lophota Hobbs & Peters, 1993
Phymocythere phyma (Hobbs & Walton, 1962)
Plectocythere crotaphis Hobbs III, 1965
Plectocythere johnsonae Hobbs & Hart, 1966
Plectocythere kentuckiensis Hobbs & Peters, 1991
Plectocythere odelli Norden, 1977
Psittocythere psitta Hobbs & Walton, 1975
Rhadinocythere serrata (Hoff, 1944)
Sagittocythere barri (Hart & Hobbs, 1961)
Sagittocythere stygia Hart & Hart, 1966
Saurocythere rhipis Hobbs III, 1969
Thermastrocythere riojai (Hoff, 1943)
Uncinocythere allenae (Hart & Hart, 1971)
Uncinocythere ambophora (Walton & Hobbs, 1959)
Uncinocythere bicuspide (Rioja, 1943)
Uncinocythere cassiensis Hart, 1965
Uncinocythere caudata (Kozloff, 1955)
Uncinocythere clemsonella (Crawford, 1961)
Uncinocythere columbia (Dobbin, 1941)
Uncinocythere cuadricuspide (Rioja, 1945)
Uncinocythere dobbinae (Rioja, 1943)
Uncinocythere equicurva (Hoff, 1944)
Uncinocythere ericksoni (Kozloff, 1955)
Uncinocythere holti Hart, 1965
Uncinocythere lucifuga (Walton & Hobbs, 1959)
Uncinocythere neglecta (Westervelt & Kozloff, 1959)
Uncinocythere occidentalis (Kozloff & Whitman, 1954)

Uncinocythere pholetera (Hart & Hobbs, 1961)
Uncinocythere simondsi (Hobbs & Walton, 1960)
Uncinocythere spathe Hart & Hart, 1971
Uncinocythere stubbsi Hobbs & Walton, 1966
Uncinocythere thektura Hart, 1965
Uncinocythere warreni Hobbs & Walton, 1968
Uncinocythere xania Hart & Hobbs, 1961
Uncinocythere xena Hart & Hart, 1971
Uncinocythere zancla Hobbs & Walton, 1963
Uncinocythere zaruri Hobbs, 1971
Waltoncythere acuta (Hobbs & Peters, 1977)

Subfamily Hartiellinae Danielopol, 1971

Hartiella dudichi (Klie, 1938)

Subfamily Microsyssitrinae Hart, Nair & Hart, 1967

Microsyssitria indica Hart, Nair & Hart, 1967
Microsyssitria nhlabane Hart & Clark, 1984

Subfamily Notocytherinae Hart & Hart, 1967

Chelocythere kalganensis Hart & Hart, 1967
Elachistocythere merista Hart & Hart, 1970
Herpetocythere acanthoides Hart & Hart, 1967
Herpetocythere australensis Hart & Hart, 1967
Herpetocythere bendora Hart & Hart, 1967
Herpetocythere gnoma Hart & Hart, 1967
Herpetocythere labidioides Hart & Hart, 1967
Herpetocythere mackenziei Hart & Hart, 1967
Hesperocythere klasteroides Hart & Hart, 1967
Hesperocythere tallanalla Hart & Hart, 1967
Hesperocythere xiphoides Hart & Hart, 1967
Laccocythere aotearoa Hart & Hart, 1971
Lichnocythere synethes Hart & Hart, 1967
Lichnocythere tubrabucca Hart & Hart, 1967
Lichnocythere victoria Hart & Hart, 1967
Notocythere antichthon Hart & Hart, 1967
Notocythere blundelli Hart & Hart, 1967
Notocythere erica Hart & Hart, 1967
Notocythere grampians Hart & Hart, 1967
Notocythere mirranatwa Hart & Hart, 1967

Notocythere rieki Hart & Hart, 1967
Notocythere synomodites Hart & Hart, 1967
Notocythere syssitos Hart & Hart, 1967
Notocythere tasmanica Hart & Hart, 1967
Riekocythere cherax Hart & Hart, 1967
Riekocythere xenika Hart & Hart, 1967

Subfamily Sphaeromicolinae Hart, 1962

Hobbsiella cirolanae (Rioja, 1951)
Hobbsiella coahuiltecae (Hobbs & Hobbs, 1973)
Hobbsiella moria (Hart, 1978)
Sphaeromicola cebennica Remy, 1948
Sphaeromicola hamigera Remy, 1946
Sphaeromicola sphaeromidicola Hubault, 1938
Sphaeromicola stammeri Klie. 1930
Sphaeromicola topsenti Paris, 1916

Comparison of two chemicals for removing an entocytherid (Ostracoda: Crustacea) species from its host crayfish (Cambaridae: Crustacea)

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International Review of Hydrobiology, *96*, 347-355

Internat. Rev. Hydrobiol.	**96**	2011	4	347–355

DOI: 10.1002/iroh.201111343

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Research Paper

Comparison of Two Chemicals for Removing an Entocytherid (Ostracoda: Crustacea) Species from Its Host Crayfish (Cambaridae: Crustacea)

key words: anaesthetic, chlorobutanol, carbonated-water, Crustacea, Ostracoda, Entocytheridae

Abstract

Entocytherids are epifauna on larger crustaceans. We assessed the effectiveness of chlorobutanol and carbonated water for removing entocytherid, *Ankylocythere sinuosa*, from crayfish, *Procambarus clarkii*. This was done using different exposure times and by assessing entocytherid and crayfish survival, together with the number of entocytherids removed. According to our results, chlorobutanol submersion of crayfishes in shorter time periods is more effective for removing entocytherids without killing the crayfish host. However, carbonated water submersion is better to extract larger amounts of living entocytherids. Finally, both chemicals are appropriate tools to establish standardised protocols for removing entocytherids from crayfish for ecological studies because the proportions of removed entocytherids per crayfish become constant whatever the entocytherid load of the crayfish.

1. Introduction

Crayfish have a broad diversity of epifauna associated with their exoskeleton, and include ciliates, temnocephalidans, free-living nematodes, branchiobdellidans and ostracods (EDGERTON *et al.*, 2002). Entocytheridae is a speciose family of ostracods with about 180 species and is ectosymbiotic primarily on crayfish. Most are native to North and Central America (Entocytherinae) and Oceania (Notocytherinae) (HART and HART, 1974). Recently, exotic entocytherids have been discovered in association with well-established populations of introduced crayfishes in Japan (SMITH and KAMIYA, 2001) and Europe (AGUILAR-ALBEROLA *et al.*, 2011).

Although many studies on entocytherids have been carried out in the United States, they are mainly taxonomic and biogeographic works (*e.g.*, HOBBS and HOBBS, 1970; HOBBS, 1971; HOBBS and PETERS, 1977; ANDOLSHEK and HOBBS, 1986), and no standardised method for removing entocytherids from their hosts has been proposed. Entocytherids are usually obtained from the bottom detritus of crayfish storage containers filled with alcohol or formalin (HART *et al.*, 1985; PETERS and PUGH, 1999). In other cases, a direct mechanical removal of entocytherids or their eggs was done by hand using tools like needles (STAMPER, 1957;

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BAKER, 1969). Only a few studies have used chemicals to detach entocytherids from crayfishes: chlorobutanol (YOUNG, 1971) and carbonated water (AGUILAR-ALBEROLA *et al.*, 2011).

Chlorobutanol (trichloro-2-methyl-2-propanol) is a common preservative of pharmacological products (AMISS and SMOAK, 1995; FURRER *et al.*, 1999) with anaesthetic properties for vertebrates (GILDERHUS and MARKING, 1987; FRANGIONI *et al.*, 1997) and invertebrates (NOGRADY and ROWE, 1993). It is usually employed in aqueous solutions of between 0.1% and 0.6% (PENNAK, 1989). YOUNG (1971) used a chlorobutanol saturated solution at 22 °C to extract entocytherids from crayfishes by immersing them for 45 mins., and achieved a 99% extraction of entocytherids per crayfish without killing crayfishes.

An alternative method used carbonated water (FISH, 1943) as fish anaesthetic. This method is attractive as it is low-priced, relatively safe for humans and the environment, and readily available (BERNIER and RANDALL, 1998; ROSS *et al.*, 2001). A more recent application has been its use for the *in vivo* extraction of epibionts from their crayfish hosts (GELDER *et al.*, 1994; MORI *et al.*, 2001). GELDER *et al.* (1994) used a 1:1 solution of stream water and carbonated bottled water for the first time to extract branchiobdellidans from living crayfishes by a 2 mins immersion. AGUILAR-ALBEROLA *et al.* (2011) adapted the method used by GELDER *et al.* for the removal of entocytherids from crayfishes by immersing crayfish in carbonated water (100%) for 15 mins.

The use of chlorobutanol or carbonated water as anaesthetics to remove entocytherids from crayfish enables the extraction of living entocytherids, plus the added possibility of not killing crayfishes. This offers new possibilities for performing experimental and ecological studies to solve doubts about this rare group of ostracods which, to date, have not been satisfactorily answered (AGUILAR-ALBEROLA *et al.*, 2011). This study aims to compare the effectiveness of chlorobutanol and carbonated water for removing entocytherid ostracods from crayfish with the best survival rates for both entocytherids and crayfish, and the largest number of entocytherids removed.

2. Methods

In February 2010, 41 red swamp crayfishes (*Procambarus clarkii* GIRARD, 1852) were captured using crayfish-baited traps from two wetlands in the Eastern Iberian Peninsula: 32 crayfishes from the Marjal de Pego-Oliva Natural Park (41°36′21.38″ N; 0° 45′37.66″ W) and 9 from the Natural Reserve Alfranca del Ebro (38°52′39.31″ N; 0° 03′18.95″ W). North American *Ankylocythere sinuosa* RIOJA, 1942 were found on the majority of crayfish in the two populations (AGUILAR-ALBEROLA *et al.*, 2011). Crayfishes were transported to the laboratory in refrigerated containers and subsequently maintained in an aquarium filled with the field water from the crayfish collection site. Two crayfish groups, with no differences in cephalotorax length ($t = -0.37$, $df = 38.6$, $P > 0.5$) and body weight ($t = -0.5$, $df = 36.09$, $P > 0.5$) per group, were employed for experimental treatments. Treatments were either carbonated water, that is, carbonic acid supersaturated water (21 crayfishes, 16 from the Pego-Oliva's Natural Park and 5 from Alfranca; the carbonated water group: CWG) or chlorobutanol (20 crayfishes, 16 from the Pego-Oliva's Natural Park and 4 from Alfranca; the chlorobutanol group: CBG).

Each crayfish belonging to CBG was consecutively submersed in three 1-L containers with 0.5 L of a 0.5% aqueous solution of chlorobutanol for 15 mins. in each container, following a modified version of the protocol by YOUNG (1971). The crayfish belonging to CWG were consecutively submersed in three 1-L containers with commercial Fuente Primavera^{TM} carbonated water (bicarbonate concentration: 297.2 mg/L; chloride: 35.8 mg/L; sulphates: 43.9 mg/L; and pH = 5.2) for 5 mins. with a full submerging time of 15 mins., following an adapted version of the protocol used by AGUILAR-ALBEROLA *et al.* (2011). All the containers were kept at a constant temperature of 23°C during the experiment. At the end of the total submersion time in both treatments, we transferred the crayfish to a container with Solan de Cabras^{TM} mineral water (bicarbonate: 285 mg/L; chloride: 7.8 mg/L; sulphates: 21.9 mg/L; and pH = 7.6) to allow living individuals to recover after stressful treatment. The containers of both chemical treatments (three containers per treatment) were individually filtered with 30-µm nytal mesh.

Sieved material was immediately deposited into 125 mL individual containers with 100 mL of Solan de Cabras^{TM} mineral water. After no anaesthetic effect remained (8 hours), we counted the number of entocytherids following each time period, and the number of living (movement) or dead (no movement) individuals. For the crayfish survival analysis, we assigned one of these three states: high mobility, low mobility, and no mobility (probably dead crayfish). Crayfish state was assessed after the first, second and third submersion periods, and again two days later. Immediately afterwards, all the crayfishes were transferred to a container with 96% ethanol, and we filtered and counted the entocytherids in the mineral water containers where crayfishes were stored for two days. We maintained crayfishes in 96% ethanol for 15 days by regularly shaking containers (once a day) to detach the remaining entocytherids from the crayfish. After this 15-day period, we filtered the ethanol and counted the entocytherids. The total number of entocytherids per crayfish was calculated as the sum of these individuals, plus those recovered from the mineral water containers, in which crayfishes remained two days after treatments, and the number of entocytherids collected during the three treatment periods. All the crayfishes were measured (cephalothorax length, between the eye basis and the posterior and nearest end of the cephalotorax on the same side) with digital callipers and wet-weighed.

χ^2 tests were used to compare the three crayfish mobility states (survival) following each chemical treatment. Entocytherids survival, expressed as a fraction of living entocytherids in relation to the total number of entocytherids per crayfish, was analysed by a linear mixed-effects ANOVA model taking a crayfish individual as a random factor, and the submersion period and type of treatment product as fixed effects factors. In addition, we compared the proportion of surviving entocytherids between groups (CWG *vs.* CBG) for both the full treatment time and the same 15 min treatment time using two Student's t-tests. To test the effects of product type (CWG *vs.* CBG) and time period on extracted entocytherid densities, we followed a linear mixed-effects ANOVA model, as described above. To do this, the number of extracted entocytherids per crayfish was previously $\log_{10}$-transformed and divided by the host crayfish $\log_{10}$-transformed weight. We used Student's t-tests to compare the extracted entocytherid densities between CWG and CBG used for the full period (15 mins. for CWG, 45 mins. for CBG), and between the two groups for one same submersion time period (15 mins.). The same analysis described above for entocytherid densities was done with the extracted entocytherid proportions; that is, the number of entocytherids extracted from the product treatment and treatment interval divided by the total number of entocytherids (summing all the individuals obtained from treatments, plus those obtained from the last two containers where crayfishes remained after treatments: mineral water after two days and 96% ethanol after 15 days). Finally for both CWG and CBG, we used linear regression models to analyse the relationship between the entocytherid densities obtained in each treatment interval and total entocytherid densities. We calculated total entocytherid densities as the sum of the entocytherids extracted after the whole treatment period and non-extracted entocytherids (that is, those individuals obtained from mineral water and from ethanol where crayfishes were stored after treatments). All these analyses were performed using *R-cran v. 2.10.1* (R DEVELOPMENT CORE TEAM, 2009).

3. Results

No differences between CWG and CBG were observed in crayfish mobility after each immersion period (first interval $\chi^2 = 1.03$, $df = 2$, $P = 0.60$; second interval $\chi^2 = 0.30$, $df = 2$, $P = 0.86$; third interval $\chi^2 = 1.70$, $df = 2$, $P = 0.43$). However, recovery of crayfish after the respective treatments was worse in those individuals submersed in chlorobutanol than in those submersed in carbonated water (Fig. 1). On the other hand, when comparing crayfish mobility between both chemical treatments for 15 mins., the anaesthetic effect was greater in the crayfishes submerged in carbonated water ($\chi^2 = 19.30$, $df = 2$, $P < 0.001$) than in those submerged in chlorobutanol, which were only slightly affected in mobility terms (Fig. 1).

A significant effect of the experimental treatment on entocytherid survival was observed (ANOVA; $F = 291.07$, $df = 40$, $P < 0.001$). Entocytherid survival remained high (mean > 0.8) at the end of the carbonated water treatment. In contrast, there was a sharp drop in entocytherid survival for chlorobutanol treatment after the first immersion period, and most entocytherids were found dead after the full treatment using this product (Fig. 2). In addition,

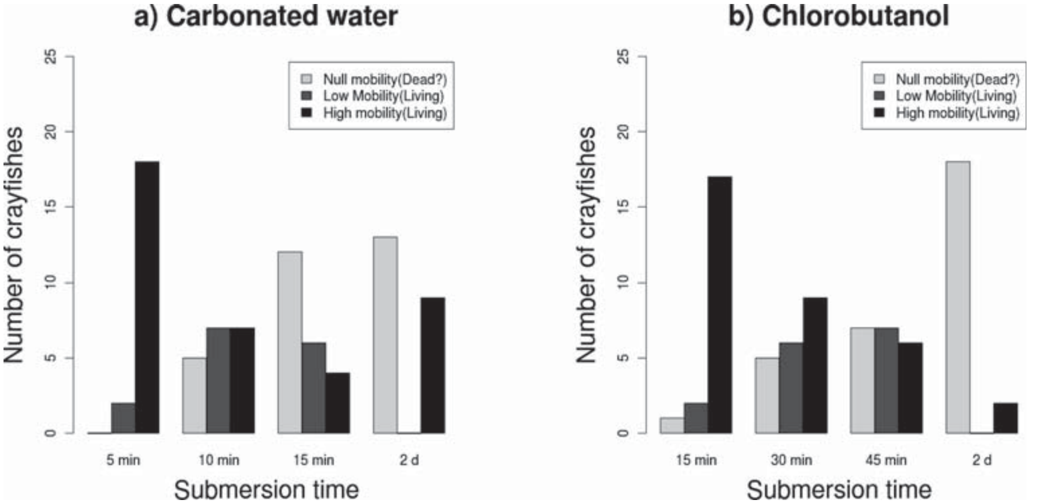

Figure 1. Crayfish survival after different experimental treatments for entocytherid extraction: (a) carbonated water (5, 10 and 15 mins.) and (b) chlorobutanol (15, 30 and 45 mins.). Three crayfish states are considered after applying treatments: high mobility, low mobility and no mobility (the crayfish is dead or too narcotised). For each product tested, mobility data were also taken two days after treatments (2 d). See the text for further explanation.

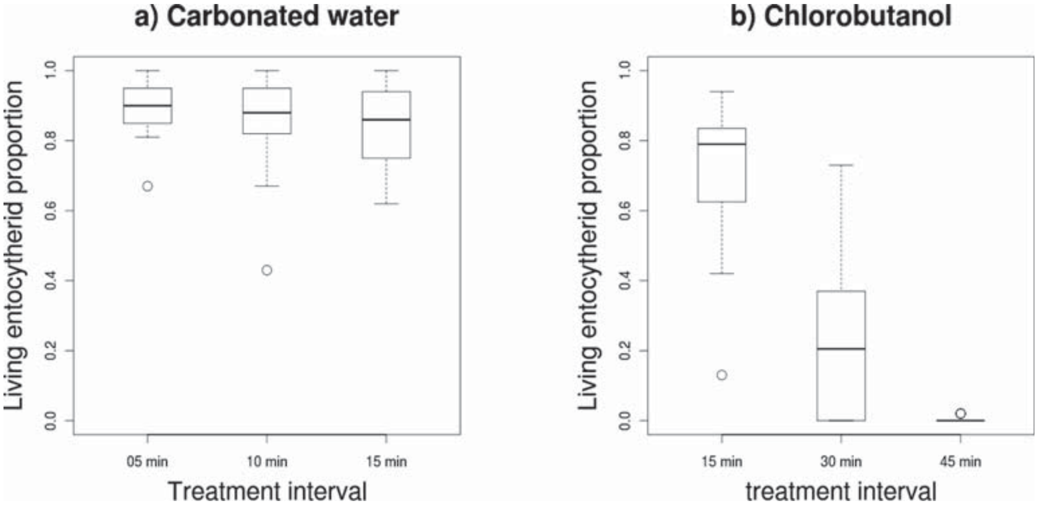

Figure 2. Comparison of the proportion of living entocytherids (in relation to the total number of extracted entocytherids) using two different methods: (a) carbonated water and (b) chlorobutanol, and three time intervals.

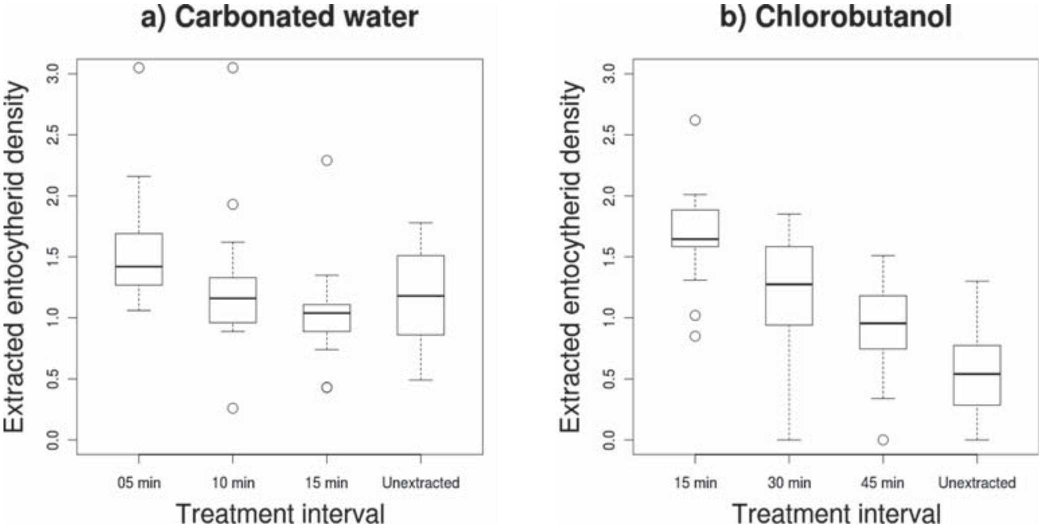

Figure 3. Extracted entocytherid densities per crayfish using two different products: (a) carbonated water and (b) chlorobutanol, and three time intervals. "Unextracted" refers to the sum of the entocytherids obtained from the mineral water and ethanol containers where crayfishes were stored after treatments. Extracted entocytherids densities per crayfish were calculated as the $\log_{10}$-transformed number of removed entocytherids per crayfish divided by the host crayfish $\log_{10}$-transformed weight.

higher entocytherid survival was observed in CWG when comparing survival for a 15 mins. submersion period ($t = 2.83$, $df = 30.644$, $P < 0.005$).

In both chemical treatments, densities of extracted entocytherids were higher in the first time interval and tended to decline progressively in subsequent intervals (Fig. 3). Treatment type did not affect entocytherid densities per crayfish (ANOVA; $F = 2.57$, $df = 40$, $P > 0.1$). According to this result, we found no differences between treatments when considering both the full treatment time ($t = -0.70$, $df = 37.29$, $P > 0.2$) and the 15-mins. submersion period ($t = 0.44$, $df = 38.55$, $P > 0.5$). However, the densities of unextracted entocytherids (Fig. 3, right-hand column), obtained from both the mineral water and 96% ethanol containers, where crayfishes were stored after treatments, were higher for the carbonated water treatment ($t = 5.32$, $df = 39$, $P < 0.001$).

Although no treatment effect was observed in the model (ANOVA; $F = 0.00$, $df = 40$, $P = 0.99$) when considering the number of extracted entocytherids in relation to the total entocytherids from the crayfish, this proportion was higher at the end of the experiment in chlorobutanol-subjected crayfishes ($t = -5.26$, $df = 23.02$, $P < 0.001$). Nevertheless, when we contemplated an equal treatment time of 15 mins., carbonated water extracted a higher proportion of entocytherids than chlorobutanol ($t = 2.63$, $df = 35.96$, $P < 0.01$). Finally, when comparing the first interval of treatments, chlorobutanol use (15 mins.) enabled the extraction of a higher proportion of entocytherids than carbonated water (5 mins.) ($t = -3.52$, $df = 29.68$, $P < 0.001$).

For both chemical treatments, the fit of the linear models relating the extracted entocytherid densities for each treatment time interval to the total entocytherid densities was very good, with an $R^2 > 0.9$ in all the time intervals, except for the first one in the chlorobutanol treatment ($R^2 = 0.88$). The regression line of the first time interval for both treatments presented a slope higher than 1 (Fig. 4), while the slope in the following intervals tended to decrease, and the regression line approached the reference straight line with intercept = 0 and

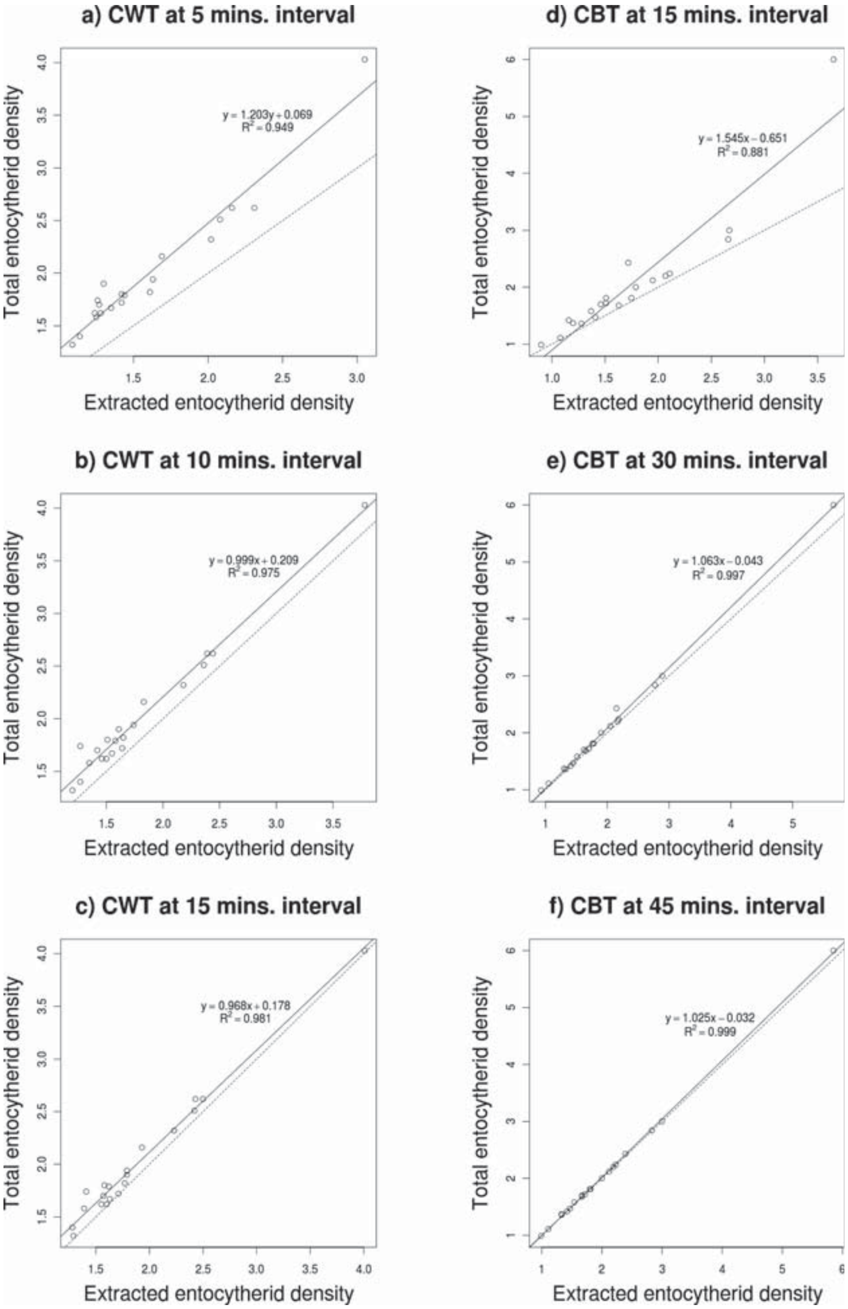

Figure 4. Linear regressions to estimate total entocytherid densities ($\log_{10}$-transformed number of removed entocytherids per crayfish divided by the host crayfish $\log_{10}$-transformed weight) from the densities obtained after applying both extraction treatments: carbonated water (CWT) (a–c), and chlorobutanol, (CBT) (d–f), at three treatment time intervals: 5, 10 and 15 mins. for the CWT (a, b and c, respectively), and 15, 30, and 45 mins. for the CBT (d, e and f, respectively). The dotted line corresponds to a reference straight line with intercept = 0 and slope = 1

slope = 1. A regression line with slope = 1 indicates treatment stability because the difference between the densities initially extracted by the tested methods and the total densities of entocytherids per crayfish remains constant, irrespectively of the variability of total entocytherid densities. In the last time period, which uses chlorobutanol, the slope and R^2 came very close to 1, indicating very good entocytherid extraction from crayfish at the end of this treatment.

4. Discussion

The use of anaesthetics to remove ectoparasites is a specific application that has been used in a few studies about fish ectoparasites, such as kinetoplastids (CALLAHAN *et al.*, 2002), and some invertebrate epifaunal groups, such as branchiobdellidans (GELDER *et al.*, 1994; MORI *et al.*, 2001) and entocytherids (YOUNG, 1971; AGUILAR-ALBEROLA *et al.*, 2011). The traditional method used for entocytherids removal consists of submerging crayfish in fixatives like ethanol or formalin. In most cases, the first objective is to preserve crayfish and to not consider entocytherids (HART and HART, 1974). Only two studies have employed specific methods to remove entocytherids from crayfishes (YOUNG, 1971; AGUILAR-ALBEROLA *et al.*, 2011). They present one main advantage if compared with traditional methods: crayfish and entocytherids can be separated without killing them.

When considering costs of materials, convenience and health risks, the carbonated method is more appropriate because it is completely innocuous and carbonated water is relatively easy to acquire from any supermarket at very low prices. However, chlorobutanol has to be bought from a specialised chemical company at a higher cost and in a solid form. Before its use, it should be prepared as a solution. Moreover, it must be used with caution because it could be harmful (VALENTOUR and SUNSHINE, 1975).

Apart from the economic and convenience aspects, and given the scientific objectives and results of our study, after comparing the two product treatments analysed herein, we found it more helpful to use carbonated water or chlorobutanol with a specific exposure time period depending on the study aims. This is mainly due to large differences as regards crayfish and entocytherid survival, and the densities and proportions of extracted entocytherids per crayfish. Elimination of entocytherids from crayfish without killing it offers new possibilities to design experiments; for example, to check the relationship between these organisms, as has been done for another epifaunal group, branchiobdellidans (LEE *et al.*, 2009), and for feather mites (FIGUEROLA *et al.*, 2003). In these cases, the main factors taken into account are crayfish survival and the proportions of removed entocytherids. According to our results, the best treatment to achieve high host survival and large proportions of removed ostracods is crayfish submersion using chlorobutanol for short periods (*e.g.*, 15 mins.) because it removes the largest relative amounts of entocytherids without killing the crayfish. However, treatment needs to be repeated for a more effective removal of entocytherids given the presence of remaining non-extracted entocytherids (Fig. 3) and the potential existence of entocytherid eggs in crayfish (STAMPER, 1957; YOUNG, 1971). In addition, crayfish submerging time should be checked and adapted to other crayfish and entocytherid species, if needed, because anaesthetic sensitivity may differ between species (SIMPSON and JOHNSON, 1996).

Obtaining living entocytherids from crayfish makes individuals available to conduct new studies on behavioural ecology which has been poorly studied in ostracods, although the ostracod chemotaxis has been clearly demonstrated (UIBLEIN *et al.*, 1996; ABE and VANNIER, 1991), a mechanism possibly involved in entocytherid host invasion. According to our results, carbonated water is better to obtain living entocytherids and offers higher entocytherid survival rates at all the submersion times considered in this study. In addition to these and other purposes, we should select large crayfishes from a population which will yield higher numbers of entocytherids.

To collect the maximum number of entocytherids from a crayfish, irrespectively of entocytherid and crayfish survival, crayfish submersion with chlorobutanol for 45 mins. is the most appropriate protocol, and one that removes practically all the entocytherids from the crayfish. This protocol is preferable to check infection frequency when studying crayfish populations with very low ostracod densities and/or prevalences, which may occur at some sites (AGUILAR-ALBEROLA *et al.,* 2011).

Finally, to carry out ecological field studies on entocytherids with spatial or temporal variability (*e.g*., niche modelling, population dynamics), a standardised entocytherid removal protocol with a known relationship between removed and total amounts of entocytherids in a crayfish is required. Entocytherid removal protocols that use both chlorobutanol and carbonated waters meet this requirement because removed entocytherid proportions remain constant for a given submerging time, whatever the entocytherid load of the crayfish. In general for these purposes, carbonated water is low-cost, more easily available and safe for the environment in case of spillages or for disposal purposes. However, using chlorobutanol at low submerging times implies the possibility of keeping the crayfish alive after entocytherid removal. This is a useful feature in some situations, for example, when working with protected native crayfish species.

5. Acknowledgements

We are grateful to CIAMA-La Alfranca, INAGA, J. A. GIL-DELGADO and the staff of the March of Pego-Oliva's Natural Park for permission and support in the field for crayfish collection. This research has been funded by the Spanish Ministry of Science and Innovation, project ECOINVADER (CGL2008–01296/BOS). A. M. is recipient of a "V Segles" predoctoral fellowship funded by the University of Valencia. HELEN WARBURTON (HyA Translations) corrected the English grammar and spelling.

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Manuscript submitted March 2nd, 2011; revised July 6th, 2011; accepted July 11th, 2011

CAPÍTOL IV

Population dynamics of an epibiont Ostracoda on the invasive red swamp crayfish *Procambarus clarkii* in a western Mediterranean wetland

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Hydrobiologia, *714*, 217–228

Hydrobiologia (2013) 714:217–228
DOI 10.1007/s10750-013-1542-5

PRIMARY RESEARCH PAPER

Population dynamics of an epibiont Ostracoda on the invasive red swamp crayfish *Procambarus clarkii* in a western Mediterranean wetland

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Received: 29 December 2012 / Revised: 12 April 2013 / Accepted: 4 May 2013 / Published online: 15 May 2013

Abstract Recently, the American entocytherid ostracod *Ankylocythere sinuosa* was discovered for the first time in Europe to inhabit widely distributed populations of the invasive American crayfish *Procambarus clarkii* in the Iberian Peninsula. Based on this finding, the aim was to describe the population dynamics of exotic entocytherids for the first time beyond their original area, and to analyse the main variables modulating temporal demographic patterns. We monitored a population of *A. sinuosa* and its host, *P. clarkii*, in the Pego-Oliva wetland (Eastern Iberian Peninsula) monthly for 1 year. Crayfish entocytherid loads strongly related to crayfish weight, moult-related exoskeleton hardening stage and water temperature. The proportion of earliest juvenile entocytherid instars, an indicator of recent hatching periods, also related to water temperature and conductivity. According to our results, the most important factors affecting entocytherid dynamics are individual crayfish moulting events, which diminish ostracod load, together with water temperature and ionic concentration, both influencing the life cycle of the exotic epibiont ostracod *A. sinuosa*.

Keywords Population ecology · Commensalism · Decapoda · Entocytheridae · Ostracod · Alien species

Introduction

The population dynamics of commensal epibionts is a poorly studied topic in ecology despite these organisms being quite common and diverse, which include a variety of taxa such as barnacles on whales or mites on bird feathers. Entocytherids (Ostracoda, Entocytheridae) constitute a family of ostracods that are epicommensals on other crustaceans. The components of the main entocytherid subfamily, the Entocytherinae, representing 83% of the 220 species of the group, are native to North and Central America where they live associated with crayfishes belonging to the Astacoidea (Hart & Hart, 1974; Mestre et al., 2012). Recently, some entocytherid species of this group have been found in other continents due to the transport of American crayfish beyond their original area for aquaculture purposes (Smith & Kamiya, 2001; Aguilar-Alberola et al., 2012). This is the case of *Ankylocythere sinuosa* (Rioja, 1942), recently discovered in the Iberian Peninsula (Aguilar-Alberola et al., 2012), which is associated with *Procambarus clarkii* (Girard, 1852), a very successful invading crayfish (Geiger et al., 2005).

Handling editor: Diego Fontaneto

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Contributions to knowledge on the population ecology of entocytherids are virtually inexistent, except for the works of Young (1971) and Hart et al. (1985). In addition, although the ecological impact of invasive crayfish has been well-recognised and studied (Rodríguez et al., 2003; Gherardi & Acquistapace, 2007; Holdich et al., 2009), the ecology of their ostracod epibionts outside the American continent remains largely unknown (Aguilar-Alberola et al., 2012).

According to current knowledge on the annual dynamics of entocytherid populations, *A. sinuosa* maintains a high reproductive rate for all seasons (Young, 1971). However, the entocytherid load per host in this species, that is, the number of entocytherids that inhabit on a crayfish individual, varies considerably all year round: it is minimum in winter, increases in spring and early summer, and peaks in late summer. In another entocytherid species, *Uncinocythere occidentalis* (Kozloff & Whitman, 1954), all seasons are favourable, except summer (Hart et al., 1985). When focusing on the proportion of juvenile and adult entocytherids, Young (1971) noted that crayfishes with large amounts of *A. sinuosa* had high proportions of immature individuals, mostly belonging to the first juvenile instar (A-7). Conversely, in crayfishes with low entocytherid loads, the proportion of adults was larger. In *A. sinuosa*, the sex ratio was biased towards females (Aguilar-Alberola et al., 2012). In many ostracods species, the sex ratio generally shows excess females, while the number of females and males is the same in others, and a larger proportion of males is rare (Cohen & Morin, 1990).

In most free-living ostracods, the growth rate and final size of adults are related to temperature, salinity and to other habitat conditions (Cohen & Morin, 1990). Generally, temperature has been considered the main variable to influence ostracod population dynamics in shallow-water habitats (Horne, 1983), as observed for instance in the cytheroidean *Cyprideis torosa* (Jones, 1850) or the cypridoidean *Heterocypris bosniaca* Petkowski et al. 2000 (Mezquita et al., 2000; Aguilar-Alberola & Mesquita-Joanes, 2011). Salinity probably affects the spatial distribution of ostracods more intensely than their population dynamics (Horne, 1983; Mesquita-Joanes et al., 2012). Some ostracods inhabiting constant environments do not show seasonality (Horne, 1983; Cohen & Morin, 1990), but most free-living ostracods show it during their life cycles, with reproduction periods restricted mostly to spring, summer or autumn.

Unlike free-living organisms, epibionts can be affected by variables directly related to their host traits or state (Threlkeld & Willey, 1993; Jovani & Serrano, 2004). Within this framework, Young (1971) proposed crayfish moult, size and reproductive cycle to be the main variables that modulate the population dynamics of entocytherid ostracods. According to Young (1971), crayfish moult could explain the broad variability of the entocytherid load that he found among crayfishes of the same size. According to this hypothesis, moulting events would trigger loss of entocytherids, and consequently after the moult event, immigration and reproduction processes would be required to recover the crayfish entocytherid load. This expectation for ostracod entocytherids of Young (1971), with epibiont loads being renewed after the host's moult, has been described in other works studying epibionts on zooplanktonic crustaceans, such as daphnids (Threlkeld & Willey, 1993; Pérez-Martínez & Barea-Arco, 2000). Furthermore, several studies have found a positive relationship between crayfish size and entocytherid load (Young, 1971; Hart et al., 1985; Aguilar-Alberola et al., 2012). In addition, according to Young (1971), *A. sinuosa* has lower densities during those periods when the number of juvenile crayfishes increases. He attributes this fact to a dilution effect caused by a spread of the entocytherid population among many immature crayfishes. Therefore, entocytherid density would depend on the host's reproductive cycle. Host gender, however, does not seem to have any effect on entocytherid load (Hart et al., 1985).

In this work, a monthly monitoring programme was carried out with an *A. sinuosa* population throughout the year in a Spanish wetland invaded by the American red swamp crayfish *P. clarkii*. With this monitoring plan, the aim was to describe, for the first time, the population dynamics of an entocytherid beyond its original distribution area, and to analyse the variables that could affect the entocytherid population ecology.

Materials and methods

Sample collection was carried out in a 400-m stretch of the Bovar canal (41°36′21.38″N; 0°45′37.66″W), located inside the Pego-Oliva Wetland Natural Park (Uriós et al., 1993). This canal holds a permanent water table and the red swamp crayfish (*P. clarkii*) can be found in it all year round. The entocytherid ostracod

A. sinuosa has been previously found at this site in a study about the distribution of the species in the Eastern Iberian Peninsula (Aguilar-Alberola et al., 2012).

During monitoring, various in situ water physicochemical parameters were measured. A digital meter (Hanna Instruments HI 98129) was employed to determine temperature (°C), conductivity (mS/cm) and pH. A Winkler test was used to measure the concentration of dissolved oxygen (mg/l). In addition, the Spanish Meteorological Agency (AEMET) provided daily precipitation and air temperature data. Crayfishes were collected monthly over one year between March 2011 and February 2012. We used 40 crayfish baited traps (40 cm × 40 cm × 80 cm) with two circular openings (8 cm in diameter) and 2 mm mesh. They were distributed regularly along the canal, separated by a 10-m distance, and were deposited at the bottom of the canal (ca. 1–2 m deep). Chicken liver was used as bait. For each sampling campaign, baited traps remained at the site overnight. If the crayfish catch did not attain a minimum of 15 individuals the next morning, traps were left at the site and were collected the following morning. After collection, the number of crayfishes per trap was counted, and juveniles and adults were differentiated. Those individuals whose total body length exceeded 6 cm were considered adults (Cano & Ocete, 2000). Smaller, juvenile crayfishes were not used for entocytherid extraction because they do not hold entocytherids, or do so but in very small amounts (pers. obs., and Aguilar-Alberola et al., 2012). Entocytherid extraction was done following the method tested by Mestre et al. (2011), as detailed hereafter. For each monthly sampling, between 15 and 20 adult crayfishes were selected randomly. These crayfishes were submerged individually in carbonated water (Fuente Primavera^{TM}) for 5 min. Then, carbonated water was filtered with a 63-µm mesh and the filter was submersed in a 50 ml jar with 96% ethanol. This method removes about 78% of entocytherids from each crayfish (Mestre et al., 2011). The selected crayfishes were weighed and sexed. The intermoult stage was also estimated using ordinal notation based on cephalothorax hardness by taking into account that the harder the cephalothorax, the longer the time since the last moulting event (Reynolds, 2002). This exoskeleton hardening stage (EHS) notation was coded as EHS 0 (very soft exoskeleton, including chelipeds), EHS 1 (soft, especially the cephalothorax lateral areas), EHS 2 (hard, but with some elasticity) and EHS 3 (very hard). Finally, crayfishes were individually marked with a small hole in one uropod. They were released back into the water in order to know if ostracods had already been removed from a collected crayfish in future recaptures so as to restore the crayfish population and to avoid disturbance effects on crayfish density if removed.

Under a high magnification stereomicroscope (Leica MZ16), all entocytherid ostracods were identified and counted following the same procedure: after filtering with a 63 µm mesh the carbonated water where crayfishes were submersed (see above), this filter was washed in a Petri dish with ethanol. The dish was carefully inspected under a stereomicroscope to measure and count all ostracods, classified onto different instars according to their size (see Aguilar-Alberola et al., 2012). Late juvenile instars (A-1) and adults were sexed according to the presence or absence of copulatory apparatus. Number of coupled male–female pairs, either mating or males guarding females (Danielopol, 1977), was noted differentiating between adult males coupled to A-1 females (i.e. 'biunguis females' according to Hobbs (1971)) and adult males with A-2 females (Aguilar-Alberola et al., 2012). [N.B.: in this paper, the term 'population' refers to the group of entocytherids that lives in the Bovar canal, and not merely the number of entocytherids inhabiting on a single crayfish, which is termed entocytherid 'load' per host herein. Entocytherid 'density' is used to refer to load per crayfish weight.]

All the graphs and statistical analyses were performed with *R* version 2.15.0 (R Development Core Team, 2012) using different packages. First, the population dynamics of entocytherids was described as follows: a constrained cluster analysis was done to group entocytherid densities [$\log_{10}$ (number of entocytherids + 1)/$\log_{10}$ crayfish weight] (according to Mestre et al., 2011) throughout the year. To that end, the *Rioja* package (Juggins, 2012) was employed with the Constrained Incremental Sum of Squares (CONISS) method. In addition, linear correlation was calculated between the juvenile entocytherid proportion and entocytherid density per crayfish. A paired *t* test was carried out to test for deviations from a balanced sex ratio. We calculated the sex ratio as the proportion of males [males/(females + males)] (Wilson & Hardy, 2002). Second, we analysed which environmental and host variables could be related to entocytherid load (number of entocytherids per crayfish) and the proportion of entocytherids belonging to juvenile instar A-7 (number of A-7 per crayfish/total number of entocytherids). We used Generalized Linear Mixed Models (GLMMs) by means of the *lme4* package (Bates et al., 2011) following Bolker et al. (2009). Entocytherid load models were constructed with a Poisson distribution and a logarithmic link function. Models for the proportion of instar A-7 individuals were constructed with a binomial distribution and a logit link function (Quinn & Keough, 2002). In the models with proportions, we merged two vectors (the number of A-7 individuals per crayfish, and the number of entocytherids that are not A-7) by means of the R command *cbind* into a single object corresponding to the response variable. Initially, general models with all the possible combinations of the following variables were obtained: month, crayfish weight and crayfish EHS. Then those models with lower Akaike Information Criterion (AIC) values—i.e. the best fit with the lowest number of variables—were chosen. In order to facilitate the understanding of the models results, the estimates with standard errors (marginalizing the random effect) were plotted together with the original data to see how well the model fits the data. In addition, the aim was to also know which variable could be related to month effects. To go about this, we constructed two other models for the two response variables with the following explanatory variables: water temperature, conductivity, oxygen concentration, number of captured crayfishes per trap per hour, and proportion of young crayfishes. All the models had a random factor corresponding to individual crayfish.

Results

Changes in the habitat followed a clear seasonal pattern (Fig. 1). The highest air temperatures were recorded in summer, reflected in the highest water temperature measured (25.4°C in July) and the lowest in February (water temperature 10.8°C). Precipitations concentrated mainly in autumn (September, October and November). Conductivity varied between 1.4 and 4 mS/cm, with maximum values recorded in dry summer months. The oxygen concentration was generally low and varied between 2.4 mg/l in August and 6.6 mg/l in February, and the pH average was 7.22 ± 0.28 (1 SD). Throughout the year, crayfish catch peaked twice in late summer (August–September) and winter (February) (Fig. 1). The juvenile crayfishes caught were less abundant than adults. Juveniles were caught all year round, except in spring (March, April and June), and were seen to be more abundant between July and November. Only three marked crayfishes were re-captured, and they were discarded for further statistical analysis of entocytherids population. For entocytherid extraction, 233 crayfishes (*P. clarkii*) were employed. Only three crayfishes had no entocytherids. Among all other individuals, only one entocytherid species was found: *A. sinuosa* (Rioja, 1942). The entocytherid load distribution was right-skewed, with 50% of crayfishes hosting less than 32 entocytherids and with a maximum value of 1,113 entocytherids. The majority of the counted ostracods were juvenile individuals, with higher proportions accounted for by the earliest instar A-7.

The cluster analysis of entocytherid density per month resulted in three groups of samples: G1 (March–June) with intermediate densities; G2 (July–October) with high densities; G3 (November–February) with low densities (Fig. 2). We detected a positive correlation between the proportion of juvenile entocytherid instars and entocytherid density per crayfish (Pearson's correlation, $r = 0.465$; $P < 0.001$). Throughout the year, the months with the highest densities also showed a higher proportion of juvenile individuals (Fig. 2).

Generally, the sex ratio of entocytherids did not significantly differ from 0.5 ($t = 0.12$; $P = 0.90$). Males dominated females only in July ($t = 2.35$; $P = 0.03$). Male–female coupled pairs were found throughout the year, but the number of pairs varied (Fig. 3). The highest average percentage of coupled pairs per crayfish (38%) was recorded in April, followed by July (17%) and August (18%). Most observed couples were adult males with A-1 females ($N = 241$), but some pairs composed of adult males with A-2 females were also observed ($N = 31$), and no male association with adult females was found. The main hatching period took place from March to September when the highest proportions of early juveniles (instar A-7) were recorded (Fig. 4). This hatching period was followed by an increase in the A-6 and A-5 proportions between May and October, an increase in more advanced juvenile instars (A-4 to A-2)

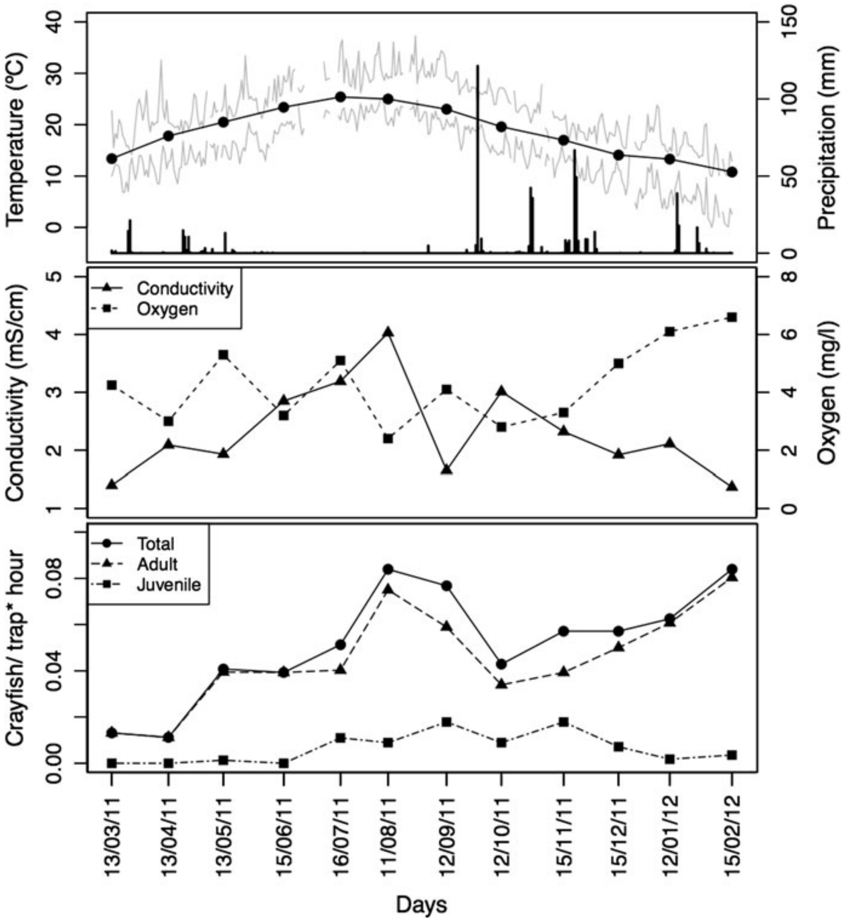

Fig. 1 Variations of the physicochemical parameters and crayfish catch throughout the sampling period, from 13 March 2011 to 15 February 2012, in the Bovar Canal (Pego-Oliva Wetland). *Upper graph* daily air temperature (minimum and maximum with *grey lines*), water temperature (*black line with dots*) and daily precipitation (*bars*). *Middle graph* conductivity and oxygen concentration. *Bottom graph* crayfish catch (crayfishes/ trap * hour)

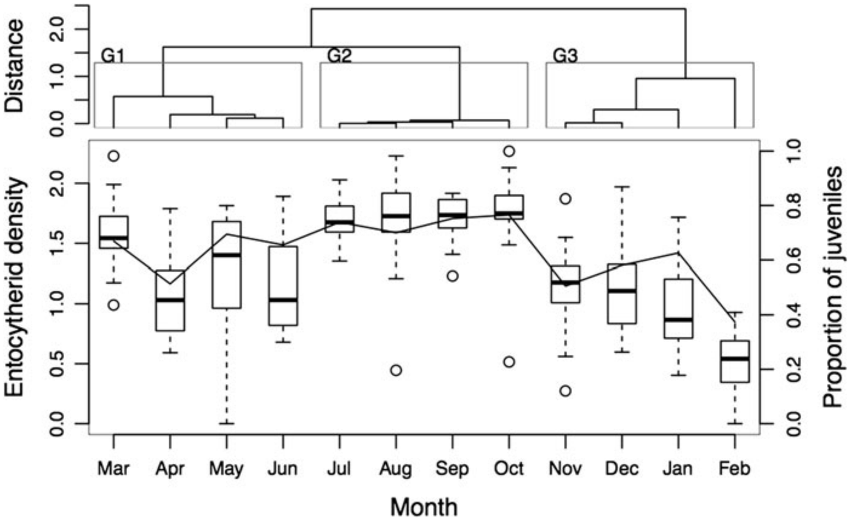

Fig. 2 At the *top*, a constrained cluster dendrogram of entocytherid density [$\log_{10}$ (number of entocytherids + 1)/$\log_{10}$ crayfish weight in g] resulting in three groups of months (G1–G3). Below a *boxplot* of entocytherid density and the proportion of juvenile entocytherids with a *line* (juvenile entocytherids/total entocytherids) per month

between July and November, and it finally resulted in the dominance of A-1 and adults between November and February. Therefore, although all instars were found throughout the year, the main growth period occurred between summer and early autumn.

The GLMMs analysis of the variables affecting population dynamics revealed that the most relevant combinations that explained entocytherid load were sampling month, crayfish weight and EHS (AIC = 1017.1). Month and EHS (AIC = 520.3) were the most important variables to account for the variation in the proportion of A-7 entocytherids (Table 1).

In the entocytherid load model (Table 2), the variance corresponding to the individual crayfishes' random effects was 0.676. Crayfish weight had a positive effect on the number of entocytherids per crayfish ($z = 9.16$; $P < 0.001$). The entocytherid load corresponding to each sampling month became significantly different from March, except for those belonging to the G2 clustering group (July–October). The largest difference corresponded to February when the smallest loads were seen (February; $z = -10.59$; $P < 0.001$). In addition, significant differences were detected between EHS 0 and other (harder, i.e. a longer time since the last moult) EHSs, which were related to greater entocytherids loads (EHS 1, $z = 2.36$; $P < 0.05$; EHS 2, $z = 3.43$; $P < 0.01$; EHS 3, $z = 3.80$; $P < 0.001$).

In the GLMMs model constructed for the proportion of A-7 juveniles (Table 3), the variance corresponding to the individual crayfishes' random effects was 0.246. Monthly significant differences were detected only in November ($z = -1.92$; $P < 0.05$) and February ($z = -3.09$; $P < 0.01$), as compared to other months when A-7 proportions where higher. The EHS effect was similar to the previous model, with a lower proportion of A-7 juveniles in the younger moult stage (EHS 0), if compared to others (EHS 1, $z = 2.34$; $P < 0.05$; EHS 2, $z = 3.12$; $P < 0.01$; EHS 3, $z = 3.24$; $P < 0.01$; Table 3). However, crayfish weight was not selected for the best model in this analysis. For the two previous models, the predicted values are presented together with the original data (excluding the random effect) in Fig. 5. The observed and predicted values showed a close pattern of variation.

Finally, the GLMMs analyses allowed us to detect which environmental variables could determine the variation between months on entocytherid load and A-7 proportions (Table 4). Two variables had significant effects on entocytherid load: water temperature, with a positive effect ($z = 6.93$; $P < 0.001$), and crayfish catch ($z = -2.95$; $P < 0.01$), with a remarkably strong negative effect (coeff = −13.99). Instead the variables for the A-7 proportions with significant effects were water temperature, with a positive effect ($z = 3.86$; $P < 0.001$), and conductivity, which had a negative effect ($z = -3.42$; $P < 0.001$), whereas crayfish catch had no significant effect. In both models, dissolved oxygen and the proportion of juvenile crayfishes appeared to have no significant effects on entocytherid population abundance and A-7 proportions.

Discussion

Throughout this 1-year study carried out in the Pego-Oliva Wetland, only one entocytherid species was found, *A. sinuosa*, which was associated with the red swamp crayfish *P. clarkii*, and with a prevalence approaching 100% for adult crayfishes. These results

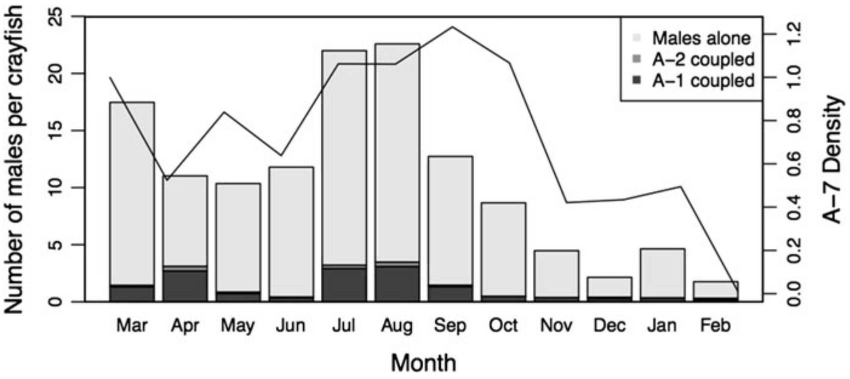

Fig. 3 *Bar graph* with the number of males associated with A-1 female, A-2 female and alone for different months. Density of A-7 entocytherids is represented by a line [$\log_{10}$ (number of A-7 + 1)/$\log_{10}$ crayfish weight in g]

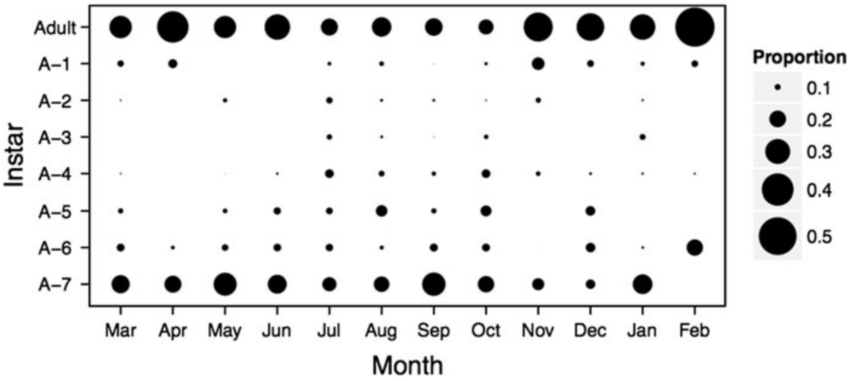

Fig. 4 *Bubble chart* showing monthly variations of the mean proportion of each entocytherid developmental instar (number of individuals of one instar/total entocytherids per crayfish), from A-7 to adult, all year round

Table 1 Main results of the GLMMs for response variables entocytherid load and proportion of stage A-7 entocytherids, which were built with combinations of explanatory variables crayfish weight, sampling month and EHS

Models	df	Entocytherid load			A-7 proportion		
		AIC	χ^2	*P*	AIC	χ^2	*P*
Weight	3	1202.6			567.1		
EHS	5	1230.8	0	1	563.3	7.83	<0.05
Weight + EHS	6	1189.3	43.44	<0.001	564.2	1.06	0.30
Month	13	1116.7	86.64	<0.001	527.4	50.79	<0.001
EHS + month	**16**	1086.5	0	1	**520.3**	**21.41**	**<0.001**
Weight + EHS + month	**17**	**1017.1**	**32.06**	**<0.001**	522.2	6.60	<0.001
Weight + month + EHS + weight:month	28	1028.1	28.74	<0.001	520.3	12.87	<0.01

The most relevant models are ordered according to degrees of freedom. The two best fitting models according to the AIC are indicated in bold. A specification of the form 'first:second' indicates an interaction term of the first with the second factor

are in agreement with the findings of Aguilar-Alberola et al. (2012) for this site and for other places in the Iberian Peninsula. The situation differs from that of North America, its original area, where one crayfish may have several entocytherids species (Hart & Hart, 1974). The poor entocytherid diversity found herein may be due to the fact that crayfishes only transported to Europe a small representation of the entocytherids inhabiting them in America. This pattern has also been found in other invasive species (Torchin et al., 2003). The high prevalence observed in this particular entocytherid species may have proven essential to explain its success in colonising exotic areas (Aguilar-Alberola et al., 2012).

Each crayfish entocytherid subpopulation differs in size and structure (Young, 1971; Hart et al., 1985; Aguilar-Alberola et al., 2012; this study). Across the observed variability, the positive correlation found between entocytherid density and the proportion of youngest juvenile stages was also observed by Young (1971) and Aguilar-Alberola et al. (2012). Furthermore, it might be an effect of the interaction among the reproductive, dispersal and mortality processes in which crayfish moult may be involved. Most probably, the highest entocytherid loads originate from recent reproductive successes that do not result in a proportional immediate increase in adult abundance within a crayfish. There may well be other factors, chiefly differential dispersal or mortality of crayfish juvenile stages, which could regulate entocytherid densities to such an extent that the crayfish moult can act as a generator of this type of processes producing entocytherid losses. Throughout the year, entocytherid density is at its minimum in winter, increases in spring, and peaks in summer and early autumn. As previously mentioned, the juvenile entocytherid proportion per crayfish is higher in those months with higher entocytherids densities, which correspond to favourable reproduction periods with high temperatures. This variation between months matches

Table 2 Results of the selected GLMM to account for crayfish entocytherid load depending on crayfish weight, sampling month and EHS

Variable	Estimate	SE	*z*	*P*
Intercept	2.019	0.344	5.87	<0.001
Weight	0.068	0.007	9.16	<0.001
Month				
April	−1.280	0.293	−4.37	<0.001
May	−0.673	0.280	−2.40	<0.05
June	−1.195	0.283	−4.22	<0.001
July	0.376	0.280	1.34	0.18
August	0.208	0.279	0.75	0.46
September	0.313	0.278	1.13	0.26
October	0.214	0.297	0.72	0.47
November	−1.226	0.294	−4.16	<0.001
December	−1.191	0.295	−4.03	<0.001
January	−1.610	0.294	−5.47	<0.001
February	−3.209	0.303	−10.59	<0.001
EHS				
1	0.848	0.323	2.63	<0.01
2	1.041	0.304	3.43	<0.001
3	1.431	0.310	4.61	<0.001

Table 3 Results of selected GLMM to account for the proportion of A-7 entocytherid juveniles depending on sampling month and EHS

Variable	Estimate	SE	*z*	*P*
Intercept	−2.186	0.311	−7.04	<0.001
Month				
April	0.218	0.231	0.94	0.35
May	0.337	0.199	1.70	0.09
June	−0.225	0.212	−1.06	0.29
July	−0.342	0.191	−1.90	0.06
August	−0.152	0.193	−0.79	0.43
September	0.317	0.186	0.17	0.09
October	−0.100	0.204	−0.49	0.62
November	−0.525	0.236	−2.22	<0.05
December	−0.469	0.244	−1.92	0.05
January	0.211	0.237	0.89	0.37
February	−3.338	1.081	−3.09	<0.01
EHS				
1	0.773	0.331	2.34	<0.05
2	0.971	0.311	3.12	<0.01
3	1.013	0.313	3.24	<0.01

Young's (1971) findings in the original distribution area. He suggested that entocytherid reproduction is high and mortality is low in spring and summer. Consequently, even though the reproduction events that generate higher entocytherid loads and juvenile instar proportions dominate during the favourable reproductive periods, the processes that result in entocytherid losses, viz. emigration, mortality and crayfish moult, prevail during unfavourable periods (winter). Nonetheless, this pattern does not seem to be universal among other entocytherids. For example, the *Uncinocythere occidentalis* population decreases in summer (Hart et al., 1985). This difference could be due to preference for low temperatures and lower heat tolerance in this species associated with the signal crayfish *Pacifastacus leniusculus* (Dana, 1852). This crayfish species prefers temperatures that are lower than 25°C (Bondar et al., 2005) and its associated entocytherids must acquire a similar preference for low temperatures in order to survive and reproduce.

In general, living as a commensal or parasite is considered as a way of life that may offer two advantages: first, environmental variation may diminish; second, there is always some food available (Poulin, 2007). This may be mostly true for endoparasites. However, commensal ostracods live as external epibionts on crayfish and, therefore, they might suffer equally the effects of water temperature or chemistry changes. Here, reduced predation pressure might be an important advantage in their way of life and not diminished environmental fluctuations. In our study, reproduction in the studied exotic entocytherid occurs all year round, as in its original distribution area (Young, 1971). However, within this uniformity, it seems that certain months are better for reproduction and development. In the Pego-Oliva wetland, the favourable reproduction period for *A. sinuosa* appears to span from April to August, when the proportion of juvenile instars increases. During these months, the largest numbers of coupled male–female pairs are followed almost immediately or with a 1 month lag by peaks in density of A-7 juveniles. In general term, entocytherid A-1 females form (pre-)mating pairs with adult males, and pairs of males with adult females are rare (Hobbs, 1971). Pairs of males with A-2 females are also infrequent, but have been previously found in the Pego-Oliva wetland (Aguilar-Alberola et al., 2012). According to our results, pairs with A-2 females are numerous when there are

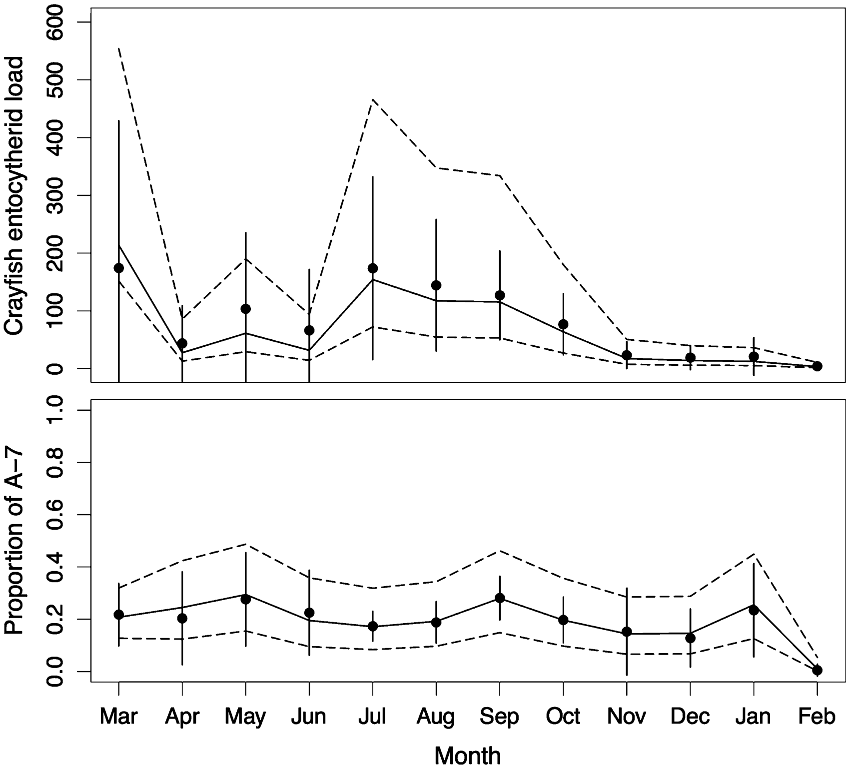

Fig. 5 Monthly variation of GLMMs estimated values together with the observed data marginalizing the random effect. *Top* GLMM results to account for crayfish entocytherid load. *Bottom* GLMM results to estimate the proportion of A-7 individuals. Monthly means predicted are represented by a *solid line* and the standard deviations by *long dashed lines*. In addition, the monthly mean for the observed data (*dots*) with the *error bars* (standard deviation) is also shown

Table 4 Main results of the GLMMs built to test the effects of environmental variables with monthly variation (water temperature, conductivity, dissolved oxygen, crayfish catch and proportion of juvenile crayfishes collected) on entocytherid load and proportion of A-7 juvenile entocytherids

Response variable	Explanatory variable	Estimate	SE	*z*	*P*
Entocytherid load	Intercept	1.062	0.690	1.54	0.12
	Temperature	0.182	0.026	6.93	<0.001
	Conductivity	−0.189	0.177	−1.07	0.29
	Oxygen	−0.002	0.095	−0.03	0.98
	Crayfish catch	−13.997	4.748	−2.95	<0.01
	Juvenile crayfish	1.638	0.958	1.71	0.09
A-7 proportion	Intercept	−1.381	0.373	−3.70	<0.001
	Temperature	0.060	0.016	3.86	<0.001
	Conductivity	−0.313	0.091	−3.42	<0.001
	Oxygen	−0.063	0.052	−1.22	0.22
	Crayfish catch	−0.455	2.784	−0.16	0.87
	Juvenile crayfish	−1.11	0.573	−1.93	0.05

more coupled males. Therefore, it is feasible that, under favourable reproduction conditions (adequate temperature, etc.), males might be less selective and not only form complexes with A-1, but also with younger females (A-2). Further research is required to know whether males really copulate with A-1 or A-2 females, or if they merely guard them to warrant subsequent copulation when females reach adulthood, as suggested by Danielopol (1977). The adult sex ratio remains at around 50% of males for most of the year, except in July when there are more males than females. These results contrast with those reported in the work by Aguilar-Alberola et al. (2012), in which females are generally more abundant than males.

According to our GLMMs, sampling period (month), crayfish weight and EHS (related to the time since the last moult event) all seem to be important explanatory variables that affect crayfish entocytherid load and population structure. The months with lesser entocytherid abundance and lower A-7 proportion correspond to the colder winter period. Young (1971) attributed the winter-related decrease in entocytherid load to a dilution effect of the entocytherid population because their spread for the colonisation of a new cohort of juvenile crayfishes appeared during this period. Consequently according to Young (1971), the host reproductive cycle may well prove more important than the physicochemical external parameters. However, the present study reveals that the external environmental conditions that do not directly relate to the host may also play an important role in the entocytherid life cycle by producing a direct effect on the reproductive rates. In our models, water temperature positively affects entocytherid load and the A-7 proportion. In most ostracods, temperature is regarded the main variable that affects growth rate and final size (Cohen & Morin, 1990). Yet although the negative effect of crayfish catch on entocytherid load found herein is in agreement with the 'dilution effect' idea proposed by Young (1971), the number of crayfish captures might be entangled with temperature. In addition, crayfish catch as an estimator of crayfish density can be modulated by crayfish activity to produce spurious results. In any case, further experiments are required to corroborate that the crayfish reproductive cycle actually affects entocytherid population variability. Conductivity negatively relates to the A-7 proportion; therefore, high conductivity probably disrupts entocytherid reproduction. High salinity can adversely influence *A. sinuosa* population maintenance and, although adults are tolerant to salinities above those measured in the field (pers. obs.), this condition can negatively affect hatching.

The positive effect of crayfish weight on their ostracod epibiont load has been previously reported in other studies on entocytherids (Young, 1971; Hart et al., 1985; Aguilar-Alberola et al., 2012). The reasons for this relationship can be that larger crayfishes offer more food, larger reproductive surfaces and longer intermoult times (Young, 1971) than smaller ones. However, this relation does not hold in some studies about epibionts of zooplanktonic crustaceans. Some authors have found positive relations (Holland & Hergenrader, 1981; Mohlenberg & Kaas, 1990; Threlkeld & Willey, 1993), while others have not (Kankaala & Eloranta, 1987; Weissman et al., 1993; Pérez-Martínez & Barea-Arco, 2000). Nevertheless, some zooplanktonic epibionts prefer species with larger bodies, such as daphnids (Barea-Arco et al., 2001; Regali-Seleghim & Godinho, 2004; Zalocar et al., 2011).

Our results of the impact of crayfish moult on entocytherid load reduction confirm the assumptions of Young (1971). Crayfish at a more advanced intermoult stage, for which more time has elapsed since the last moult, tend to have more entocytherids. The moult process, which is more frequent in younger crayfish, causes loss of entocytherids; consequently, the number of entocytherids increases as the intermoult time accumulates until the next entocytherid loss event after moulting. In the field, four recent crayfish exuviae were collected, three of which contained living entocytherids (pers. obs.), thus supporting the aforementioned explanation. In addition, the GLMM model for the proportion of A-7 entocytherid stages indicates that recently moulted crayfish (EHS 0) have a lower A-7 proportion than those at older intermoult stages. Entocytherids loss is, therefore, more intense in the early juvenile instars of the ostracod than more advanced stages, including adults, probably because the latter can better avoid remaining on older exuviae by moving to new crayfish exoskeletons (Young, 1971). This could explain the lower A-7 proportion noted in recently moulted crayfishes. Yet there is another possible hypothesis to explain this phenomenon; i.e. that the crayfish moult produces entocytherid loss which similarly affects all the entocytherid developmental stages, but the following recolonisation of 'empty' crayfish is produced mainly by older instars. In any case, crayfish moult affects the total loss of eggs attached on its exoskeleton (Young, 1971). Therefore, some time is needed for eggs to be replaced with new ones, which will hatch later in a new cohort of A-7 ostracods.

The Entocytheridae represent an interesting model taxon to study the ecology and behaviour of epibionts, although very few studies are currently available. Presently, there are many questions on their ecology that remain unanswered. For instance, host crayfish moult implies loss of ostracods, but we do not yet know whether this loss is partial or complete, or which ostracod instars are more affected. We do not know many details about their dispersal and colonisation mechanisms, or whether or not exotic species could colonise native European crayfish populations, such as *Austropotamobius pallipes* (Lereboullet, 1858), and implications of such a transfer on the conservation of the native species remain a mystery. Our results help provide an understanding of the ecology of epicommensal organisms, such as entocytherids, and the relationships they establish with their hosts, particularly within the invasion ecology framework, as they enable us to understand how combinations of associated alien species respond to recently colonised environments.

Acknowledgments We are indebted to Josep Antoni Aguilar-Alberola for his suggestions, knowledge and help with the fieldwork. We thank the staff of the Pego-Oliva Wetland Natural Park for their support, and the State Meteorological Agency (AEMET) for providing us with climatological data. This work has been supported by the ECOINVADER project (CGL2008-01296/BOS) funded by the Spanish Ministry of Science and Innovation and partially by the University of Valencia (project UV-INV_AE112-66304). English grammar and spelling have been checked by Helen Warburton (HyA). We would like to thank two anonymous reviewers and the associate editor for their constructive comments and help.

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CAPÍTOL V

Factors affecting abundance and host occupancy of a non-parasitic freshwater ectosymbiont (Ostracoda, Entocytheridae) inhabiting the invasive crayfish *Procambarus clarkii*

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Freshwater Biology. In review

Factors affecting abundance and host occupancy of a non-parasitic freshwater ectosymbiont (Ostracoda, Entocytheridae) inhabiting the invasive crayfish *Procambarus clarkii*.

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April 17, 2014

Abstract

1. Symbiosis represents a widespread and successful lifestyle essential to understand ecological interactions and evolutionary questions, so as to solve biological conservation issues. However, their research have been mainly focused on parasites. Three general patterns in parasite ecology have been proposed: 1) Aggregation; 2) positive and tight correlation between the mean symbiont abundance and its variance and 3) positive correlation between abundance and host occupancy of symbionts. The factors affecting abundance and host occupancy within symbiont species can be grouped into host features and environmental conditions. According to research in parasite ecology, environmental conditions seem to play a minor role.
2. We checked the three most recognised parasite patterns on a non-parasitic freshwater ectosymbiont and analysed the effects of host features (i.e. sex, body size, density and ecdysis) and environmental conditions (i.e. climate and water chemistry) on abundances and host occupancy of the symbiont. Our species model was an exotic ectocommensal ostracod, *Ankylocythere sinuosa*, inhabiting the invasive crayfish *Procambarus clarkii* in Europe. We sampled 373 crayfishes from 26 Spanish localities. We modelled both abundance per occupied host (i.e. intensity) and host occupancy (i.e. prevalence) by using zero-altered models and a backward model selection of host and environmental variables, and assessed model performance through graphical analyses of Pearson residuals and the relationship between observed and fitted values of the finally selected model.
3. We observed the three aforementioned parasite patterns in *A. sinuosa*. The strongest effect on abundances of *A. sinuosa* corresponded to conductivity (with negative effect), followed by host density (positive effect) and ecdysis (negative effects), and variables related to water chemistry, i.e. NH_4^+ concentration, alkalinity$/(Cl^- + SO_4^{2-})$ and Cl^-/SO_4^{2-} ratios, with moderate effects. Host occupancy was affected mainly by crayfish density (positive effect), and also by climatic conditions (i.e. max. T of warmest month, annual precipitation and precipitation seasonality).
4. Our results suggest that some "parasite rules" could actually be general "symbiont rules". Moreover, our symbiont model was markedly affected by climatic conditions at a regional scale acting on host occupancy, and water chemistry at a more reduced, local level, acting on symbiont abundances. This supports the hypothesis of the outstanding role of environmental conditions influencing population parameters of non-parasitic freshwater ectosymbionts. Physiological tolerances to highly unstable environmental factors, but also their dependence on greatly variable external food sources, are probably the reasons of the high sensitivity to environmental conditions of this symbiont type.

Keywords— commensal symbionts, environmental effects, exotic species, host features, population patterns

Introduction

Symbionts, that is organisms "living together" with other species (generally larger, termed the hosts) without implication of cost or benefit, represent a successful life style, given their broad taxonomical range and their ubiquity. Living together with a host can provide important advantages to the symbiont, like protection against predation, environmental or resource stability and higher dispersal capacity. Symbionts play indeed a significant role in ecology of invasions and conservation (Torchin et al., 2003; Strauss et al., 2012). Research in population ecology of symbionts has been mainly focused on parasites, while non-parasitic symbionts have generally received little attention.

In symbiont population ecology, the term infrapopulation refers to all the individuals of a symbiont species inhabiting a host (Poulin, 2007b). Abundance, prevalence and intensity are the most commonly used parameters to characterize a symbiont population, where abundance is defined as the mean number of symbionts per host (including uninfected), prevalence is the percentage of infected hosts in a population, whereas intensity is the mean number of symbionts per infected host (i.e. mean infrapopulation size). As these are terms derived from a parasitologist focus, we would rather use more general terms adapted from concepts of habitat selection (Lele et al., 2013): abundance per occupied host (i.e. intensity) and host occupancy (i.e prevalence).

A considerable effort has been made looking for general patterns in parasite ecology. The most well established "parasite rule" is the aggregated pattern of parasite abundances (Poulin, 2013). The positive and tight correlation between the log-transformed mean abundance and its log variance is other recurrent pattern found both between (Shaw & Dobson, 1995) and within (Matthee & Krasnov, 2009) parasite species. The slope of this relationship (i.e. the Taylor's slope) is species-specific, and it is positively correlated with the degree of aggregation (Matthee & Krasnov, 2009). The second pattern is explained by the trade-off of parasites between their increased probability to become lost at higher degrees of aggregation due to the increased host mortality rates in overly infested hosts, and their decreased mating chances in lower aggregation levels (Shaw & Dobson, 1995). In general, symbiont infrapopulations are subjected to density-dependent regulation processes, such as intraspecific competition for food and space. However, parasite abundances are strongly affected by their specific mechanisms: parasite-induced host mortality and host-induced parasite mortality (or reduction in fecundity) through immune responses (Stanko et al., 2006). The Taylor's slope has been suggested as an inverse indicator of the powerful of some of the regulation mechanisms typical of parasite-host systems (i.e. parasite-induced host mortality) (Anderson & Gordon, 1982). Lastly, the positive correlation between host occupancy and abundance among parasite species (Morand & Guégan, 2000) or populations within a species (Matthee & Krasnov, 2009) is another common pattern observed in parasites, that is a particular version of the widely documented positive relationship between local abundance and geographical distribution of free-living organisms (Gaston, 1996; Gaston et al., 1997).

The factors affecting abundance and host occupancy in a symbiotic species can be grouped into host features and environmental conditions. One of the most important sources of variability in abundance and host occupancy of symbiont populations is the heterogeneity in the rates of symbiont acquisition and loss by host individuals (Poulin, 2007b). There are two main sources of host heterogeneity: differences among hosts in the degree of exposure to symbionts and in their susceptibility to develop a symbiont infrapopulation. Parasitic symbionts are a special case where the second source, that is susceptibility to infection, can be determined by host immune responses or defensive behaviours, e.g. grooming against ectoparasites (Stanko et al., 2006). In general, for all symbiont types, there are two host features that are specially important: host body size and population density. To explain their effect on symbiont abundance and host occupancy, some authors invoked the metapopulation theory referring to patch size and connectivity (Poulin, 2007a). Bigger hosts improve habitat suitability through increasing spatial resources and, often linked to older host age, through larger exposure times to symbionts (Poulin, 2013). Both mechanisms favour an increase of symbiont abundances and host occupancy with increasing host body size. Some exceptions occur, for example, when older hosts acquire resistance against parasitic symbionts (Poulin, 2013). However, in general, the referred pattern is very common. In symbionts with a direct cycle, epidemiological models predict an increase in abundance and host occupancy with higher host densities (Anderson & May, 1978). However, this does not seem to be the rule in ectoparasites, for which contradictory cases with negative or lack of relationship have been recorded. A negative trend in host occupancy appears in symbionts with low transmission rates compared with host reproductive and dispersal rates, so that a percentage of the new born or dispersing hosts remain "unused" (Stanko et al., 2006). The same negative trend arises in symbiont abundances when reproductive rates of host population overcome that of symbiont, producing a "dilution effect" as a result of colonizations of new hosts (Krasnov et al., 2007). On the other hand, some host events represent a potential threat for symbionts to be removed from the host, such as ecdysis in crustaceans (Fernandez-Leborans, 2010).

Environmental conditions can affect symbiont fecundity or survival indirectly through the effects on the host (Kadlec et al., 2003) or directly (DeWitt et al., 2013).

At a temporal scale, seasonality in symbiont abundances is frequent, especially in aquatic ecosystems, and it can be linked to fluctuations in environmental conditions, such as temperature or salinity (Tucker et al., 2001; Castillo-Escrivà et al., 2013), even though sometimes it results from symbiont adjustments to their host cycle (Blanco & Frías, 2001). In spite of this, some works suggest that local environmental conditions play a minor role in population ecology of parasites compared with intrinsic parasite features (Krasnov et al., 2005; Poulin, 2006). Moreover, at regional scales and intraspecific level, host heterogeneity is considered as the most important source of variability in the abundances of symbiont populations (Poulin, 2013). Nevertheless, the relative importance of environmental conditions on host occupancy and abundances of symbionts might depend on different factors. Ectosymbionts are expected to be more exposed to external environment than endosymbionts. Freshwater environments are highly variable compared with marine or terrestrial ones (Hairston Jr & Bohonak, 1998). Therefore, freshwater ectosymbionts are expected to be markedly affected by environmental conditions. The symbiont-host relationship could also influence the dependence of symbionts on environmental conditions. Firstly, non-parasitic symbiont infrapopulations are expected to be less constrained due to the lack of regulatory mechanisms specific of parasite-host relationships, like parasite-induced host mortality, providing more variability in abundances to be modulated by abiotic conditions. Secondly, non-parasitic symbionts do not usually feed on host items (e.g. skin, blood...), being more dependent on external food sources susceptible to be affected by environmental conditions. Accordingly, we expect greater sensitivity to environmental conditions in non-parasitic symbionts. Therefore, we hypothesize that environmental conditions acquire special importance to explain the variability in host occupancy and abundance of non-parasitic freshwater ectosymbionts.

Abundance and probability of occurrence of freshwater free-living ostracods are affected by temperature and water chemistry (Mesquita-Joanes et al., 2012). Entocytherids are an ostracod family constituted by freshwater ectosymbionts commensal on other crustaceans. Among these, *Ankylocythere sinuosa* is an American entocytherid recently found in Europe associated with the invasive crayfish *Procambarus clarkii* (Aguilar-Alberola et al., 2012). The invasive success of *P. clarkii* is, in part, due to its broad tolerance to abiotic conditions (i.e. temperature and water chemistry) and high dispersal capabilities (Siesa et al., 2011). In this survey, we aimed to know: 1) if a non-parasitic freshwater ectosymbiont follows the three general ecological patterns observed in parasites (i.e. aggregation, tight positive correlation between log mean-variance in abundances and positive correlation occupancy-abundance) and 2) the relative importance of host features and environmental conditions affecting host occupancy and abundance of a non-parasitic freshwater ectosymbiont. To analyse those patterns, we modelled abundance per occupied host and host occupancy of *A. sinuosa* exotic populations associated with *P. clarkii* in SW Europe, according to host features (i.e. sex, body size, density and ecdysis) and environmental conditions (i.e. climate and water chemistry). The selected species pair was specially adequate for these purposes because *P. clarkii* tolerances offer a wide range of abiotic conditions susceptible to affect a freshwater ostracod and, on the other hand, the lack of other entocytherid species in European populations of *P. clarkii* (Mestre et al., 2013) avoided undesired effects of interspecific interactions.

Methods

Field and laboratory methods

The field samples were collected in the Iberian Peninsula and the Balearic Islands. We selected 26 sampling sites (Table 1) with presence of *P. clarkii*, widely distributed along the study area (Fig. 1). To minimize the seasonality effect on host occupancy and abundance of *A. sinuosa* (Castillo-Escrivà et al., 2013), we concentrated the sampling effort between April and June, during the 2010-11 seasons. From each sampling site, we set between 10-20 bait traps (40 cm × 40 cm × 80 cm) regularly distributed (10-m distance between traps) in the water body littoral area overnight. The next day we collected the *P. clarkii* individuals and recorded the number of crayfishes per trap. Afterwards, we selected a maximum of 20 crayfishes with a postorbital cephalothorax length > 10 cm for the collection of entocytherids, taking into account that juvenile crayfish are usually devoid of entocytherids (Castillo-Escrivà et al., 2013). The selected crayfishes were subjected to a standardised protocol of entocytherid removal consisting on submersion of each crayfish in a container filled with carbonated water during 15 min (Mestre et al., 2011). After the treatment, crayfishes were transferred to another container with 96 % ethanol. To isolate the entocytherids from each crayfish, the carbonated water from each container was individually filtered with a 63 µm mesh and the retained content was stored into 50-mL containers filled with 96 % ethanol. In the lab we sexed and weighted the crayfishes and measured their postorbital cephalothorax length. We estimated the crayfish intermoult stage, establishing a code based on body and cephalothorax hardness (Table 2; Castillo-Escrivà et al., 2013). The entocytherid samples were checked for species identification following Hart & Hart Jr (1974), and individuals of each developmental instar were counted, and sexed in the case of adults.

We measured in situ the water oxygen concentration, pH, temperature and conductivity of the sampled water body with portable probes and collected water and sediment samples. The water samples were analysed to determine the

Table 1. Location of the 26 sampling sites registered in this study, indicating the locality name, coordinates (latitude and longitude in WGS84 datum), altitude (in meters), type of the water body sampled, sampling date, the number of crayfishes (*Procambarus clarkii*) checked for entocytherid (*Ankylocythere sinuosa*) presence and abundance (with a total of 373 crayfishes sampled), and the median, 25 % and 75 % quantiles of the entocytherid abundance per crayfish (Nento) for each locality.

Site code	Locality	Coordinates (E°-W°,N°)	Altitude (m)	Water body	Sampling date	N crayfish	Nento median	Nento quantiles	Mean abundance	Prevalence
LOC002	Oliva	(-0.0555, 38.8775)	0	Canal	2010-04-28	15	88	(46, 135.5)	101.9	1
LOC003	Puçol	(-0.2715, 39.6207)	0	Canal	2010-04-30	20	58.5	(11.8, 140.3)	91.2	0.95
LOC004	Valparaíso	(-6.2882, 41.9952)	853	Reservoir	2010-05-06	9	8	(6, 16)	33.3	1
LOC005	Carucedo	(-6.7838, 42.4879)	495	Lake	2010-05-07	14	18.5	(4.5, 32.5)	27.6	1
LOC006	Chozas de Arriba	(-5.7139, 42.518)	893	Lagoon	2010-05-07	11	43	(11.5, 202.5)	165.4	1
LOC007	Vivares	(-5.8978, 39.0948)	279	Canal	2010-05-15	20	26.5	(11.8, 81)	70.4	1
LOC008	Valdebótoa	(-6.9160, 38.9958)	183	Pond	2010-05-15	17	9	(5, 14)	17.3	0.94
LOC009	Rosalejo	(-5.4980, 39.9440)	270	Stream	2010-05-15	5	3	(3, 18)	21.4	0.8
LOC010	Sarrià de Ter	(2.8126, 42.0077)	55	Stream	2010-05-20	20	76	(24.5, 136.8)	88.9	1
LOC011	Navarcles	(1.9040, 41.7309)	262	Stream	2010-05-20	10	5.5	(4, 11.5)	7.8	1
LOC012	Granollers	(2.2767, 41.5841)	107	River	2010-05-21	8	55.5	(23.8, 74.5)	54.1	1
LOC013	Quero	(-3.2275, 39.5422)	666	River-stream	2010-05-26	19	33	(16.5, 48.5)	35.5	1
LOC014	Socuéllamos	(-2.8597, 39.3469)	674	River	2010-05-27	4	5	(3, 7.5)	5.5	0.75
LOC018	Mequinenza	(0.3170, 41.3938)	61	River	2010-06-02	20	77.5	(49.5, 127.3)	130.5	1
LOC019	Alpicat	(0.5354, 41.6246)	203	Reservoir	2010-06-03	20	164.5	(80.3, 201.5)	154.6	1
LOC021	Sa Pobla	(3.064, 39.7899)	4	Canal	2010-06-09	4	0	(0, 0)	0	0
LOC022	Sòller	(2.7939, 39.7873)	745	Reservoir	2010-06-09	11	1	(0, 4)	2.3	0.55
LOC024	Sa Pobla	(3.0632, 39.7914)	3	Canal	2010-06-10	6	0	(0, 0)	0	0
LOC025	Puerto Real	(-6.1326, 36.5578)	15	Canal	2010-06-16	20	247.5	(154, 378.8)	299.5	1
LOC026	Palacios	(-5.9913, 37.1524)	7	Canal	2010-06-16	20	184	(92.5, 262.8)	193.4	0.95
LOC027	Padul	(-3.6052, 37.0075)	720	Canal	2010-06-18	16	202	(89.8, 311.3)	220.9	1
LOC028	Cubillas	(-3.6887, 37.2728)	612	River	2010-06-18	20	25	(18.8, 57.5)	49.7	1
LOC029	Robledo	(-2.3660, 38.8032)	923	Canal	2010-06-23	11	6	(2, 22.5)	13.9	0.82
LOC032	El Palmar	(-0.3147, 39.3268)	2	Pond	2011-04-20	20	124	(46.5, 284.3)	164.6	1
LOC034	Torreblanca	(0.2123, 40.2009)	4	Canal	2011-07-19	13	0	(0, 0)	1	0.23
LOC035	Torreblanca	(0.1914, 40.1899)	0	Canal	2011-07-19	20	3	(1, 4.3)	11.1	0.95

Table 2. Variables considered for modelling abundance per occupied host and host occupancy in European exotic populations of the entocytherid *Ankylocythere sinuosa* inhabiting the crayfish host *Procambarus clarkii*. The mean and standard deviation (SD) of the numeric variables are presented. *The crayfish weight was used as an explanatory variable in the regression part of the zero-altered (ZA) models, and as offset (with a $\log_e$-transformation) in the Poisson, Negative Binomial and count part of ZA models. **For the variable "nento", we provide the median and quantiles 25 % and 75 %.

Variable	Abbreviation	Type	Modelling role	Units	Mean	SD	Description
Number of entocytherids per crayfish	nento	Discrete	Response	Individuals	30**	(7, 13)**	Number of entocytherids inhabiting a crayfish.
Crayfish weight	CrWeight	Continuous	Offset and explanatory*	g	19.7	10.6	Crayfish host wet weight.
Crayfish sex	CrSex	Categorical	Explanatory	—	—	—	Sex of the crayfish host: male or female.
Crayfish moult	CrMoult	Categorical	Explanatory	—	—	—	Crayfish host intermoult stage: 0 = Crayfish body completely and extremely soft, including the chelas; 1 = Cephalothorax sides soft and very elastic; 2 = Cephalothorax sides hard but slightly elastic; 3 = Cephalothorax sides hard and totally rigid.
Crayfish density	CrDens	Continuous	Explanatory	crayfish/trap	2.27	1.74	Crayfish density index, i.e. mean number of crayfishes captured per trap.
Conductivity	cond	Continuous	Explanatory	$\mu S\,cm^{-1}$	1.95	2.95	Electric conductivity of the sampled water body.
Chlorophyll *a*	chlora	Continuous	Explanatory	$\mu g\,L^{-1}$	10.8	13.0	Clorophyll *a* concentration in the water.
Alkalinity	alk	Continuous	Explanatory	meq L^{-1}	3.50	1.59	(Bi-)carbonate alkalinity of the water.
Cl^-	Cl	Continuous	Explanatory	$mg\,L^{-1}$	380	746	Chloride concentration.
SO_4^{2-}	sulph	Continuous	Explanatory	$mg\,L^{-1}$	235	268	Sulphate concentration.
O_2	ox	Continuous	Explanatory	$mg\,L^{-1}$	7.01	3.19	Oxygen concentration.
NH_4^+	ammon	Continuous	Explanatory	$mg\,L^{-1}$	0.44	0.889	Ammonium concentration.
NO_3^-	nitr	Continuous	Explanatory	$mg\,L^{-1}$	23.0	32.4	Nitrate concentration.
PO_4^{3-}	phosph	Continuous	Explanatory	$mg\,L^{-1}$	0.116	0.158	Phosphate-P concentration.
Ratio alkalinity/($Cl^- + SO_4^{2-}$)	RatioAlkAn	Continuous	Explanatory	—	1.28	1.83	Ratio between alkalinity (meq L^{-1}) and the sum of chloride and sulphate (meq L^{-1}).
Ratio Cl^-/SO_4^{2-}	RatioClS	Continuous	Explanatory	—	2.71	3.70	Ratio between chloride (meq L^{-1}) and sulphate (meq L^{-1}).
Ratio NO_3^-/NH_4^+	RatioNitrAmmon	Continuous	Explanatory	—	127	165	Ratio between nitrate (meq L^{-1}) and ammonium (meq L^{-1}).
Habitat type	habitat	Categorical	Explanatory	—	—	—	Water body type, with two levels: lentic or lotic.
Sediment organic matter	SedOM	Continuous	Explanatory	—	0.074	0.049	Index LOI550 of the percentage of organic matter in the sediment of the water body.
Sediment humidity	SedH	Continuous	Explanatory	—	0.930	0.391	Proportion of water content in the sediment.
Maximum temperature	Tmax	Continuous	Explanatory	°C	30.6	2.56	Maximum temperature of the warmest month in the sampled locality.
Minimum temperature	Tmin	Continuous	Explanatory	°C	4.05	2.51	Minimum temperature of the coldest month.
Annual precipitation	precip	Continuous	Explanatory	mm	520	105	Annual precipitation in the sampled locality.
Precipitation seasonality	PrecipSeas	Continuous	Explanatory	%	44.2	11.5	Coefficient of variation of monthly precipitation.

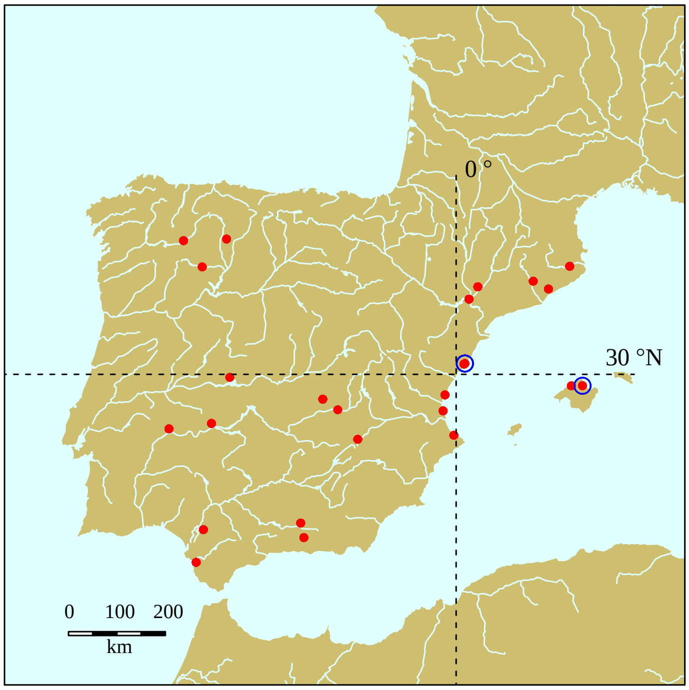

Figure 1. Study area in the Iberian Peninsula and Balearic Islands. We represented the hydrographic network in light blue. The 26 sampling sites of this survey are represented by red dots, and those surrounded by a blue circumference include two close localities. The map has a Mollweide equal-area projection.

concentration of anions (alkalinity, Cl^- and SO_4^{2-}) and nutrients (NH_4^+, NO_3^- and PO_4^{3-}) with standard methods (APHA-AWWA-WEF, 2005). We also estimated the chlorophyll *a* concentration based on Jeffrey & Humphrey (1975). Finally, we estimated the sediment water and organic matter content, with the LOI method (Heiri et al., 2001). The climatic data were obtained from WorldClim (Hijmans et al., 2005).

Checking for patterns of *A. sinuosa* abundance and host occupancy

We checked *A. sinuosa* abundances for aggregated distribution, by using the variance-to-mean ratio (Krebs, 1999). We analysed the relationship between the $\log_e$-transformed mean abundance and the $\log_e$ variance of the abundances of *A. sinuosa* populations from the 26 localities sampled, through a linear regression analysis. Finally, we also analysed the relationship between abundance per occupied host and host occupancy of *A. sinuosa* populations through a logistic regression.

Modelling abundance per occupied host and host occupancy of *A. sinuosa*

Model selection

We modelled abundance per occupied host and host occupancy of *A. sinuosa* populations by initially considering 22 explanatory variables (Table 2). To avoid problems relating to collinearity (Dormann et al., 2012), we analysed the correlations between all the paired combinations of explanatory variables and, for all the pairs with an $|r| > 0.7$, we removed one of the correlated variables according to a biological criterion. We used crayfish weight as a measure of crayfish size (see Fig. S1 in Supporting Information), and included the natural logarithm of crayfish weight as an offset variable in the count models to fix the effects of host size in abundances (Zuur et al., 2009). During the model type selection, we worked with a full model, that is a model with all the explanatory variables except those removed in the collinearity analysis. As the response variable showed a distribution with a large proportion of zero counts (see Fig. S2), we assumed the following model possibilities: zero-altered Poisson (ZAP) and zero-altered Negative Binomial (ZANB). ZA models have two components: the count part that analyses the entocytherid abundance per occupied host with a Zero-Truncated distribution and a "log" link function, and the regression part that analyses host occupancy with a Binomial distribution and a "logit" link function. This kind of models has greater capacity to predict the absences in response variables characterized by a zero-inflated distribution due to intrinsic reasons (i.e. there are not false absences due to methodological issues), and is specially useful for modelling symbiont population parameters because allows us to integrate both abundance per occupied host and host occupancy within the same model frame (Heinzmann & Torgerson, 2008). ZANB models are required for response variables with additional overdispersion in the non-zero data. We compared both ZAP and ZANB models using a Likelihood Ratio Test (LRT) to decide which one to use. We also compared the ZAP, ZANB and a Negative Binomial (NB) model using a Poisson as null model through the Akaike Information Criterion (AIC) and the zero capturing (i.e. the capacity of the model to predict the observed zero values), in order to make sure we selected the best option. Finally, comparisons between non-nested models (e.g. NB against ZANB) were made through a Vuong non-nested hypothesis test (Vuong, 1989).

Selection of explanatory variables and interactions

The hypothesis testing strategy to find the optimal model was a backward selection (Zuur et al., 2009), starting from the full model (i.e. with all the explanatory variables in both model components) and using a LRT testing procedure. When we obtained the optimal model without interactions, we continued with the selection of interactions, following the same backward procedure, starting from a model that included all the possible paired variable interactions between those variables selected in the optimal model without interactions (we did not contemplate higher level interactions to avoid an excessive model complexity). In this manner, we finally obtained an optimal model with interactions. The interactions were classified into synergistic, antagonistic and buffering, following Cohen et al. (2003).

Model assessment

The model assessment was based on a graphical analysis of Pearson residuals (Zuur et al., 2009) and the linear relationship between observed (y_i) and fitted ($\hat{y}_i$) values, applied to the optimal model with interactions. We checked Pearson residuals for the existence of outliers and temporal patterns. On the other hand, we did a linear regression of y_i versus $\hat{y}_i$ and used the slope (m) and intercept (b) of the fitted line to assess the model consistency and bias, by testing the hypotheses of a model with consistent predictions (H_o: $m = 1$) and not biased (H_o: $b = 1$). The coefficient of determination (r^2) was used to know the proportion of variation of y_i explained by the $\hat{y}_i$ values as a measure of the explanatory capacity of the model. We also calculated the root mean squared deviation (RMSD) that shows the mean deviation of $\hat{y}_i$ with respect to y_i, in the same units as the response variable, i.e. number of entocytherids per host (Piñeiro et al., 2008).

The statistical analyses were made with R (R Core Team, 2013). Additionally to standard R packages, we also used MASS for NB models (Venables & Ripley, 2002), PSCL (Jackman, 2012) to do ZA models and Vuong tests, and the LRT tests were made through LMTEST (Zeileis & Hothorn, 2002).

Results

Patterns of *A. sinuosa* host occupancy and abundance

The general value of host occupancy was 91.15 % (N = 373). We found higher levels of host occupancy in most of the localities (Table 1). Moreover, the abundance of *A. sinuosa* among occupied hosts was highly aggregated ($s^2/y = 190.5$). Approximately 20 % of the hosts harboured 67 % of the whole population of *A. sinuosa*. There was a positive correlation between the $\log_e$-abundance and the $\log_e$-variance of the abundance of *A. sinuosa* (Taylor's slope = 1.67; SE = 0.10; $t = 16.07$; $df = 22$; $P < 0.01$; Fig. 2a). Furthermore, the relationship showed a tight fit ($r^2 = 0.92$). Finally, there was a positive correlation between abundance

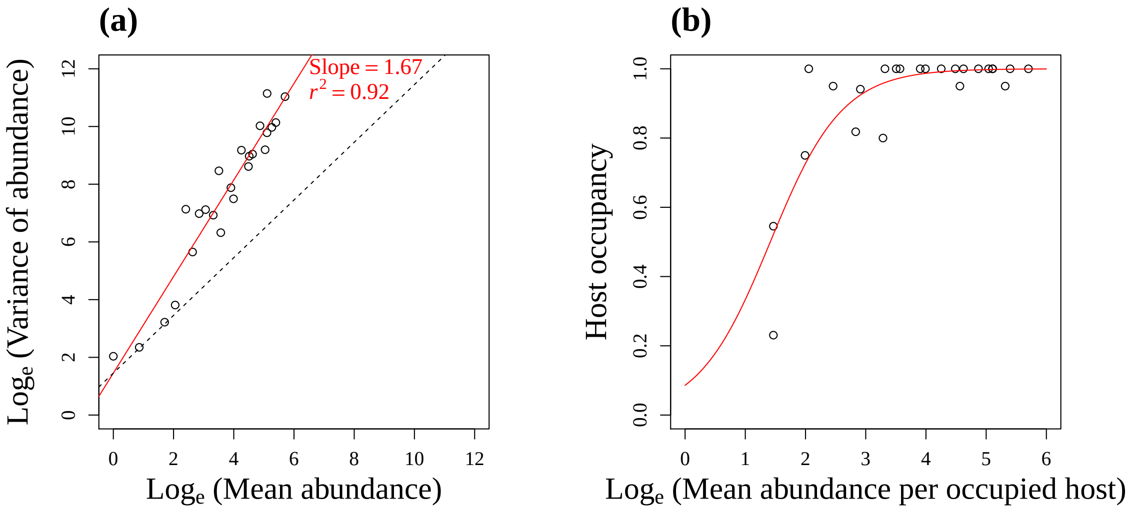

Figure 2. Patterns in abundance and host occupancy of European exotic populations of the entocytherid *Ankylocythere sinuosa*, commensal on the invasive crayfish *Procambarus clarkii*: **(a)** Relationship between $\log_e$ variance of abundance and $\log_e$ mean abundance. The solid line (in red) corresponds to the fitted regression and the dashed line is a reference regression line with slope (m) equal to 1. **(b)** Relationship between abundance per occupied host and host occupancy. We represented de fitted curve of the logistic regression. The information about the 24 localities included in these analyses is shown in Table 1 (the two localities without occupied hosts, i.e. LOC021 and LOC024, were removed).

per occupied host and host occupancy (Estimate $= 1.67$; SE $= 0.29$; $Z = 5.82$; $P < 0.01$; Fig. 2b).

Abundance per occupied host and host occupancy of *A. sinuosa*

We checked a total of 373 crayfishes for entocytherid presence and abundance. The descriptive statistics (*mean* and SD) of all variables considered for the modelling process are shown in Table 2.

Model selection

The results of the model selection through the AIC (Table 3) show a great improvement of NB and ZANB models compared with their respective Poisson and ZAP, indicating a high level of overdispersion in the non-zero values of the response variable. Moreover, the ZA models were better predicting the observed zero values, getting a 100 % of zero captures, compared with the 21 % for Poisson and 73 % for NB. Finally, a comparison of NB with ZANB using the Vuong test evidenced the superiority of ZANB (Vuong statistic $= -3.74$; $P < 0.01$). Therefore, we selected the ZANB models that were proved as better dealing with overdispersion and excess of zero counts in our data.

Table 3. Comparison between model types for modelling abundance and host occupancy of European populations of the exotic entocytherid *Ankylocythere sinuosa* inhabiting the crayfish *Procambarus clarkii*, using Akaike Information Criterion (AIC) and zero count capturing. Models compared are zero-altered Poisson (ZAP), zero-altered Negative Binomial (ZANB), Negative Binomial (NB) and Poisson. We used the Poisson as null model for the comparison through the AIC. The number of observed zeros in our data were 33.

	Poisson	NB	ZAP	ZANB
AIC	21235	3616	19980	3582
ΔAIC	0	-17618	-1254	-17653
Zero captures	7	24	33	33

Selection of explanatory variables and interactions

As a result of the selection of explanatory variables, we obtained an optimal model with seven variables for the count part and five variables for the logistic part (See table S1 for more detailed information about the process; the selected variables are shown in Table 4). During the collinearity analysis, three variables were removed due to collinearity with others ($r > 0.7$): "chlora" and "Cl" with "cond", and "SedH" with "SedOM". We decided to keep "cond" and "SedOM" because "cond" affects osmotic regulation in crustaceans and "SedOM" could be related with the food provisioning of entocytherids during their dispersal phase. The selected interaction terms for the final optimal model with interactions were six for the count part, and zero for the logistic part (more detailed information in table S2; the selected interactions are shown in Table 5).

Fixed effects for entocytherid abundance per occupied host

The set of explanatory variables affecting the entocytherid abundance per occupied host was composed by four variables related with water physico-chemical features, two variables of host features and one climatic variable (Table 4, count part). According to the *beta* values of the model without interactions (Table 4, count part), the variable showing the strongest effect was the water electric conductivity ($|beta| = 1.05$) followed by both variables related to the host, crayfish intermoult stage (levels 2 and 3) and crayfish density, all with *beta* values around 0.5. The three remaining physico-chemical variables, i.e. NH_4^+ concentration and both alkalinity/($Cl^- + SO_4^{2-}$) and Cl^-/SO_4^{2-} ratios, had absolute values of *beta* between 0.23 and 0.31. The slightest effect corresponded to the climatic variable, precipitation seasonality ($|beta| = 0.18$). The RR values (Table 4, count part) show an expected decrease of around a 30 % of entocytherid abundance for an increase in 1 $\mathrm{mS\,cm^{-1}}$ in water conductivity (RR = 0.70; 95 % CI: 0.64 − 0.77); an approx. increase of 64–70 % of abundance for crayfish moulting stages 2 and 3 compared with stage 0 (RR (stage 2) = 1.69; 95 % CI: 1.01 − 2.82; RR (stage 3) = 1.64; 95 %

CI: 0.97 – 2.79); an increase of 35 % for an increase of one crayfish captured per trap (RR = 1.35; 95 % CI: 1.27 – 1.44); an increase of 41 % per increase of $1\,\mathrm{mg\,L^{-1}}$ in NH_4^+ concentration (RR = 1.41; 95 % CI: 1.25 – 1.59); a decrease of 12 % per increase of one unity in ratio alkalinity/($Cl^- + SO_4^{2-}$) (RR = 0.88; 95 % CI: 0.82 – 0.94); and an increase of 6.7 % per increase of one unity in ratio Cl^-/SO_4^{2-} (RR = 1.07; 95 % CI: 1.02 – 1.11). The resulting model with interactions contained 6 interactions in the count part (Table 5). All interactions were composed by pairs of physico-chemical variables, with the exception of two interactions where crayfish density was present.

Table 4. Summary of fixed effects for the zero-altered Negative Binomial (ZANB) optimal model without interactions to analyse host occupancy and abundance per occupied host in European exotic populations of the entocytherid *Ankylocythere sinuosa* inhabiting the crayfish host *Procambarus clarkii*. The count part of the model follows a truncated negative binomial distribution with a "log" link function to model abundance per occupied host. The logistic part has a binomial distribution with "logit" link function to model host occupancy. Model terms are named using the variable abbreviations in Table 2. The beta coefficients (*beta*) are the standardized coefficients of the term effects, indicating an additive change in the logarithm (log) of the response variable per 1 SD increase in the predictor. The relative risk (RR; for count part) and the odds ratio (OR; for binomial part) represent the multiplicative change in the mean abundance per occupied host (RR) and host occupancy (OR) of entocytherids for an increase of a unit in the predictor (the units of each predictor are shown in Table 2). Finally, we also provide the unstandardized coefficients (Estimate) with their 95 % confidence intervals (95 % CI), their Z-statistic (*Z*) and p-values (*P*). The dispersion parameter of the ZANB was $k = 1.095$. *Significant P (< 0.05).

Model part	Model Term	*beta*	RR and OR	Estimate	95 % CI	*Z*	*P*
Count	Intercept	0.668	—	-0.167	(-0.815, 0.481)	-0.505	0.614
	CrMoult 0	0	1	0	—	—	—
	CrMoult 1	0.034	1.034	0.033	(-0.489, 0.557)	0.127	0.899
	CrMoult 2	0.525	1.691	0.525	(0.012, 1.038)	2.007	0.044*
	CrMoult 3	0.497	1.644	0.497	(-0.030, 1.025)	1.847	< 0.001*
	CrDens	0.526	1.353	0.302	(0.238, 0.366)	9.307	< 0.001*
	cond	-1.048	0.701	-0.355	(-0.454, -0.256)	-7.036	< 0.001*
	ammon	0.305	1.409	0.343	(0.222, 0.464)	5.561	< 0.001*
	RatioAlkAn	-0.234	0.880	-0.128	(-0.197, -0.059)	-3.651	< 0.001*
	RatioClS	0.241	1.067	0.065	(0.024, 0.106)	3.091	0.002*
	PrecipSeas	0.175	1.015	0.015	(0.006, 0.024)	3.306	< 0.001*
Logistic	Intercept	7.951	—	-25.716	(-39.592, -11.841)	-3.632	< 0.001*
	CrWeight	0.687	1.067	0.065	(0.009, 0.120)	2.291	0.022*
	CrDens	7.371	68.988	4.234	(2.665, 5.803)	5.289	< 0.001*
	Tmax	2.304	2.457	0.899	(0.437, 1.361)	3.810	< 0.001*
	Precip	1.577	1.015	0.015	(0.007, 0.023)	3.608	< 0.001*
	PrecipSeas	-3.244	0.754	-0.283	(-0.404, -0.162)	-4.58	< 0.001*

Fixed effects for probability of host occupancy

The selected variables for modelling the host occupancy in *A. sinuosa* were three climatic and two features related to host (Table 4, logistic part). According to the standardised coefficients (*beta*; Table 4, logistic part), the variable with the strongest effect on host occupancy was crayfish density ($beta = 7.37$), followed by the climatic variables, with absolute values between 1.5 and 3, and the slightest effect corresponded to crayfish weight ($beta = 0.69$). Following the criterion based on odds ratios (OR) of our optimal model without interactions (Table 4, logistic part), there was a positive greater effect of crayfish density, with a mean increase of 69 times the odds of entocytherid presence in a host per each increase of one crayfish captured per trap (OR = 68.99; 95 % CI: $14.36 - 331.25$). Regarding the effects of climatic variables, an increase of 1 °C in maximum temperature of warmest months produces a 2.5 times odds increase (OR = 2.46; 95 % CI: $1.54 - 3.90$); for an increase of 1 mm of annual precipitation we expect an increase of 1.5 % in the odds (OR = 1.02; 95 % CI: $1.01 - 1.02$); finally, there is an expected 24 % decrease in odds for each increase of 1 unit of precipitation seasonality (OR = 0.75; 95 % CI: $0.67 - 0.85$). No significant interactions were selected for the logistic part (Tables S2 and 5, logistic part).

Table 5. Summary of fixed effects of interactions for the zero-altered Negative Binomial (ZANB) optimal model with interactions to analyse abundance per occupied host and host occupancy in European populations of the entocytherid *Ankylocythere sinuosa* inhabiting the crayfish host *Procambarus clarkii*. The count part of the model follows a truncated negative binomial distribution with a "log" link function to model abundance per occupied host. The logistic part has a binomial distribution with "logit" link function to model host occupancy. Model terms are named using the variable abbreviations in Table 2, and ":" as symbol of interaction. The relative risk (RR) represents the multiplicative change in the effect of a predictor for an increase of a unit in the other interacting predictor (the units of each predictor are shown in Table 2). We also provide the interaction type (synergistic, antagonistic or buffering), the unstandardised coefficients (Estimate) with their 95 % confidence intervals (95 % CI), their Z-statistic (*Z*) and p-values (*P*). No interactions were selected for the logistic part. The dispersion parameter of this ZANB was $k = 1.38$. We did not provide main effects for individual predictors of this model because, in the presence of interactions, they are very difficult to interpret. Synergistic interactions are those where both predictors affect the response variable in the same direction and their combined effect is stronger than additive. In antagonistic interactions, both variables affect the response variable in the same direction but their combined effect is lower than additive (Cohen et al., 2003). Finally, buffering interactions are composed by two predictors of opposite sign, and the increase of one predictor weakens the effect of the other. *Significant *P* (< 0.05).

Model part	Model Term	RR	Type	Estimate	95 % CI	*Z*	*P*
Count	CrDens : ammon	10.43	Synergistic	2.35	(1.55, 3.14)	5.80	< 0.001*
	CrDens : RatioClS	0.75	Antagonistic	-0.29	(-0.37, -0.20)	-6.56	< 0.001*
	cond : ammon	7.87	Buffering	2.06	(1.49, 2.64)	7.07	< 0.001*
	cond : RatioAlkAn	15.44	Antagonistic	2.74	(2.02, 3.45)	7.51	< 0.001*
	ammon : RatioAlkAn	1427.14	Buffering	7.26	(5.25, 9.28)	7.06	< 0.001*
	RatioAlkAn : RatioClS	1.53	Buffering	0.42	(0.32, 0.52)	8.13	< 0.001*

Model assessment

Pearson residuals of our optimal model with interactions showed a skewed distribution (Median $= -0.33$; 1Q $= -0.71$; 3Q $= 0.43$; Min $= -1.20$; Max $= 10.88$). The distribution of Pearson residuals along the fitted values did not show clear general patterns, although some unclear small patterns were present (Fig. 3a). Pearson residuals (ε^P) evidenced the existence of two outliers in the model (Fig. 3a, S3a): CR037 ($y_{CR037} = 216$; $\hat{y}_{CR037} = 21.40$; $\varepsilon^P_{CR037} = 9.19$) and CR403 ($y_{CR403} = 161$; $\hat{y}_{CR403} = 14.96$; $\varepsilon^P_{CR403} = 10.88$), both corresponding to crayfishes with moderate infrapopulation sizes belonging to localities with very low mean abundances (Table 1: LOC004 for CR037 and LOC035 for CR403).

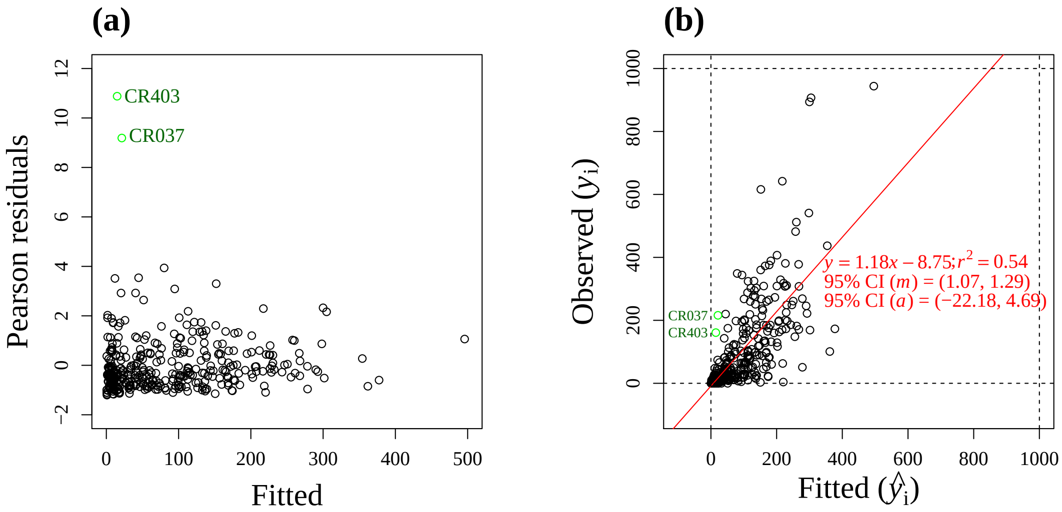

Figure 3. Assessment of the zero-altered negative binomial (ZANB) model with interactions for analysing abundance per occupied host and host occupancy of the entocytherid *Ankylocythere sinuosa* on crayfish *Procambarus clarkii*: **(a)** Plot of Pearson residuals versus fitted values. **(b)** Plot of observed (y_i) versus fitted ($\hat{y}_i$) values of the model (units are number of entocytherids within a host). The continuous line (in red) is the fitted line of the linear model fitting both variables. We also provide its equation and the 95 % confidence intervals (95 % CI) of slope (m) and intercept (a). We added dashed lines highlighting the 0 and 1000 values for both variables to remark the trend of the fitted line in relation to an $m = 1$ and $a = 0$ of a theoretical perfect fit. The total number of crayfishes analysed in the ZANB model were 373. The outliers of the model (in green) are labelled using a codification for the corresponding crayfish (CR037 and CR403). Crayfish CR037 belongs to the locality LOC004; CR403 was collected from LOC035 (information about localities in Table 1).

The linear model fitting of y_i versus $\hat{y}_i$ (Fig. 3b) showed that more than 50 % of the variance of y_i is explained by $\hat{y}_i$ ($r^2 = 0.54$). The intercept (b; Fig. 3b) was not significantly different from zero ($Z = -1.28$; $df = 371$; $P = 0.20$), indicating no evidence for model bias, but the slope (m; Fig. 3b) was different from one ($Z = 3.20$; $df = 371$; $P < 0.01$), so that there was evidence for model inconsistency.

However, the same ZANB model fitted with the dataset without both outliers did not show evidence for model inconsistency ($m = 1.11$; 95 % CI = $[0.99, 1.22]$; $Z = 1.87$; $df = 369$; $P = 0.06$). On the other hand, the mean deviation of $\hat{y}_i$ with respect to y_i was 94 entocytherids per crayfish (RMSD = 93.86). This mean value was inflated by few data with greater negative $\hat{y}_i - y_i$ values given that the majority of values are compressed within a ratio of 100 units around the 0 (Fig. S3b). That few data belong to the crayfishes with greater amount of entocytherids that were underestimated by the ZANB model. Indeed, our model showed a trend to underestimate the few larger values of y_i ($>$ 500 entocytherids per crayfish; Fig. 3b), and a slight trend to overestimate the main body of data (Fig S3b). We did not find differences in predictions between crayfishes sampled in different months (see Fig. S4).

Discussion

Patterns of *A. sinuosa* abundance and host occupancy: following parasite rules

As we have shown, the ostracod *A. sinuosa*, a non-parasitic freshwater ectosymbiont, followed three general patterns in parasite ecology (Poulin, 2007a; Matthee & Krasnov, 2009): high level of aggregation, positive and tight correlation between log mean abundance and log variance of abundance and positive correlation between abundance per occupied host and host occupancy.

A variance-to-mean ratio and a Taylor's slope very far from the value of unity indicated that *A. sinuosa* abundance per occupied host was highly aggregated. Actually, c. 67 % of the whole population of *A. sinuosa* was concentrated on 20 % of the hosts, a value slightly lower than the quantitative pattern commonly observed in parasites by other authors, with 20 % of hosts harbouring 80 % of their parasites (Poulin, 2007b). However, this "twenty-eighty rule" does not apply to all parasite systems, and it depends on other interrelated infection parameters, such as host occupancy. Accordingly, in our case, the lower percentage could be explained by the higher global value of host occupancy of *A. sinuosa* populations (91 %) favouring a greater dispersion of entocytherids among available hosts. Regarding the intraspecific relationship between log mean abundance and log variance of abundance, the values of Taylor's slope and r^2 are very similar to those observed in some ectoparasitic arthropods (Matthee & Krasnov, 2009). Therefore, this constrained relationship, indeed with a high fit ($r^2 = 0.92$), not only occurs in parasites, but also in non-parasitic symbionts, at least at the intraspecific level. This finding has important consequences, as the widely accepted explanation for this pattern is the trade-off between parasite-induced host mortality at higher levels of aggregation and the decrease of mating encounters of symbionts at lower abundance levels (Shaw & Dobson, 1995). As parasite-induced host mortality is a regulatory mechanism of symbiont abundances exclusively affecting parasitic symbionts, our results suggest that other different mechanisms are able to constrain the upper limits of symbiont infrapopulations to produce the pattern. There are three possible mechanisms for regulating entocytherid infrapopulations: symbiont intraspecific competition (Mori et al., 2001; Hayashi & Ichiyanagi, 2005), host-induced symbiont removal through behavioural responses (i.e. grooming activity; Jones & Lester, 1996) and host-induced removal through ecdysis (Walton & Hobbs Jr, 1971; Young, 1971; Castillo-Escrivà et al., 2013). High levels of host occupancy probably favoured the invasive success of *A. sinuosa* by increasing the chance to be present in the founding crayfish population, and protecting its invasive populations from global disappearance in the new area (Bush & Kennedy, 1994). Additionally, the high levels of host occupancy suggest a high efficacy of symbiont transmission. Supporting this possibility, Young (1971) experimentally demonstrated the horizontal transfer of entocytherids among hosts in a few days without requiring physical contact between them. Nevertheless, a positive correlation between abundance per occupied host and host occupancy indicates that entocytherids have some limitations in their off-host mobility, so that at lower mean abundances, the probability of host encounters decrease and some hosts remain uncolonised (Pennuto et al., 2002).

Factors affecting abundance per occupied host and host occupancy of entocytherids: environmental conditions matter

Abundance per occupied host and host occupancy of *A. sinuosa* were differently affected by the factors analysed in this survey, composed by host features and environmental conditions. Regarding the abundance per occupied host of *A. sinuosa*, the strongest effect corresponded to water conductivity. Water electric conductivity is used as a surrogate of salt concentration, which challenges the physiology of freshwater crustaceans. Indeed, microcrustacean species richness generally decreases with increasing salinity in non-marine aquatic environments (Jensen et al., 2010). Some ostracod species can evolve osmotic regulation mechanisms to tolerate wide conductivity ranges (Mesquita-Joanes et al., 2012), and this could be the case of *A. sinuosa*, given that conductivity does not seem to significantly affect host occupancy. Nevertheless, osmoregulation has energetic costs so that an increase of conductivity might reduce symbiont reproductive rates being reflected in a decrease of the mean infrapopulation size in localities with higher conductivities. A second set of explanatory variables strongly affecting abundance per occupied host were host density and ecdysis. The positive effect of host density indicates that host reproductive rates do not overcome those of the symbiont. Nevertheless, although a "dilution effect" of symbiont abundances was not observed at the spatial level of this survey, *A. sinuosa* infrapopulations show annual fluctuations characterized by a decrease during winter that has been attributed to this phenomenon (Young, 1971; Castillo-Escrivà et al., 2013). The convergence of two population events during the winter season might explain this pattern: firstly, a strong decrease in symbiont reproductive activity and, secondly, the appearance of a new cohort of juvenile crayfishes with appropriate sizes to be colonized by the entocytherids. Our findings therefore suggest that *A. sinuosa* completely reestablishes its infrapopulations during the following spring so that the dilution effect is only seasonal. The negative effects of host ecdysis observed here are in accordance with other surveys focused on the same symbiont species (Young, 1971; Castillo-Escrivà et al., 2013). Indeed, there is wide evidence about the impact of host ecdysis on non-parasitic ectosymbionts (Jones & Lester, 1996; Mori et al., 2001; Fernandez-Leborans, 2010). In our case, as host ecdysis does not seem to affect host occupancy, this means that crayfish does not lose all the entocytherids through a moult event, or a fast recolonization of the crayfish individual proceeds soon after the moult process. Regarding the first possibility, some ectosymbionts have behavioural adaptations to avoid their loss during the moult (Itani et al., 2002). However, in our case, at least certain proportion of entocytherids are unable to avoid their removal through moult. For example, Castillo-Escrivà et al. (2013) observed entocytherids inhabiting an old exuvia collected in the field. The second alternative of a fast recolonization could be favoured by the protective habit of burrowing crayfishes to moult into a shelter cohabiting a while with the old exuvia, as has been proposed for branchiobdellids (Mori et al., 2001). Unlike host ecdysis, crayfish sex was not selected in our models. Walton & Hobbs Jr (1971) showed that females of the crayfish *Cambarus bartonii* harboured heavier entocytherid loads than males, attributing those differences to the reduction of annual moult events in females due to reproduction. In our symbiont-host species association, there is not evidence of differences in symbiont loads due to crayfish sexual variation in moulting strategies, in accordance to the absence of differences between crayfish genders also observed by Aguilar-Alberola et al. (2012). A third set of predictors, composed by other water chemistry variables had moderate effects on symbiont abundances per occupied host. Two of them, i.e. alkalinity/($Cl^- + SO_4^{2-}$) and Cl^-/SO_4^{2-} ratios, can interfere on physiological processes of ostracods such as carapace calcification or ionic regulation (Mezquita et al., 1999), and are considered important factors in defining ostracod species niches (Mesquita-Joanes et al., 2012). In contrast, the other variable affecting positively ostracod densities, i.e. NH_4^+ concentration, is probably linked to symbiont nutrient sources. A moderate increase of NH_4^+ would favour the growth of the rich biota adhered to the host exoskeleton (Edgerton et al., 2002) that might serve as food for the symbiont ostracod. Moreover, according to the selected interactions of NH_4^+ concentration with other variables, an increase in food availability would enhance the positive effects of host density and reduce the negative effects of conductivity and ratio alkalinity/($Cl^- + SO_4^{2-}$). So, in conditions of abundant food, *A. sinuosa* could be favoured by higher host densities and be more tolerant to water chemistry. The great number of interactions between variables related to water chemistry indicate a complexity of their effects. Finally, the fact that only one climatic variable showed significant effects, with a small intensity, suggest that climatic conditions are mostly irrelevant for explaining inter-population variability in *A. sinuosa* abundances per occupied host at the geographic scale considered.

The main factor affecting host occupancy, with the strongest positive effect, was host density. So, *A. sinuosa* follows the pattern predicted by epidemiological models for symbionts with direct cycle, explained by an increase in the probability of host encounters as a result of a reduction of distances between hosts and an increased density of dispersal symbionts in the off-host environment. Moreover, the pattern also indicates that transmission rates of *A. sinuosa* are well synchronized with reproductive and dispersal rates of its host, so that an increase in host recruitments do not lead to an increased proportion of unoccupied hosts due to limitations in symbiont transmissibility (Stanko et al., 2006). This idea provides additional support to the hypothesis of high transmission efficacy in *A. sinuosa*. Following host density in importance, with moderate effects, climatic conditions appear as the second set of explanatory variables affecting host occupancy of *A. sinuosa*. Thus, the probability of presence of *A. sinuosa* within an individual host depends on a climatic profile so that is favoured by higher maximum temperatures and annual precipitation, and lower precipitation seasonality. Moreover, as there is a link between host occupancy and local extinction of a symbiont, i.e. lower levels of host occupancy in a locality increase the probability of extinction of symbiont population in that locality (Poulin, 2007a,b), our results suggest the possibility of restrictions in *A. sinuosa* geographical distribution due to climatic conditions. Along this line, Mestre et al. (2013), based on global distribution data for *A. sinuosa*, predicted an invasive scenario where *A. sinuosa* had much less extended climatically suitable areas in Europe than *P. clarkii*, so that some European regions with inappropriate climatic conditions for *A. sinuosa* could be inhabited by *P. clarkii* populations without the symbiont. Finally, host body size was found to have a slight positive effect on host occupancy. Smaller body size implies smaller infrapopulation sizes due to reduced spatial resources, increasing the probability of infrapopulation extinction. Furthermore, smaller hosts are often younger, having more frequent moult events and shorter intermoult stages, negatively affecting symbiont abundance (Castillo-Escrivà et al., 2013). Therefore, the balance between symbiont losses through moulting processes and symbiont recruitments during the intermoult stage falls towards the losses (Young, 1971; Mori et al., 2001).

Regarding the anomalies shown by the model assessment, a possible hypothesis to explain the outliers and the underestimation of crayfishes with high levels of symbiont abundance could be the stochastic variability in demographic parameters (i.e. birth rates), that may generate overdispersion in abundances of symbionts with direct cycle and direct reproduction within the host (Damborenea, 1998; Krasnov et al., 2005). An alternative explanation for the outliers is host dispersal. Symbiont dispersal abilities are related to host dispersal range (Whiteman & Parker, 2004). Accordingly, *P. clarkii* populations are influenced by crayfish dispersal movements at distances up to 2.5 km (Siesa et al., 2011).

All in all, our results are in accordance with the hypothesis of the outstanding role of environmental conditions influencing population parameters of non-parasitic freshwater ectosymbionts. Previous studies focused on the effect of environmental conditions on population parameters in similar symbiont systems support this idea (Martín et al., 2005; DeWitt et al., 2013), with one exception (Pennuto et al., 2002). In our symbiont system, climatic conditions and water chemistry are important sources of spatial variability of *A. sinuosa* population parameters. Consequently, a commensal freshwater symbiont living on a crustacean exoskeleton seems to be influenced by the high environmental instability characteristic of temperate freshwater ecosystems, so that within-host habitat does not offer an entire independence from the external environment, which affects host occupancy through climatic conditions at a regional scale, and symbiont abundances through water chemistry at a more reduced, local level. Physiological tolerances to such highly unstable environmental factors, but also their dependence on greatly variable external food sources as well, are probably the main reasons for the high sensitivity of populations to environmental conditions in our symbiont model. In this sense, a further evolutionary step of a commensal symbiont towards a greater population stability, less dependent on external environmental fluctuations, would be to start feeding directly from host items, evolving to a host-symbiont parasitic relationship (Hairston Jr & Bohonak, 1998).

Conclusions

Ankylocythere sinuosa, the studied freshwater non-parasitic ectosymbiont, followed three "parasite rules", suggesting the idea of extending the scope of these patterns to talk about "symbiont rules". Furthermore, although the role of environmental conditions as factors influencing symbiont population parameters has been generally considered irrelevant compared with symbiont and host features, we showed how a non-parasitic freshwater ectosymbiont is markedly affected by climate and water chemistry. Therefore, the importance of environmental conditions depends on the symbiont system considered, so that some particular symbiont models, e.g. non-parasitic freshwater ectosymbionts, could be specially sensitive to environmental variability. To conclude, research on non-parasitic symbionts and a wider view of symbiont ecology not exclusively focused on parasites would contribute to discern between general implications of a symbiont life and those specific of the symbiont-host relationship, regarding ecological aspects such as the existence of characteristic patterns in population parameters, the degree of independence from external environment or the kind of mechanisms regulating symbiont abundances.

Acknowledgements

We wish to thank Josep Antoni Aguilar-Alberola, Maria Antón, Marco Arruej, Andreu Castillo, Andreu Escrivà, Jose Antonio Gil-Delgado, Cristina Molina, Adrián Ponz, Josep R. Roca, Olivier Schmit, Luís Valls and Laia Zamora for their help in the field. This research was funded by the Spanish Ministry of Science and Innovation Project ECOINVADER (CGL2008-01296/BOS) and the University of Valencia ("V-Segles" predoctoral grant to A. Mestre). We thank sampling permission from the regional Spanish governments of Castilla-La Mancha, Castilla-León, Extremadura, Aragón, Andalucía, Navarra, Illes Balears, Catalunya, and C. Valenciana.

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Supporting Information

Table S1. Details about selection of explanatory variables for modelling abundance per occupied host and host occupancy in European exotic populations of the entocytherid *A. sinuosa* inhabiting the crayfish host *P. clarkii*.

Table S2. Details about selection of pairs of interactions between variables for modelling abundance per occupied host and host occupancy in European exotic populations of the entocytherid *A. sinuosa* inhabiting the crayfish host *P. clarkii*.

Figure S1. Relationship between weight and cephalothorax length in *P. clarkii* individuals obtained in this survey.

Figure S2. Frequency distribution of *A. sinuosa* abundances among *P. clarkii* hosts sampled in this survey.

Figure S3. Half-normal plot of Pearson residuals and frequency distribution of the differences between fitted and observed values ($\hat{y}_i - y_i$) of the optimal model to analyse abundance per occupied host and host occupancy.

Figure S4. Monthly distribution of Pearson residuals of the optimal model to analyse abundance per occupied host and host occupancy.

Table S1. Selection of explanatory variables for modelling abundance per occupied host and host occupancy in European exotic populations of the entocytherid *Ankylocythere sinuosa* inhabiting the crayfish host *Procambarus clarkii*, using a zero-altered Negative Binomial (ZANB) model. We used a backward selection method, and the number of steps to find the optimal model were four. For each ZA model part and predictor, we indicate the abbreviated variable name (for variable full name and description see Table 2), if it was selected or not for the final optimal model, the last backward step for this variable in which it was decided to be removed or selected and, finally, the χ^2 statistic, degrees of freedom (df) and p-value (P) of the LRT test for this final step. *Predictors added in the second step of the variable selection because of the impossibility to build an initial full model with all the predictors due to its high complexity. **Significant P (< 0.05).

Model part	Predictor	Selected?	Last step	Last LRT χ^2	df	P
Count	CrSex	No	Step 1	2.2404	1	0.134
	CrMoult	Yes	Step 4	15.859	3	0.001**
	CrDens	Yes	Step 4	82.435	1	< 0.001**
	cond	Yes	Step 4	40.343	1	< 0.001**
	chlora	No	Collinearity	—	—	—
	alk	No	Step 2	3.418	1	0.064
	Cl	No	Collinearity	—	—	—
	ox	No	Step 1	3.4286	1	0.0641
	ammon	Yes	Step 4	34.411	1	< 0.001**
	nitr	No	Step 1	2.401	1	0.121
	phosph	No	Step 2	0.016	1	0.901
	sulph	No	Step 1	0.629	1	0.428
	RatioAlkAn*	Yes	Step 4	12.056	1	0.001**
	RatioClS*	Yes	Step 4	10.671	1	0.001**
	RatioNitrAmmon*	No	Step 2	0.817	1	0.366
	habitat	No	Step 2	0.427	1	0.513
	SedOM	No	Step 2	1.433	1	0.231
	SedH	No	Collinearity	—	—	—
	Tmax	No	Step 1	1.973	1	0.160
	Tmin	No	Step 1	3.369	1	0.066
	precip	No	Step 1	2.534	1	0.111
	PrecipSeas	Yes	Step 4	10.856	1	0.001**
Regression	CrSex	No	Step 1	0.532	1	0.466
	CrWeight	Yes	Step 4	5.911	1	0.015**
	CrMoult	No	Step 3	6.543	3	0.088
	CrDens	Yes	Step 4	80.421	1	< 0.001**
	Cond	No	Step 1	1.297	1	0.255
	chlora	No	Collinearity	—	—	—
	alk	No	Step 2	0.904	1	0.342
	Cl	No	Collinearity	—	—	—
	ox	No	Step 1	0.318	1	0.573
	ammon	No	Step 2	2.862	1	0.091
	nitr	No	Step 2	3.561	1	0.059
	phosph	No	Step 1	2.336	1	0.126
	sulph	No	Step 1	2.543	1	0.111
	RatioAlkAn*	No	Step 2	0.001	1	0.973
	RatioClS*	No	Step 2	0.245	1	0.620
	RatioNitrAmmon*	No	Step 2	0.331	1	0.565
	habitat	No	Step 2	0.161	1	0.689

Continued on next page

Table S1 – ***Continued from previous page***

Model part	Predictor	Selected?	Last step	Last LRT χ^2	df	P
	SedOM	No	Step 1	2.977	1	0.084
	SedH	No	Collinearity	—	—	—
	Tmax	Yes	Step 4	19.091	1	< 0.001**
	Tmin	No	Step 1	2.936	1	0.087
	precip	Yes	Step 4	15.835	1	< 0.001**
	PrecipSeas	Yes	Step 4	32.701	1	< 0.001**

Table S2. Selection of interactions for the optimal model to analyse the abundance per occupied host and host occupancy in European exotic populations of the entocytherid *Ankylocythere sinuosa* inhabiting the crayfish host *Procambarus clarkii*, using a zero-altered Negative Binomial (ZANB) model. We considered all the possible interactions between the selected variables for the optimal model without interactions, and followed a backward selection protocol. After 3 steps, we obtained a final optimal model with interactions that contained six interactions for the count model part and zero interactions for the regression part. In the table, we named each interaction using the variable abbreviations in Table 2, and ":" as symbol of interaction. For each interaction, we indicate the corresponding model part, if it was or not selected for the optimal model with interactions, the last backward step for this interaction in which it was removed or selected, and the χ^2 statistic, degrees of freedom (df) and p-value (P) of the LRT test for this final step. *Significant P (< 0.05).

Model part	Interaction	Selected?	Last step	Last LRT		
				χ^2	df	P
Count	CrMoult : CrDens	No	Step 1	7.149	3	0.067
	CrMoult : cond	No	Step 1	6.928	3	0.074
	CrMoult : ammon	No	Step 1	0.767	3	0.857
	CrMoult : RatioAlkAn	No	Step 1	3.422	3	0.331
	CrMoult : RatioClS	No	Step 1	1.895	3	0.594
	CrMoult : PrecipSeas	No	Step 1	2.490	3	0.477
	CrDens : cond	No	Step 1	1.087	1	0.297
	CrDens : ammon	Yes	Step 3	31.691	1	< 0.001*
	CrDens : RatioAlkAn	No	Step 1	0.151	1	0.697
	CrDens : RatioClS	Yes	Step 3	44.129	1	< 0.001*
	CrDens : PrecipSeas	No	Step 1	0.475	1	0.491
	cond : ammon	Yes	Step 3	47.282	1	< 0.001*
	cond : RatioAlkAn	Yes	Step 3	51.034	1	< 0.001*
	cond : RatioClS	No	Step 1	3.228	1	0.072
	cond : PrecipSeas	No	Step 1	0.114	1	0.736
	ammon : RatioAlkAn	Yes	Step 3	46.126	1	< 0.001*
	ammon : RatioClS	No	Step 2	0.233	1	0.629
	ammon : PrecipSeas	No	Step 2	0.013	1	0.908
	RatioAlkAn : RatioClS	Yes	Step 3	59.919	1	< 0.001*
	RatioAlkAn : PrecipSeas	No	Step 1	2.174	1	0.140
	RatioClS : PrecipSeas	No	Step 1	1.745	1	0.187
Regression	CrWeight : CrDens	No	Step 1	0.092	1	0.762
	CrWeight : Tmax	No	Step 1	0.861	1	0.354
	CrWeight : precip	No	Step 1	0.989	1	0.320
	CrWeight : PrecipSeas	No	Step 1	0.67	1	0.413
	CrDens : Tmax	No	Step 1	0.822	1	0.365
	CrDens : precip	No	Step 1	3.081	1	0.079
	CrDens : PrecipSeas	No	Step 1	1.584	1	0.208
	Tmax : precip	No	Step 1	1.282	1	0.258
	Tmax : PrecipSeas	No	Step 1	3.264	1	0.071
	precip : PrecipSeas	No	Step 1	2.332	1	0.127

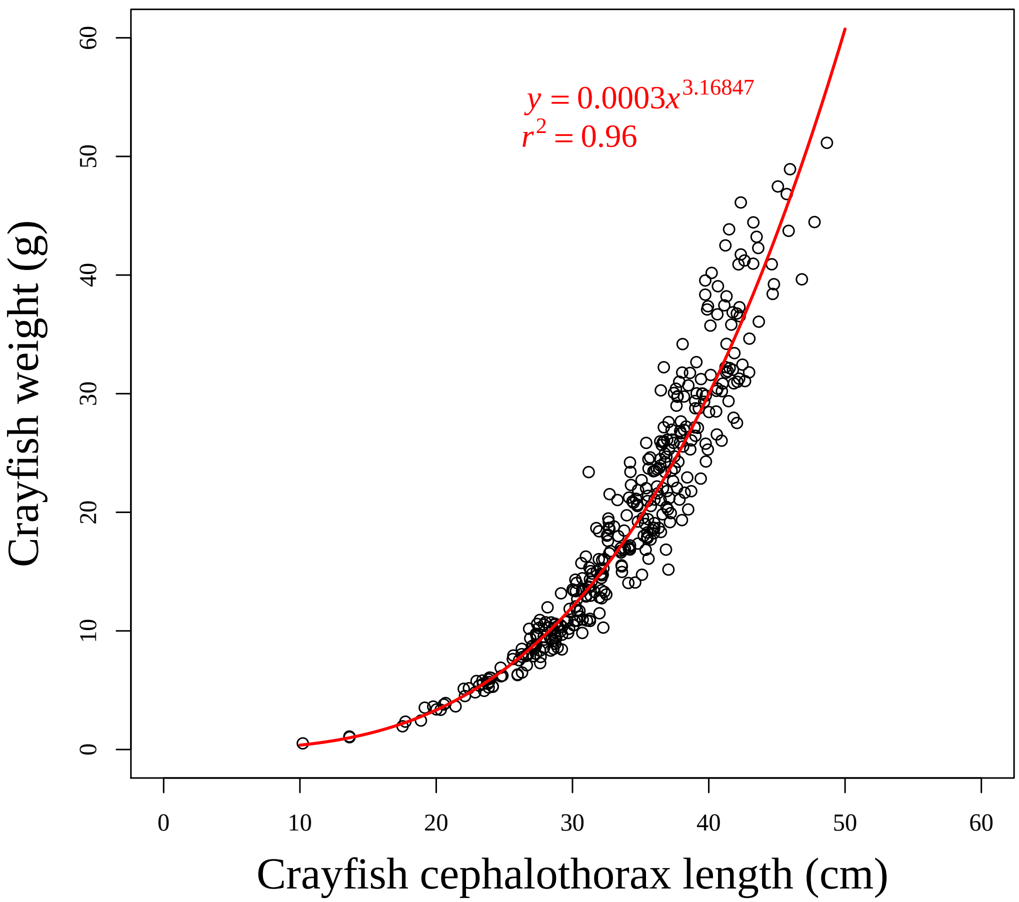

Figure S1. Relationship between the weight and the postorbital cephalothorax length of the red swamp crayfish, *Procambarus clarkii*, considering those individuals used for this survey (N = 369; four crayfishes are not in this graph due to the absence of data for their cephalothorax length). In red, we added a fitted curve, its equation and its coefficient of determination (r^2). The tight fit of the model indicates that weight is a good estimator of host size (≈ volume) in our study organism.

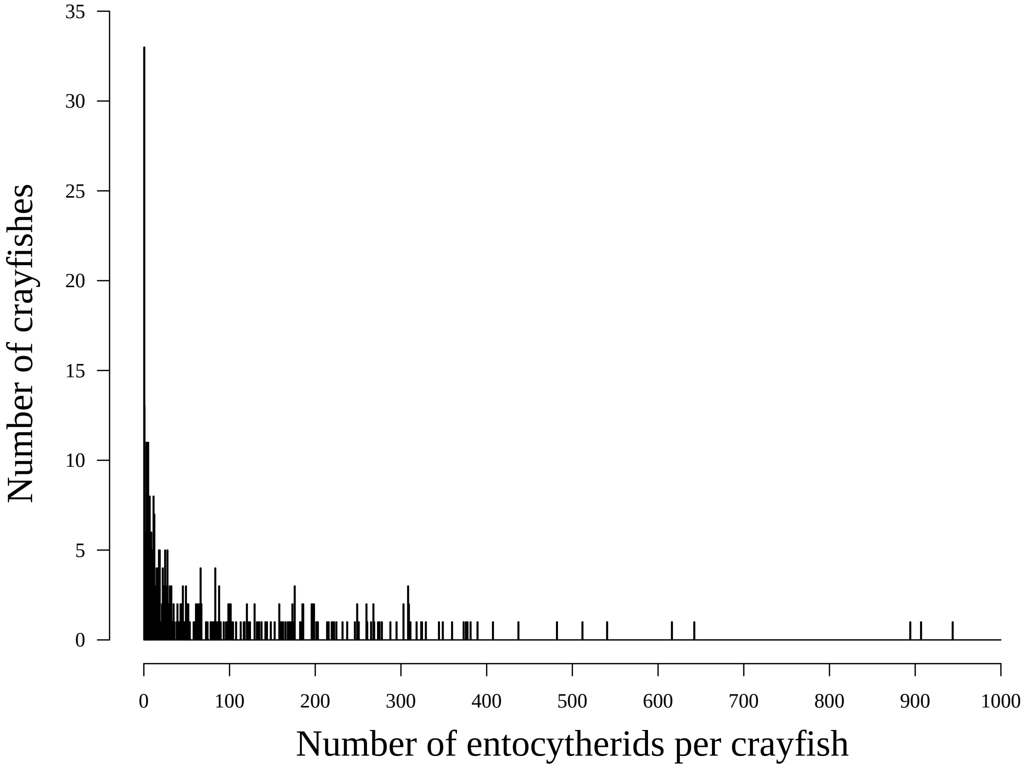

Figure S2. Frequency distribution of entocytherid abundance per crayfish host for the entocytherid *Ankylocythere sinuosa* by considering all 373 crayfishes (*Procambarus clarkii*) checked in this study, showing a zero-inflated pattern with 33 crayfishes without entocytherids.

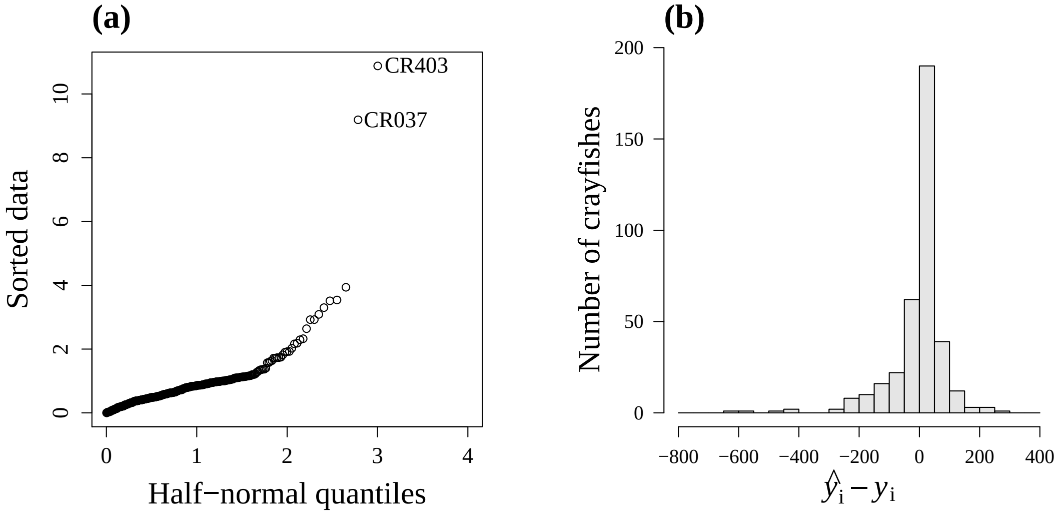

Figure S3. Assessment of the zero-altered negative binomial (ZANB) model with interactions for analysing abundance per occupied host and host occupancy of the entocytherid *Ankylocythere sinuosa* on crayfish *Procambarus clarkii*: **(a)** Half-normal plot of Pearson residuals. The outliers of the model are labelled using a codification for the corresponding crayfish (CR037 and CR403). Crayfish CR037 belongs to the locality LOC004; CR403 was collected from LOC035 (information about localities in Table 1). **(b)** Frequency distribution of the differences between fitted and observed values ($\hat{y}_i - y_i$) in our model. The total number of crayfishes analysed in the ZANB model were 373.

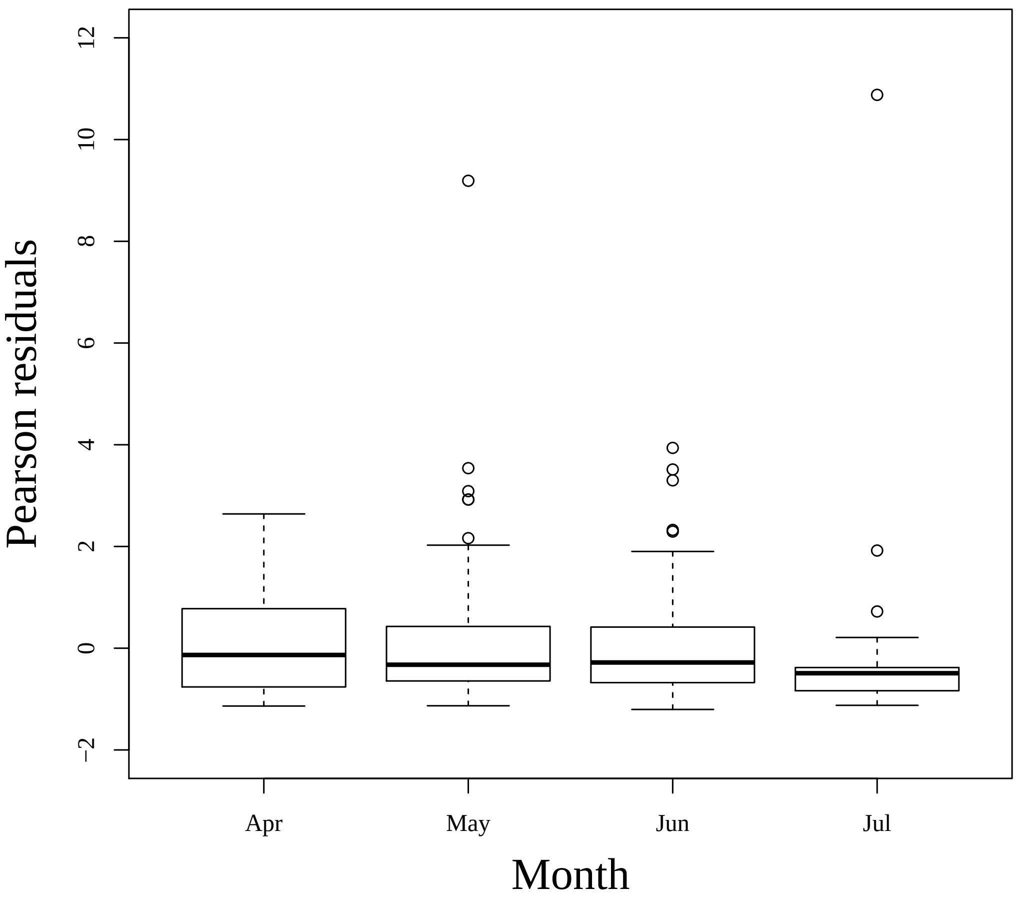

Figure S4. Monthly variation of Pearson residuals of the zero-altered negative binomial (ZANB) model with interactions for analysing abundance per occupied host and host occupancy of the entocytherid *Ankylocythere sinuosa* inhabiting the crayfish host *Procambarus clarkii*. The sampling campaign was done between April-June, during the 2010-11 seasons (Table 1). The total number of crayfishes analysed through the ZANB model were 373.

CAPÍTOL VI

Invasion biology in non-free-living species: interactions between abiotic (climatic) and biotic (host availability) factors in geographical space in crayfish commensals (Ostracoda, Entocytheridae)

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Ecology and Evolution, *3*, 5237-5253

Ecology and Evolution

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Invasion biology in non-free-living species: interactions between abiotic (climatic) and biotic (host availability) factors in geographical space in crayfish commensals (Ostracoda, Entocytheridae)

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Keywords
Biological invasions, BAM diagrams, ecological niche models, host availability.

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Funding Information
Research funded by the Spanish Ministry of Science and Innovation Project ECOINVADER (CGL2008-01296/BOS) and the University of Valencia ("V-Segles" predoctoral grant to A. Mestre). AK acknowledges Project CENAKVA CZ.1.05/2.1.00/01.0024.

Received: 6 September 2013; Revised: 22 October 2013; Accepted: 27 October 2013

***Ecology and Evolution* 2013; 3(16): 5237–5253**

doi: 10.1002/ece3.897

Abstract

In invasion processes, both abiotic and biotic factors are considered essential, but the latter are usually disregarded when modeling the potential spread of exotic species. In the framework of set theory, interactions between biotic (*B*), abiotic (*A*), and movement-related (*M*) factors in the geographical space can be hypothesized with BAM diagrams and tested using ecological niche models (ENMs) to estimate *A* and *B* areas. The main aim of our survey was to evaluate the interactions between abiotic (climatic) and biotic (host availability) factors in geographical space for exotic symbionts (i.e., non-free-living species), using ENM techniques combined with a BAM framework and using exotic Entocytheridae (Ostracoda) found in Europe as model organisms. We carried out an extensive survey to evaluate the distribution of entocytherids hosted by crayfish in Europe by checking 94 European localities and 12 crayfish species. Both exotic entocytherid species found, *Ankylocythere sinuosa* and *Uncinocythere occidentalis*, were widely distributed in W Europe living on the exotic crayfish species *Procambarus clarkii* and *Pacifastacus leniusculus*, respectively. No entocytherids were observed in the remaining crayfish species. The suitable area for *A. sinuosa* was mainly restricted by its own limitations to minimum temperatures in W and N Europe and precipitation seasonality in circum-Mediterranean areas. *Uncinocythere occidentalis* was mostly restricted by host availability in circum-Mediterranean regions due to limitations of *P. leniusculus* to higher precipitation seasonality and maximum temperatures. The combination of ENMs with set theory allows studying the invasive biology of symbionts and provides clues about biogeographic barriers due to abiotic or biotic factors limiting the expansion of the symbiont in different regions of the invasive range. The relative importance of abiotic and biotic factors on geographical space can then be assessed and applied in conservation plans. This approach can also be implemented in other systems where the target species is closely interacting with other taxa.

Introduction

Biotic and abiotic factors in invasion processes

Dramatic impacts of alien species on invaded ecosystems have prompted interest to scientifically understand invasion processes in order to prevent their harmful effects (Strayer et al. 2006; Young and Larson 2011). Invasive species have a combination of attributes that facilitate their arrival and establishment in a novel region (Sol 2007; Karatayev et al. 2009). But several external factors are also involved in an invasion success, usually classified into abiotic, biotic, and dispersal factors. Although some authors give more importance to dispersal factors such as propagule pressure in accounting for the success or failure of an invasion event (e.g., Lockwood et al. 2005), abiotic and biotic factors have been shown as important elements in invasion biology.

The role of the abiotic conditions in invasion biology is evident, and physical suitability for an invader obtained from environmental predictors, mainly climatic, has been considered as good predictor of invasibility (Williamson 1996). Several studies also show that spatial and temporal heterogeneity and physical disturbances, usually related to abiotic conditions (like climatic or geographical), may facilitate the establishment of invasive species (Melbourne et al. 2007). Another example of the importance of abiotic factors in invasion biology is the effect of climate change on the invasion processes (Hellmann et al. 2008; Rahel and Olden 2008).

In spite of the wide use of climatic conditions to predict the regions susceptible to be invaded by exotic species, biotic interactions have also been shown as important elements limiting the species distributions (Guisan and Thuiller 2005). Indeed, biotic interactions are considered a key factor in biological invasions (White et al. 2006; Roy and Handley 2012). Biotic factors such as community complexity, the existence or absence of enemies (predators, competitors, parasites, and pathogens), and mutualisms or commensalisms with other species may facilitate or hamper the establishment of an invader in a novel area (Mooney and Cleland 2001; Sakai et al. 2001; Prenter et al. 2004; Davis 2009; Engelkes and Mills 2011). For example, the Enemy Release Hypothesis proposes a facilitation of the invasion success due to loss of negative interactions from the native range, including competition, predation, or parasitism, during the early invasive stages of the displacement to the novel area (Sax and Brown 2000; Torchin et al. 2003; Roy et al. 2011). But those symbionts that get to remain with the exotic species during the invasive process have also an important role. Host jump, a key element in the evolution of non-free-living organisms (Poulin 2007), is also essential in invasion biology. An invasion event offers new biogeographic and evolutionary opportunities to the symbionts accompanying an invasive host. The process of symbiont transmissions from invasive to native hosts, also called "spillover" (Kelly et al. 2009), is considered an important threat for native species conservation (Roy and Handley 2012; Strauss et al. 2012). [NB: This work employs the term "symbiosis" with its broad meaning of organisms living in association, including positive (mutualism), negative (parasitism), and neutral (commensalism) interactions, following Sapp (1994). The terms "symbiont" and "non-free-living species" are employed for a smaller organism living in symbiosis with a larger species, termed the "host"].

The ecological niche in set theory and BAM diagrams

According to the niche concept proposed by Hutchinson (1957), "an n-dimensional hypervolume is defined, every point in which corresponds to a state of the environment which would permit the species S_1 to exist indefinitely." The potential niche is the range of environmental conditions available in the geographical space associated with positive intrinsic growth rates. The realized niche is the portion of the potential niche without biotic and/or dispersal constrictions. We want to highlight the distinction between the environmental space, linked to the niche concept, and the geographical space, composed of grid cells covering a particular region, associated with the geographical distribution of species (Peterson et al. 2011).

Based on the application of set theory (Hrbacek and Jech 1999) to niche concepts, BAM diagrams (Soberón and Peterson 2005) offer a framework to configure different hypothetical interactions between biotic (B), environmental or abiotic (A), and movement-related or dispersal (M) factors in the geographical space, which can be applied to invasion biology (Jiménez-Valverde et al. 2011). In this framework, A is the geographical area in which the environment is suitable at a given time, and where the intrinsic growth rate of the species would be positive; B is the geographical area where biotic interactions are favorable for species' existence, and M is the geographical area that is accessible to the species. In these models, the geographical area occupied by the species (G_o) is that with suitable environmental conditions for species existence, favorable biotic interactions, and accessible for the species ($\mathrm{A} \cap \mathrm{B} \cap \mathrm{M}$). Here, A represents the geographical area where the environmental conditions belong to the environmental space of the potential niche, and G_o is the projection of the realized niche in the geographical space. Therefore, the BAM diagrams link the environmental space of the niche theory with the geographical space of the species distributions.

We can hypothesize the different possible interactions between *A* and *B* by means of BAM diagrams. Only three interactions are possible (Fig. 1): (1) *A* contains *B* ($B \subset A$ or $(A\backslash B \neq \varnothing) \wedge (B\backslash A = \varnothing)$), (2) *B* contains *A* ($A \subset B$ or $(A\backslash B = \varnothing) \wedge (B\backslash A \neq \varnothing)$), and (3) a partial overlap between *A* and *B* ($(A\backslash B \neq \varnothing) \wedge (B\backslash A \neq \varnothing)$). In a theoretical context in which there are no restrictions by accessibility (i.e., *M* contains *A* and *B*, $(A \cup B) \subset M$), two areas of the BAM framework of Soberón and Peterson (2005) characterize the three cases: G_{BI} is the geographical area accessible and presenting favorable environmental conditions but inappropriate biotic conditions, and *BI* is the environmentally unsuitable but biotically appropriate area. In this theoretical context, G_{BI} is the portion of *A* that remains out of *B* ($G_{BI} = A\backslash B$), and *BI* is the portion of *B* that does not coincide with *A* ($BI = B\backslash A$); therefore, in the first case when *A* contains *B*, only G_{BI} (but not *BI*) will appear; in the second case when *B* contains *A*, only *BI* will appear; finally, in the third case of a partial overlap between *A* and *B*, both area types, G_{BI} and *BI*, will be present. So, G_{BI} and *BI* can be used to identify which model of interaction between *A* and *B* fits or is closer to the case of the exotic species analyzed, through the evaluation of their relative proportion. Moreover, they also represent areas where the species is specifically absent due to abiotic (*BI*) or biotic (G_{BI}) factors, so that these factors are acting as specific barriers against the species expansion into those areas.

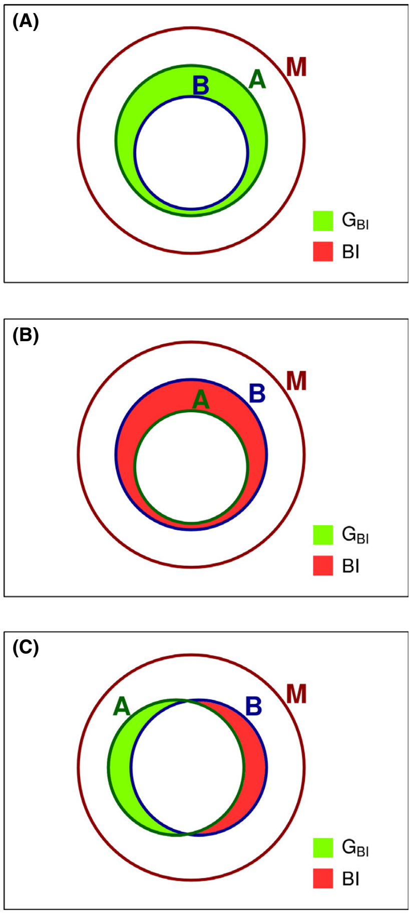


Figure 1. BAM diagrams adapted from Jiménez-Valverde et al. (2011) representing the three possible interactions between environmental and biotic factors in the geographical space of a species distribution model for invasive species when the species has no dispersal limitations ($(A \cup B) \subset M$). Represented by circles, *A* is the geographical area with suitable environmental conditions, *B* the area where biotic interactions allow species existence, and *M* is the accessible area for the species. G_{BI} is the available geographical area with favorable environmental conditions, but inappropriate biotic conditions ($G_{BI} = A\backslash B$) and *BI* the area with unsuitable environmental, but appropriate biotic conditions ($BI = B\backslash A$) for the species. Within this model frame, the three possible interactions between *A* and *B* are as follows: (A) *A* includes *B* ($B \subset A$ or $(A\backslash B \neq \varnothing) \wedge (B\backslash A = \varnothing)$), (B) *B* includes *A* ($A \subset B$ or $(A\backslash B = \varnothing) \wedge (B\backslash A \neq \varnothing)$), and (C) a partial overlap between *A* and *B* ($(A\backslash B \neq \varnothing) \wedge (B\backslash A \neq \varnothing)$). Colors for G_{BI} and *BI* as in Fig. 5.

Ecological niche models and set theory

Ecological niche models (ENMs) have proven useful in providing statistical tools to predict the environmentally suitable areas for the invasion by an exotic species (Thuiller et al. 2005), a practical approach that has been widely used recently (e.g., Reshetnikov and Ficetola 2011). The predictions are based on modeling the relation between species occurrence data and environmental predictors. Although biotic factors may also affect species distributions, most ENMs are based only on physical predictors because the high complexity of biotic interactions makes their inclusion in an ENM approach difficult. Nonetheless, some studies consider biotic interactions in their analyses, by adding biotic predictors or constraining the model predictions to the presence of interacting species (e.g., Heikkinen et al. 2007; Meier et al. 2010; Schweiger et al. 2012). Recently, novel techniques have incorporated biotic interactions into ENMs through modeling multi-species interactions by means of interaction matrices (Kissling et al. 2012). On the other hand, the application of ENMs to invasion biology is subject to methodological uncertainties derived from doing predictions across space and time. In this sense, the development of ensemble ENM techniques has represented a useful progress in order to assess the modeling uncertainty (Capinha and Anastácio 2011; Capinha et al. 2011).

ENMs can be applied in a theoretical framework of BAM models to analyze the interactions between *A* and *B* in the geographical space of exotic species that are strongly affected by a particular interaction with other species, for example, the dependence on the presence of a specific host, prey, or mutualist, or the absence of a particular predator or parasite. To do so, as we are not focused on *M*, the assumption of the absence of dispersal factors affecting the study region may facilitate the BAM analyses. So, our species model should have accessibility to all the areas of the study region. Secondly, we need to limit the set of factors involved on *A* and *B*. Climatic conditions are a good choice to characterize *A* when we work at large extension and coarse resolution scales (Elith and Leathwick 2009). The *B* factors would be limited to the presence of positively interacting species (a host, prey, or mutualist) or its absence if interacting negatively (i.e., a predator or parasite). Once we have established the theoretical framework and the geographical scale (large extension and coarse resolution for climatic variables characterizing *A*), the next step is to use ENMs to estimate *A* and *B* areas. *A* can be estimated, in a practical way, predicting the climatically suitable areas for the exotic species in the study region, through ENM analysis and using the global occurrence dataset of the species and global climatic information. The estimation of *B* areas can be carried out in the same way, but predicting the climatically suitable (for a positive interaction) or unsuitable (for a negative interaction) areas for the interacting species. Consequently, we will need global occurrence data for these species. Finally, combining both predictions, representing the *A* and *B* areas in the geographical space of our study region, we will be able to highlight the proportion and distribution of the G_{BI} and *BI* areas that will allow to diagnose which interaction model follow *A* and *B* in our target species, and to identify areas where climatic conditions and/or biotic interactions with other species may be acting as specific barriers against the expansion into those areas.

Study system: entocytherid ostracods and their host crayfish

Invasive crayfish species are known to cause important harms to the native biota from the invaded site (McCarthy et al. 2006; Matsuzaki et al. 2009; Olden et al. 2011). A well-known impact in Europe was the “spillover” effect caused by the oomycete *Aphanomyces astaci* (Schikora, 1906), carried by American exotic crayfish and becoming one of the main problems for native European crayfish conservation (Gil-Sánchez and Alba-Tercedor 2002). The impact of *A. astaci* on European native crayfish is a typical case of the so-called naive host syndrome: a novel host receiving an exotic symbiont might be severely affected due to lack of history-evolved resistance (Taraschewski 2006; Mastitsky et al. 2010). Crayfishes have a rich associated biota (Edgerton et al. 2002), including entocytherids. The Entocytheridae is an ostracod family constituted entirely by epicommensal species on other crustaceans (Hart and Hart 1974). Entocytherinae, the main subfamily of the group with 183 species, are native from North and Central America living on Cambaridae and Astacidae crayfishes. Recently, two American exotic entocytherid species associated with invasive crayfish were cited in Europe and Japan: *Ankylocythere sinuosa* (Rioja, 1942), found in some localities of the E Iberian Peninsula, associated with *Procambarus clarkii* (Girard, 1852) (Aguilar-Alberola et al. 2012) and *Uncinocythere occidentalis* (Kozloff and Whitman, 1954), cited in a few German and Japanese localities living on *Pacifastacus leniusculus* (Dana, 1852) (Smith and Kamiya 2001; Grabow and Martens 2009; Grabow et al. 2009). In their native range, both entocytherid species have been found in 47 different host species in the case of *A. sinuosa* and three different species of crayfish in the case of *U. occidentalis* (Mestre and Mesquita-Joanes 2013), suggesting that they are not very host specific as seems to be common in the group (Mestre et al. in press). Although both exotic crayfish species have a much longer history in Europe (more than 35 years), entocytherids had not been previously detected, probably because they are tiny (<0.5 mm in length) and apparently not harmful to their hosts. On the other hand, we found no previous comprehensive study, which has checked the presence of Entocytheridae (native or exotic) in European native crayfish.

Exotic entocytherids and crayfishes are particularly adequate to analyze the interactions between *A* and *B* in the geographical space. The total dependence of the entocytherids on their crayfish hosts allows to easily estimate *B* as the crayfish host species presence. Moreover, due to the long invasion history of exotic crayfish in Europe with multiple introduction events by humans in many European countries (Holdich 2002), we can simplify our BAM models assuming the absence of dispersal barriers for these organisms in Europe. Finally, the low host specificity shown by the exotic entocytherids points to the possibility of restriction by host dependence in the invaded range, because they suffer a reduction in host availability from multiple crayfish host species in the native range to just a few exotic crayfish host species in the invaded range.

Set theory approach: dominance of biotic or abiotic factors in the invasion process?

Symbiont organisms associated with invasive hosts can join them to invaded areas, although a filtering selection in initial invasive stages occurs, as stated by the Enemy Release Hypothesis (Torchin et al. 2003). Having overcome the filters, they must accompany their hosts in the expansive phase. Then two questions arise: Are exotic symbionts able to travel with their hosts wherever they go or could they have physiological limitations preventing them from doing so? Alternatively, could they be limited by their host's tolerances to colonize all the potential areas they are physiologically able to invade (Wharton and Kriticos 2004)? Regarding the last question, the host climatic restrictions are susceptible to constrain the potential distributions of symbiotic organisms in new invaded areas because exotic symbionts often suffer a reduction in host availability from a number of hosts in their native range to just a few or only one invasive host. We can deal with this issue by analyzing the interactions between *A* (as limited to climatic factors) and *B* (reduced to host availability) in the geographical space using the set theory approach. In this context, the three different models of interaction between *A* and *B* proposed above correspond to the different possibilities that we can find in a symbiont–host system. The first model, where *A* includes *B*, would represent a case where the symbiont has broader abiotic tolerance than its host, so its distribution is simply determined by host availability. In contrast, the second and opposite model, where *B* includes *A*, represents a case where the symbiont has a tolerance to abiotic conditions much more restricted than their hosts', facing a climatic barrier to invade a region. Finally, the third and intermediate model outcome with a partial overlap between *A* and *B* represents a case where there is a spatial segregation between both restriction types, affecting different regions of the geographical space.

Aims and research strategy

To establish an initial evaluation of the distribution of crayfish-living entocytherids in Europe, we carried out the first extensive sampling campaign on native and exotic European crayfish species using specific entocytherid sampling techniques. Furthermore, the main aim of our survey was to evaluate the interactions between abiotic (climatic) and biotic (host availability) factors in geographical space for exotic symbionts, using ENM techniques combined with a theoretical framework based on set theory. To this end, we used as model organisms the exotic entocytherids found in Europe (*A. sinuosa* and *U. occidentalis*) and their hosts (*P. clarkii* and *P. leniusculus*). For each exotic entocytherid species, we carried out the following steps: (1) We established the theoretical framework based on the BAM models proposed by Soberón and Peterson (2005), specifying the model assumptions; (2) we estimated *A* and *B* areas through ENM modeling; (3) we combined the predicted *A* and *B* through a raster operation highlighting the G_{BI} and *BI* areas, and, finally, (4) we diagnosed the model of interaction between *A* and *B* that followed each entocytherid species analyzed assessing the relative proportion and distribution of G_{BI} and *BI*.

Methods

Field and laboratory methods

In order to evaluate the distribution of crayfish-living entocytherids in Europe, we sampled 12 crayfish species from 93 widely distributed European localities. Eight crayfish species were considered exotic, and four were native to Europe (Table 1). Crayfishes, caught with bait traps or hand nets, were subjected to entocytherid removal protocols based on submerging specimens in anesthetic liquids (carbonated water or chlorobutanol), as discussed and tested in Mestre et al. (2011). In some other cases, we checked the bottom of the container where crayfish were previously preserved in ethanol. Whatever the protocol used, the liquid (carbonated water, chlorobutanol, or ethanol) where crayfishes were submerged was filtered through a 63-μm mesh-sized filter, and the content retained was stored in ethanol. *A posteriori*, these samples were checked in the laboratory under a stereomicroscope, and the entocytherid species found were identified following Hart and Hart (1974). The copulatory apparatuses of selected adult males were drawn using a camera lucida, and SEM and light microscope photographs of adults were also taken to ascertain identifications. Our spatial analyses were mostly focused on both entocytherid species recently found in Europe, *Ankylocythere sinuosa* (Rioja, 1942), cited in association with *Procambarus clarkii* (Girard, 1852) and *Uncinocythere occidentalis* (Kozloff and Whitman, 1954), living on *Pacifastacus leniusculus* (Dana, 1852).

Applying set theory

BAM diagrams were applied by considering *A* the European geographical areas with suitable environmental (climatic) conditions for entocytherid species, *B* the European areas where host presence allows the existence of entocytherid symbionts, and *M* the European accessible areas for the species. It was assumed that: (1) Mobility-related limitations (i.e., physical dispersal barriers) do not exist for entocytherids and crayfishes in Europe. In set theory notation, we can express this assumption as: $((A \cup B) \subset M) \wedge ((A_H \cup B_H) \subset M_H)$ (*H* subscripts indicate the parameters related to the host; those without

Table 1. Summary of crayfish species checked for entocytherid occurrences in Europe. For each species, we indicate its status in Europe (native or exotic), the number of individuals (N crayfish) and localities (N localities) sampled, and the number of sites with presence of entocytherids belonging to species *Ankylocythere sinuosa*, *Uncinocythere occidentalis,* or an unidentified species.

Crayfish species	Crayfish status	N crayfish	N localities	*A. sinuosa*	*U. occidentalis*	Unidentified species
Astacus astacus (Linnaeus, 1758)	Native	53	6	0	0	0
Astacus leptodactylus Eschscholtz, 1823	Native	142	11	0	0	0
Astacus sp.	Native	10	1	0	0	0
Austropotamobius pallipes (Lereboullet, 1858)	Native	87	5	0	0	0
Austropotamobius torrentium (Schrank, 1803)	Native	15	2	0	0	0
Cherax destructor Clark, 1936	Exotic	7	1	0	0	0
Cherax quadricarinatus Martens, 1868	Exotic	7	2	0	0	1
Orconectes limosus (Rafinesque, 1817)	Exotic	103	4	0	0	0
Orconectes virilis (Hagen, 1870)	Exotic	48	6	0	0	0
Pacifastacus leniusculus (Dana, 1852)	Exotic	183	18	0	9	3
Procambarus acutus (Girard, 1852)	Exotic	40	2	0	0	0
Procambarus clarkii (Girard, 1852)	Exotic	495	39	28	1	3
Procambarus fallax (Hagen, 1870)	Exotic	4	1	0	0	0

refer to their symbionts). This assumption is based on the long invasion history of both hosts, *P. clarkii* and *P. leniusculus*, in Europe with multiple introduction events by humans in many European countries (Holdich 2002); (2) the only B factors considered are the adequate abiotic conditions for host presence, that is, $B = A_H$; and (3) the climatic predictors used in the ENM analyses are good estimators of A and A_H. In this model frame, three possible interactions between A and B exist (Fig. 1): (1) A includes B, $B \subset A$, or $(A \setminus B \neq \emptyset) \wedge (B \setminus A = \emptyset)$; (2) B includes A, $A \subset B$, or $(A \setminus B = \emptyset) \wedge (B \setminus A \neq \emptyset)$; (3) A partial overlap between both A and B, $(A \setminus B \neq \emptyset) \wedge (B \setminus A \neq \emptyset)$. Two areas in the models characterize these three cases: $G_{BI} = A \setminus B$ are the available geographical areas with favorable environmental conditions, but inappropriate biotic conditions for entocytherids, which in our models were estimated as the climatically suitable areas for the entocytherid but unsuitable for the host, representing those geographical areas where the symbiont is specifically restricted by host availability; $BI = B \setminus A$ areas with unsuitable environmental conditions, but appropriate biotic conditions, estimated in our models as the climatically unsuitable areas for the entocytherid and suitable for the host, representing those areas where the symbiont is specifically restricted by its own climatic tolerances. Consequently, G_{BI} is present in cases (1) and (3), and BI in (2) and (3) (Fig. 1).

Data sources for the ENMs

Occurrence data

The occurrence data for ENM analyses were extracted from three sources: (1) Own data reported in this work; (2) a worldwide database of entocytherid species and their hosts built by Mestre et al. (2012, in press) from published sources; and (3) the Global Biodiversity Information Facility (GBIF; http://data.gbif.org). After checking and cleaning occurrences to remove duplicate and erroneous points, and subsampling oversampled states or countries (i.e., U.K. and Sweden for *P. leniusculus*) following the same protocol as Iguchi et al. (2004), the number of occurrences, representing the global range of the four species studied, was 281 for *A. sinuosa*, 75 for *U. occidentalis*, 266 for *P. clarkii,* and 307 for *P. leniusculus*. We did not use real absences, as suggested by Jiménez-Valverde et al. (2011) because they are conflictive data, among other reasons, due to the difficulty, in most cases, to have a complete certainty that the species is absent, as may occur in entocytherid populations with low prevalences (Aguilar-Alberola et al. 2012).

Environmental data

Environmental predictors were restricted to climatic variables, considered more determinant on large extension and coarse resolution scales (Elith and Leathwick 2009). Climatic data were obtained from WorldClim (Hijmans et al. 2005). Datasets at a 5-arcmin resolution were selected. To avoid problems relating to collinearity between predictors (Dormann et al. 2012), only four climatic variables were utilized: minimum temperature of the coldest month (MinT); maximum temperature of the warmest month (MaxT); annual precipitation (AnPrec), and precipitation seasonality (i.e., coefficient of variation, PrecSeas). The selection of the variables was based on the fact that they reflect thermal limits and water environmental availability, consistently relating to important physiological attributes in our organisms, that is, thermoregulation and hydric stress. The effect of these climatic variables on the large-scale distribution of both crayfish species treated is well supported (Capinha et al. 2012). Regarding the entocytherids, both temperature and hydroperiod are important variables affecting the population dynamics of Ostracoda (Mesquita-Joanes et al. 2012), and the strong effects of temperature have been shown in the entocytherid species *A. sinuosa* (Castillo-Escrivà et al. 2013). A previous analysis of collinearity between the selected predictors on our occurrence species data was carried out based on graphical tools from the R (R Core Team 2013) RASTER package (Hijmans and van Etten 2012) and calculations on correlations between variables. No graphical evidence for collinearity was found, and all the paired combinations of predictors showed an $|r| < 0.7$ in all the climatic datasets for the four species studied.

ENM analyses

ENM modeling

We applied ENMs to predict the climatically suitable areas for each entocytherid species as an estimation of *A* areas of the BAM models, and the climatically suitable areas for each corresponding host species, to estimate *B* areas. The ENMs were built with BIOMOD2 (Thuiller et al. 2013), and the raster management was implemented using RASTER. Geographical resolution was the same for both models and predictions, determined by the environmental raster data, that is, five arcmin. The extension for the models was global, but European (12°W–60°E; 30°N–75°N) for predictions. ENMs were designed using the world occurrences of each species by also including the invaded range to improve their predictive ability in invaded areas (Broennimann and Guisan 2008; Capinha et al. 2011).

We applied ensemble modeling techniques with worldwide random selection of pseudo-absences (with the same number than the occurrences), data splitting into 70% for model calibration and 30% to test ENMs, and using eight different algorithms: generalized linear model (GLM), generalized additive model (GAM), generalized boosting model (GBM), artificial neural network (ANN), classification tree analysis (CTA), flexible discriminant analysis (FDA), multiple adaptive regression splines (MARS), and random forest (RF). In BIOMOD2, we used the default algorithm parameters. We repeated the modeling process 800 times, combining ten pseudo-absence selections ×8 algorithms ×10 calibrating-testing repetitions obtaining, as a result, 800 individual projections. Afterward, we averaged those individual projections built from the same pseudo-absence selection and calibrating-testing repetition, but different algorithm, obtaining 100 ensemble projections. For this, we applied a weighted average giving more weight to those algorithms with better performance according to the area under the curve (AUC) parameter (Capinha and Anastácio 2011). Finally, the 100 ensemble projections were averaged to get a final consensus projection, showing the probability of species presence in Europe according to the climatic predictors.

Assessing ENM performance

Three different aspects of ENM predictive performance were assessed: the performance of the climatic predictors, the test data predictive ability, and the ENM uncertainty. The performance of the predictors was analyzed with generalized linear models (GLMs) of binomial family with a "logit" link function, where the response variable was a dataset with the occurrence data and a pseudo-absence selection, and the explanatory variables were the climatic predictors. The test data fitting assessment was carried out using the AUC parameter, based on receiver operating characteristic (ROC) plots, representing the probability that the classifier (ENM) will rank a randomly chosen positive instance higher than a randomly chosen negative instance (Fawcett 2006), which reflects the relation between true-positive (well-predicted occurrence) and false-positive (absence predicted as presence) prediction rates (Peterson et al. 2011). We tested the effects of the algorithm type on the AUC results, through GLMs with the binomial family and a "logit" link function. ENM predictive uncertainty was also assessed by plotting the SD of the probabilities of species presence of the 100 ensemble projections, as in Capinha and Anastácio (2011).

Integration of ENM predictions and set theory to estimate the relative importance of abiotic and biotic factors

Once *A* and *B* areas were estimated through the ENM predictions, we combined both areas to obtain the G_{BI} and *BI* areas for each entocytherid species, used for the diagnosis. For this, the consensus projection for each species was transformed into presence–absence binary data. Threshold selection was based on threshold optimization by the ROC method (Thuiller et al. 2013). Optimized threshold values from the evaluation of the ensemble models were averaged to obtain a consensus threshold per species. Then, we combined the binary consensus projection of each entocytherid (representing *A*) and its respective host (representing *B*) by a subtraction raster operation to highlight the G_{BI} and *BI* areas for both symbiont species. Finally, we evaluated each case based on the relative proportion and distribution of G_{BI} and *BI* in the geographical space of Europe.

Results

Exotic entocytherids found in Europe

As a result of our field survey (Table 1; see also Table S1 in Supporting Information), two entocytherid species were detected: *Ankylocythere sinuosa* and *Uncinocythere occidentalis*. A general view of a mating pair and morphological details of the latter species is shown in Fig. 2 (Aguilar-Alberola et al. 2012 already presented pictures of exotic populations of *A. sinuosa*). New detailed morphological information on the male copulatory apparatus of some of these European populations of both species is provided in the SI (see Fig. S1). Occurrences of *A. sinuosa* were widely distributed in the Iberian Peninsula (Fig. 3C), while those of *U. occidentalis* were located in NE Iberian Peninsula, Central and N Europe (Fig. 4C). In all cases, host species were *P. clarkii* for *A. sinuosa* and *P. leniusculus* for *U. occidentalis*, with one exception, a locality where *U. occidentalis* was found inhabiting *P. clarkii*, in a small pond in N Spain, where all four species cohabited (LOC039 in Table S1). More specifically, both entocytherid species were found inhabiting the same *P. clarkii* specimen. For the remaining crayfish species sampled, no evidence was found for entocytherid occurrences in any case (Table 1), except for an unidentified entocytherid associated with *Cherax quadricarinatus* Martens, 1868, from a pet shop in Spain (LOC094 in Table S1).

ENM predictions for the climatic suitability of exotic entocytherids and their hosts in Europe

According to our consensus projections (Figs 3A,B and 4A,B), *A. sinuosa* was the species with the most limited climatically suitable European areas, restricted to circum-Mediterranean regions and some areas around the Black and Caspian Seas (Fig. 3A), while the climatically suitable areas for its host *P. clarkii* included, apart from these, a wider region of W Europe (Fig. 3B). In contrast, the climatically suitable areas for *U. occidentalis* and *P. leniusculus* occupied most of Europe, excluding the SW Iberian Peninsula, the highest altitudes of mountain ranges, N Fennoscandia, and the coastal lowlands around the Mediterranean region (Fig. 4A,B).

ENM assessment

Performance of the climatic predictors

According to our GLM results in regard to the effect of the climatic predictors on the probability of species presence (see Table S2), *A. sinuosa* is limited by the lower minimum temperatures (MinT: *Coef.* = 0.006; df = 557; $Z = 2.422$; $P < 0.05$), prefers climates with low annual precipitation (AnPrec: *Coef.* = −0.001; df = 557; $Z = -2.038$ $P < 0.05$), and is negatively affected by precipitation seasonality (PrecSeas: *Coef.* = −0.093; df = 557; $Z = -10.299$ $P < 0.001$), whereas its host, *P. clarkii*, tolerates the extreme temperatures (MaxT: *Coef.* = 0.05; df = 527; $Z = 2.106$ $P < 0.05$, MinT: *Coef.* = −0.012; df = 527; $Z = -8.067$ $P < 0.001$), as well as the precipitation seasonality (PrecSeas: *Coef.* = 0.036; df = 527; $Z = 8.436$ $P < 0.001$). The species *U. occidentalis* shows a preference for lower minimum temperatures (MinT: *Coef.* = −0.005; df = 145; $Z = -2.171$ $P < 0.05$), while its host, *P. leniusculus*, shows limitations in extreme temperatures (MinT: *Coef.* = 0.010; df = 609; $Z = 7.582$ $P < 0.001$, MaxT: *Coef.* = −0.011; df = 609; $Z = -4.839$ $P < 0.001$) and with high precipitation seasonality (PrecSeas: *Coef.* = −0.033; df = 609; $Z = -7.926$ $P < 0.001$). The only GLM with a nonadequate fit according to the residual deviance (*Dev.*) was the model for *U. occidentalis* (*Dev.* = 193.13; df = 145; $P(>\chi^2)<0.001$).

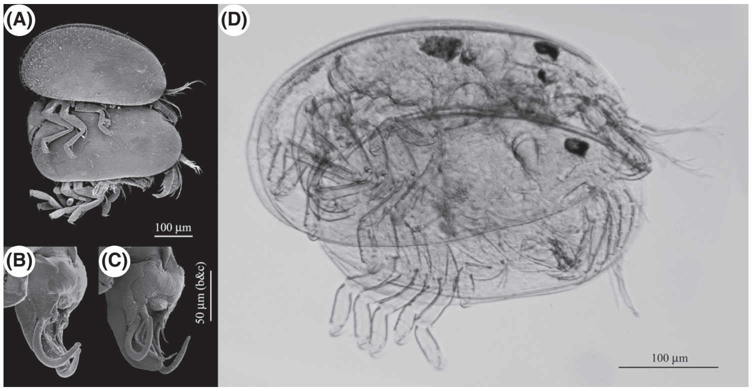
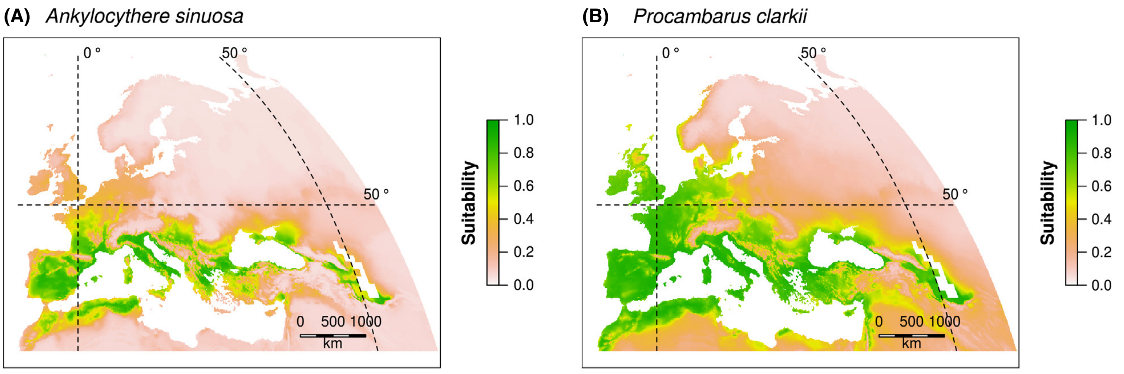
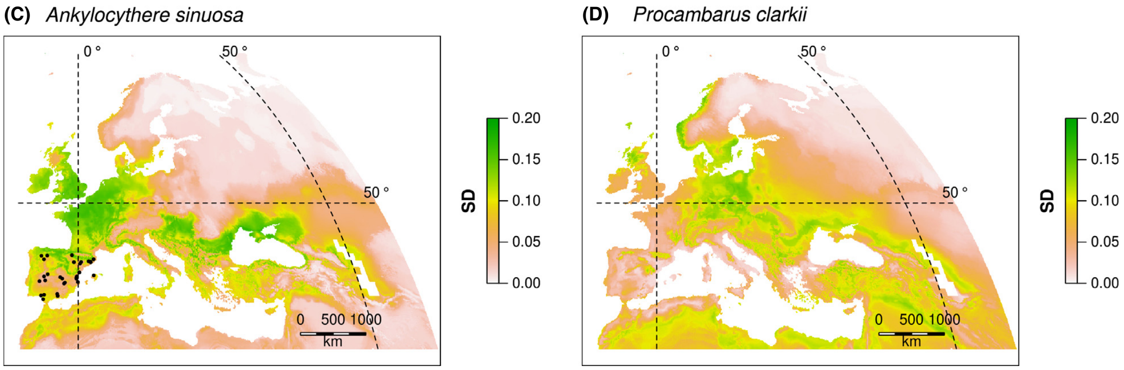


Figure 2. (A–C) Scanning electron microscope (SEM) and (D) stereomicroscope photographs of *Uncinocythere occidentalis* specimens from (A–C) LOC047 and (D) LOC039 (for information about locality codes, see Table S1 in Supporting Information). (A) Mating pair of an adult male (top) and an A-1 female (bottom); (B,C) copulatory organs of adult males in (B) lateral and (C) sublateral views; (D) mating pair of an adult male (top) and an A-2 female (bottom). A-1 refers to the last developmental instar prior to the adult, and A-2 to the juvenile instar prior to A-1.



(A) *Ankylocythere sinuosa* **(B)** *Procambarus clarkii*



(C) *Ankylocythere sinuosa* **(D)** *Procambarus clarkii*

Figure 3. (A,B) Consensus projections obtained from combining the 800 ecological niche models for (A) *Ankylocythere sinuosa* and (B) *Procambarus clarkii* using ensemble modeling techniques, showing the potential climatic suitability for both species in Europe (12°W–60°E; 30°N–75°N). (C,D) Variability among the 100 ensemble projections used to build the consensus projection for (C) *Ankylocythere sinuosa* and (D) *Procambarus clarkii*. Black dots in (C) are localities with *A. sinuosa* occurrences from our field survey. The maps have a 5-arcmin resolution and a Mollweide equal-area projection.

AUC and uncertainty assessments

The mean AUC values for those individual ENMs built using the same algorithm were higher than 0.8 in all cases (model types and species), with the highest scores obtained with GAMs (Table 2). The mean AUC values of the ensemble models were higher than 0.95 for all species (Table 2). The GLM results showed that the algorithm type significantly affected the AUC parameter in the ENMs of four species: *A. sinuosa* (*Null Dev.* = 195.99; *Dev.* = 101.03; df = 792; $P(>\chi^2)<0.001$), *U. occidentalis* (*Null Dev.* = 1737; *Dev.* = 1117; df = 792; $P(>\chi^2)<0.001$), *P. clarkii* (*Null Dev.* = 539.55; *Dev.* = 265.91; df = 792; $P(>\chi^2)<0.001$), and *P. leniusculus* (*Null Dev.* = 209.9; *Dev.* = 87.669; df = 792; $P(>\chi^2)<0.001$) (see Table S3 for further details on the algorithm effects estimates). However, the model of U. occidentalis has the greater proportion of deviance not explained by the algorithm type (*Dev./Null Dev.*×100 = 64%). In concordance, the SD of the AUC values for the ensemble models showed the highest value in the species *U. occidentalis* (SD = 0.008) (Table 2). Therefore, *U. occidentalis* ENMs presented the greatest predictive instability, according to the AUC assessment.

In the uncertainty assessment, the SD values of the probability of species presence of the ensemble projections remained below 0.2 for all species (Figs 3C,D and 4C,D). *Uncinocythere occidentalis* (Fig. 4C) was the species with more extended areas with higher uncertainty.

Integration of ENM predictions and set theory to estimate the relative importance of abiotic and biotic factors

Both combinations of entocytherid–host binary consensus projections followed two different patterns (Fig. 5). The *sinuosa–clarkii* combination had larger areas with a cli-

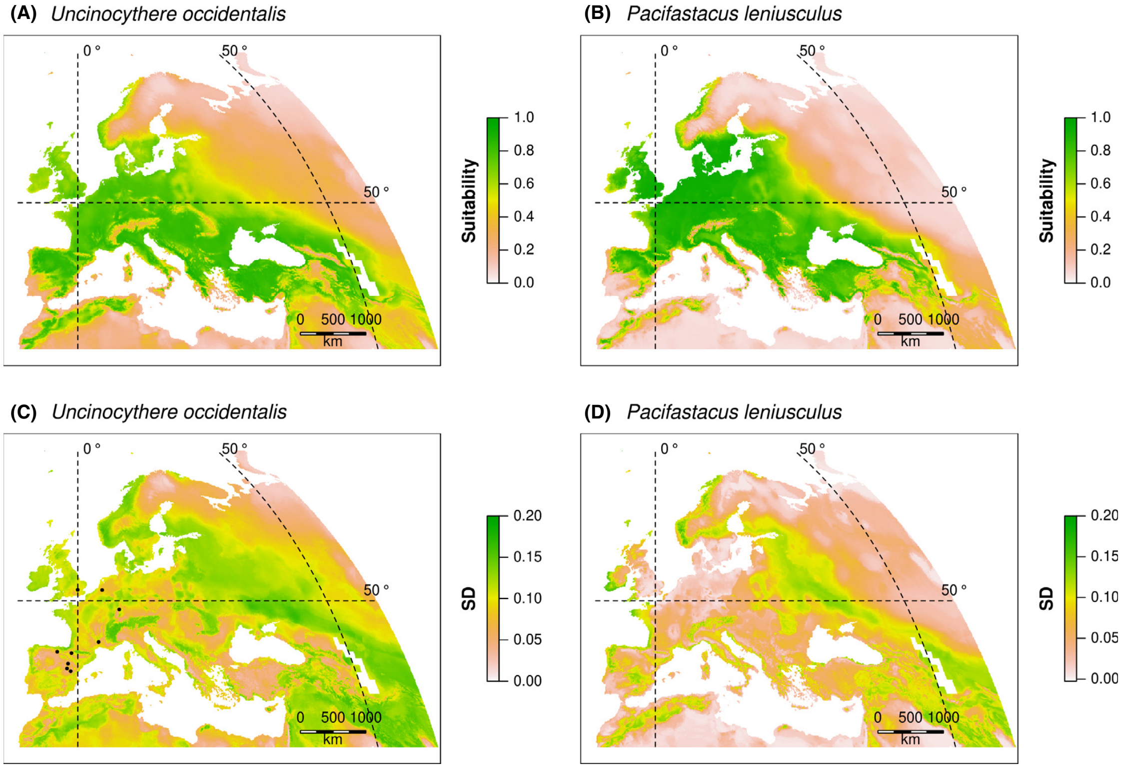


Figure 4. (A,B) Consensus projections obtained from combining the 800 ecological niche models for (A) *Uncinocythere occidentalis* and (B) *Pacifastacus leniusculus* using ensemble modeling techniques, showing the potential climatic suitability for both species in Europe (12°W–60°E; 30°N–75°N). (C,D) Variability among the ensemble projections used to build the consensus projection for (C) *Uncinocythere occidentalis* and (D) *Pacifastacus leniusculus*. Black dots in (C) are localities with *U. occidentalis* occurrences from our field survey. The maps have a 5-arcmin resolution and a Mollweide equal-area projection.

matic restriction for the entocytherid (*BI*) in W Europe and circum-Mediterranean regions, with only a few small areas with a restriction by host availability (G_{BI}) around the Black Sea (Fig. 5A). In the *occidentalis–leniusculus* species pair, G_{BI} occupied a wide range of S European, N African, and Middle East areas, while the *BI* areas appeared mainly in N Europe around the Baltic Sea with some small and diffuse areas in Central Europe associated with the highest altitudes of mountain chains (Fig. 5B). Both symbiont species followed two different distributional patterns of G_{BI} and *BI* areas, with a predominance of *BI* areas in the case of *A. sinuosa*, closer to the set model where *B* includes *A* (Figs 1B and 5A; case 2 in the Methods section). On the other hand, *U. occidentalis* presented a more balanced proportion of G_{BI} and *BI* areas, in accordance with a theoretical model with a partial overlap between *A* and *B* (Fig. 1C; case 3 in the Methods section).

Discussion

In this work, after carrying out the first comprehensive evaluation of the presence and distribution of entocytherids inhabiting crayfishes (exotic and native) in Europe, we were surprised by the low number of species found, which included only two exotic but widely distributed species. For these two species, and according to the main objective of this survey, that is, to compare the influence of biotic and abiotic factors in the spread of invasive symbionts, we analyzed the interactions between their climatically suitable area (*A*) and the suitable area according to host availability (*B*) using ENM techniques in a set theory framework, following Soberón and Peterson (2005). Therefore, for both ostracod symbionts, *A. sinuosa* and *U. occidentalis,* we first estimated their *A* and *B* areas (according to their climate envelopes and their exotic crayfish hosts' *P. clarkii* and *P. leniusculus*) through ENM

Table 2. Mean and SD values of the area under the curve (AUC) of the 100 individual ecological niche models carried out with the same algorithm for *Ankylocythere sinuosa*, *Uncinocythere occidentalis*, *Procambarus clarkii*, and *Pacifastacus leniusculus.*

Species	GLM		GAM		GBM		ANN		CTA		FDA		MARS		RF		EM	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
A. sinuosa	0.979	0.012	**0.989**	0.006	0.975	0.010	0.953	0.030	0.943	0.020	0.978	0.010	0.981	0.010	0.983	0.009	0.993	0.002
U. occidentalis	0.911	0.038	**0.974**	0.024	0.917	0.038	0.881	0.081	0.841	0.056	0.910	0.045	0.907	0.047	0.943	0.030	0.980	0.008
P. clarkii	0.919	0.022	**0.950**	0.016	0.935	0.018	0.881	0.039	0.883	0.027	0.923	0.022	0.920	0.023	0.948	0.016	0.968	0.004
P. leniusculus	0.966	0.015	**0.984**	0.009	0.974	0.009	0.947	0.020	0.931	0.022	0.970	0.013	0.973	0.012	0.982	0.008	0.991	0.002

The modeling algorithms used were generalized linear model (GLM), generalized additive model (GAM), generalized boosting model (GBM), artificial neural network (ANN), classification tree analysis (CTA), flexible discriminant analysis (FDA), multiple adaptive regression splines (MARS), and random forest (RF). The highest mean AUC values are shown in bold. The last two columns are the mean and SD values of AUC for the ensemble models (EM) used to get the consensus projection for each species.

modeling. The combination of *A* and *B* predictions allowed estimating the G_{BI} and *BI* areas, which resulted largely different between the two focus species and consequently highlight the importance of both biotic and abiotic factors in the expansion processes of exotic species with tight biological interactions.

Crayfish-hosted entocytherids in Europe

We evidenced the widely ranging presence of two exotic entocytherid species, *Ankylocythere sinuosa* and *Uncinocythere occidentalis*, in W Europe, previously observed in some locations of E Iberian Peninsula in the case of *A. sinuosa* (Aguilar-Alberola et al. 2012), and in a German locality for *U. occidentalis* (Grabow et al. 2009). Both species have been cited in association with more than one host species in their native range (Mestre and Mesquita-Joanes 2013), including those observed in Europe, *P. clarkii* and *P. leniusculus,* respectively. Notably, both entocytherid species have been observed living on crayfish species belonging to two different families, Cambaridae and Astacidae, showing a broad taxonomic range of hosts. No more entocytherid species were found on the exotic crayfishes sampled in this study across Europe. In contrast, all the sampled American exotic crayfishes had been previously found with entocytherid associates in their native ranges, for example, 27 entocytherid species associated with *Procambarus acutus* (Girard, 1852) (Mestre and Mesquita-Joanes 2013). Moreover, *P. clarkii* and *P. leniusculus* have all been found to be associated with four other entocytherid species (Mestre and Mesquita-Joanes 2013). Our results agree with Torchin et al. (2003) about the effects of strong filters acting on parasites and other symbionts such as entocytherids in early invasive stages.

The absence of native European entocytherids associated with autochthonous crayfish reminds of a similar pattern for another group of crayfish ectosymbionts: the Temnocephalidae. These Platyhelminta are widely distributed in the Neotropical, Ethiopian, Oceanic, and Oriental regions. However, in Europe, a few species are found living as symbionts on cave prawns and shrimps, but not on native crayfish (Gelder 1999). This absence of native ectosymbionts might facilitate the expansion of recently introduced species through host jump given the absence of competitors in their biotic niche. Nevertheless, exotic entocytherids have not been found in native European crayfish hitherto. The main probable reason is that the crayfish plague (*A. astaci*) hinders the coexistence of alien and native crayfish populations because the latter quickly extinguish locally when infected with this parasite. Another additional explanation might rely on the small numbers and high isola-

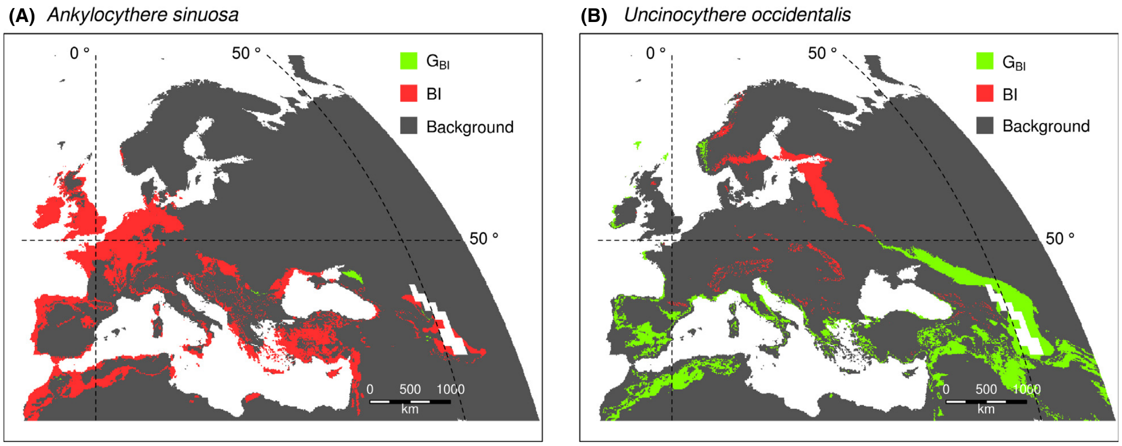


Figure 5. Combined entocytherid-host binary transformed consensus projections for species pairs (A) *Ankylocythere sinuosa* and *Procambarus clarkii,* (B) *Uncinocythere occidentalis* and *Pacifastacus leniusculus*, showing those areas climatically suitable for the symbiont but unsuitable for its host (G_{BI}), and the climatically unsuitable areas for the symbiont and suitable for the host (*BI*), in Europe (12°W–60°E; 30°N–75°N). The maps have a 5-arcmin resolution and a Mollweide equal-area projection (see Fig. 1 and text for definitions of the G_{BI} and *BI* areas; colors for G_{BI} and *BI* as in Fig. 1).

tion of populations in native crayfish metapopulations, which makes their potential colonization by exotic entocytherids difficult.

Evaluation of the relative importance of climate and host availability in the geographical space of exotic symbionts

In our models about interactions between climate and host availability in the geographical space of exotic entocytherids in Europe, we showed two species with different distributional patterns of G_{BI} and *BI* areas. *Ankylocythere sinuosa* had a predominance of *BI* areas, being closer to the model where *A* is included in *B* and therefore this species seems to be mainly restricted by its own climatic tolerances. As shown by the analysis of the predictors, the climatic restrictions of *A. sinuosa* related to *BI* may be due to the limitations to lower minimum temperatures mainly affecting the *BI* areas of W and N Europe, and the precipitation seasonality in *BI* circum-Mediterranean areas. This model suggests the existence of potential invading areas with lower minimum temperatures or higher precipitation seasonality where the host, *P. clarkii*, with a wider tolerance to these climatic variables, could lose its entocytherid symbionts, with the consequent lost of the hypothetical benefit or harm caused by their interaction. On the other hand, *U. occidentalis* has a more balanced proportion of G_{BI} and *BI* areas, fitting with the model of a partial overlap between *A* and *B*. Both areas affect different European regions, having a spatial segregation of both restriction types at a high scale level. G_{BI} of *U. occidentalis*, occupying S European, N African, and Middle East areas, may be related to limitations of *P. leniusculus* to higher precipitation seasonality and maximum temperatures. The interpretation of the limitations related to the *BI* areas of *U. occidentalis* is more difficult to ascertain due to the reduced fit shown by the GLM model for the effect of the climatic predictors on the probability of presence of this species. So, in this model of a partial overlap between *A* and *B*, the G_{BI} areas imply the existence of potential areas that *U. occidentalis* may invade if it is able to jump to other exotic or native crayfish species with a better tolerance to higher precipitation seasonality and maximum temperatures than *P. leniusculus*. This possibility cannot be excluded given the group's low host specificity, that is, one entocytherid species can inhabit more than one host species (Hart and Hart 1974), including crayfish hosts belonging to different families, as was also evidenced in this study in which we found a locality where *U. occidentalis* was also associated with *P. clarkii*, a host having those requirements (tolerance to higher precipitation seasonality and maximum temperatures).

We showed an example of spatial analyses combining ENM and a BAM theoretical framework, applied to the evaluation of the relative importance of climate and host availability in the geographical space of exotic symbionts. Both area types that characterize our models, that is, G_{BI}, where the symbiont is specifically restricted by the host availability, and *BI*, where it is specifically restricted by its own climatic tolerances, apart from their capacity to act as ecological barriers against the symbiont geographical expansion, may have other implications in the invasive process of symbionts and their hosts. In *BI* areas, typical of a model where the symbiont has a tolerance to abiotic conditions much more restricted than their hosts (*B* includes *A*), climatic barriers could act as a host "cleaning" so that the host could lose its symbiont, with the consequent loss of hypothetical benefits or harms derived from such association that may affect the invasive capacity of the host in these areas. On the other side, G_{BI} areas, characterizing the model where the symbiont has broader abiotic tolerance than its host (*A* includes *B*), may be potentially invaded by the exotic symbiont in the case of a hypothetical host jump to other host species (native or exotic), an event that may derive on a conservation issue threatening the native host species through the "spillover" effects (Roy and Handley 2012; Strauss et al. 2012). Practically, all species have symbiotic organisms affecting them. So, this type of research approach contributes to better understanding the invasive processes and could be applied to conservation plans of native species as potential hosts of exotic symbionts.

In particular, the crayfish–symbiont system has special interest in crayfish conservation. Taking into account the hypothetical jump of exotic entocytherids to European native crayfish, although the main hypothesis for the entocytherid–crayfish relationship is commensalism, this has not been rigorously dealt with, and the line between commensalism and parasitism is often very narrow (Poulin 2007). Moreover, even if it is demonstrated that they are strictly commensal, the role of entocytherids as vectors for parasites and diseases is another possibility that should be considered. Indeed, a rich fauna has been observed in association with ostracods (Mesquita-Joanes et al. 2012), which can act as intermediate hosts of parasites (e.g., Grytner-Ziecina 1996; Moravec 2004). In this sense, we wish to draw attention to the chance of a hypothetical host jump of exotic entocytherids to European native crayfish. Given the low host specificity of entocytherids (Hart and Hart 1974) and the experimentally tested horizontal transfer between adult crayfishes (Young 1971), this jump is quite likely. The potential negative effects of this event on crayfish conservation remain unknown. In this sense, we showed the role of climate and host availability as limiting factors to the expansion of the exotic entocytherid species and identified the new potential areas that the entocytherid could invade if a host jump to native crayfish would occur, information that can be used to get a better assessment of the process.

Approach limitations and recommendations

An important issue of these methods and, in general, in ENM approaches applied to invasion biology, comes from *A* being calculated by ENMs based on environmental predictors without considering biotic interactions, which are actually modulating the species distribution where those predictors are obtained from. Therefore, we do not estimate *A*, but we actually estimate $A \cap B_{GR}$, where B_{GR} represents the suitable geographical areas for species existence according to all the biotic interactions within the global range (the same applies to A_H). For example, our estimation of *BI* for *A. sinuosa* and *U. occidentalis* could be an overestimation of the real *BI* due to the existence of geographical restrictions within their native range caused by competition with other entocytherids, considering that five different species have been found associated with each of both native *P. clarkii* and *P. leniusculus* populations. So in Europe, the lack of competitors would allow the exotic entocytherids to invade part of those overestimated *BI* areas from data obtained mainly from native regions affected by intraspecific competition. In that case, the estimated *A* in our models would actually correspond to the climatically suitable European areas for the entocytherid by considering all the hosts it inhabits and restrictions from competitive interactions with other entocytherids within the global range ($A \cap B_{GR}$) (the same may occur in A_H). Actually, this is a general issue of ENMs, and in most datasets, environmental effects are confounded with those of competitors and mutualists (Elith and Leathwick 2009). The inclusion of occurrence data from invasive ranges, as we did here, and the design of laboratory experiments about species tolerances against environmental predictors may help to rigorously estimate the *A* areas of the BAM geographical space in order to minimize this problem.

The ENM uncertainty assessment reveals that the G_{BI} and *BI* geographical areas coincide in most cases with those areas with higher predictive uncertainty (compare Fig. 5 with Figs 3C,D and 4C,D). Probably, the reason is because these areas are usually close to the boundaries of the predicted species distributions, more susceptible to be predictively unstable. Therefore, the estimation of G_{BI} and *BI* is especially sensitive to ENM accuracy. Consequently, these methods should be based on ENMs with good performance. Along these lines, our ENM assessment based on three ENM performance aspects (i.e., predictors performance, AUC, and uncertainty assessments) give us evidences of weak ENM performance for *U. occidentalis* models: This was the only species with an inadequate fit of the climatic predictors and showed the highest predictive instability according to the AUC assessment through the GLMs (larger proportion of deviance not explained by the algorithm type) and higher ENM predictive uncertainty based on variability shown by the ensemble projections (wider areas with higher variability). These results strongly suggest that our estimation of G_{BI} and BI for this species could be affected by the bad performance of the ENMs for *U. occidentalis*, probably due to the lower number of occurrences available for this species.

As we have shown, a good ENM assessment is essential to analyze the interactions between abiotic and biotic factors in the geographical space. Assessing the performance of the ENM predictors provides useful information about the effects of each individual predictor for each species and can be combined with the results of niche models to better understand which specific variable could be involved on the restrictions present in the different G_{BI} and BI areas. The use of two different approaches to assess ENM performance based on ensemble modeling techniques (i.e., AUC and uncertainty assessments) gives stronger support to our results and, finally, the uncertainty is specially valuable because it helps us to locate those areas with higher predictive instability, and then, we can compare them with the G_{BI} and BI areas to assess the reliability of our estimations.

The methodological approach presented in this work, focused on a symbiont–host system, can also be applied to other systems where the target species is strongly affected by interactions with other species. The range of possibilities may include different kinds of mutualisms, predators with a strong dependence on a specific prey, or species having incompatibilities with the presence of some specific predators, parasites, or competitors. The data required to develop this kind of models are a global occurrence dataset for the interacting species and a global climatic dataset of a large extension and coarse resolution scale. The first step of the analyses through the implementation of set theory is especially important, because it allows a wide variety of theoretical contexts to adapt our models to a particular biological question proposed, for example, the inclusion of dispersal barriers affecting the species expansion through the use of M, or the consideration of more than one interacting species to estimate B. The generalization of our approach to species without tight biotic relationships would require a higher development of this methodology because, in those cases, the B areas do not depend only on the presence of the interacting species, but other parameters would be implied, such as the species densities or the existence of interactions between the environmental conditions and the effect of the biotic interaction. Finally, when applying this kind of models, we do not have to lose the perspective that we deal with dynamic systems (Larson and Olden 2012; Lu et al. 2013).

Acknowledgments

We wish to thank Bart Achterkamp, J.M. Aguilar, Maria Antón, Marco Arruej, Eliott and Scott Birner, Andreu Castillo, Andreu Escrivà, Sara Farreras, Gregorio Herrera, Joaquín Guerrero, Sara Lapesa, Cristina Molina, Pilar Ore, Tomáš Policar, Adrian Ponz, Alba Remolar, Josep R. Roca, Olivier Schmit, Robin Smith, Jorge Urbano, Luís Valls, Renate Walter, and Laia Zamora for their help during fieldwork, sample treatment, or other aspects of this survey. This research was funded by the Spanish Ministry of Science and Innovation Project ECOINVADER (CGL2008-01296/BOS) and the University of Valencia ("V-Segles" predoctoral grant to A. Mestre). AK acknowledges Project CENAKVA CZ.1.05/2.1.00/01.0024. We thank sampling permission from the regional Spanish governments of Castilla-La Mancha, Castilla-León, Extremadura, Aragón, Andalucía, Navarra, Illes Balears, Catalunya, and C. Valenciana. We would like to thank three anonymous reviewers and the editors of *Ecology and Evolution* for constructive suggestions to previous versions of the manuscript.

Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Drawings of male copulatory organs of the entocytherid species found during the field survey.
Table S1. Detailed European crayfish data checked for entocytherids in this work.
Table S2. GLM results for the performance of the climatic predictors.
Table S3. GLM results for the fixed algorithm effects on AUC.

Supporting Information

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Table S3. GLM results for the fixed algorithm effects on AUC.

Additional references

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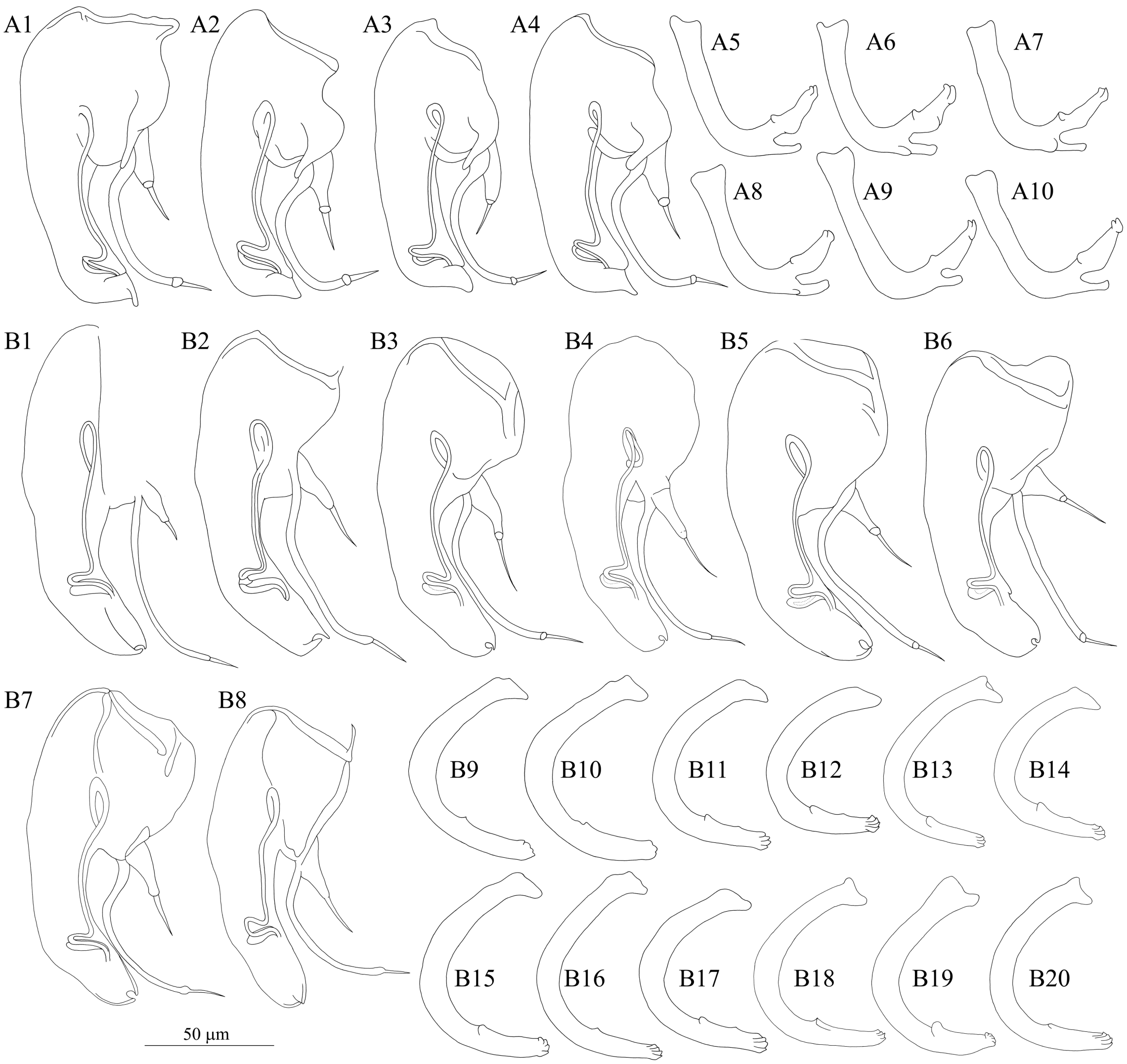

Figure 1. Variation in male copulatory organs of (A) *Ankylocythere sinuosa* and (B) *Uncinocythere occidentalis* in different European localities from this work, with additional comparative drawings belonging to specimens from the native range and other invasive ranges. (A1-4, B1-8) are drawings of the whole copulatory complex and (A5-10, B9-20) are details of the clasping apparatuses (see Hart & Hart, 1974, for the morphological nomenclature), in lateral views. Specimens A1, A2, A5 and A6 are from Baton Rouge, Louisiana (personal collection); (A3, A8) from LOC027 (Table S1 contains information about locality codes); (A4, A10) from LOC017; (A7) from LOC016; (A9) from LOC005; (B1, B9) are drawings adapted from Hart & Hart (1974) belonging to American specimens; (B2, B10) adapted from Hiruta & Smith (2001) for Japanese exotic specimens; (B3, B11, B12) from LOC041; (B4, B13, B14) from LOC039; (B5, B15) from LOC040; (B6, B16, B17) from LOC044; (B7, B18) from LOC045; (B8, B20) from LOC047; (B19) from LOC046. Note: male copulatory organs are taxonomically relevant as they are used to identify entocytherid species (Hart & Hart, 1974).

Table S1. Detailed data on crayfish samples obtained for checking entocytherid occurrences in Europe carried out in this work. The data fields are: crayfish species; a unique code established to identify each locality; a brief description of the sampling site; name of the sampled locality and state; sampling day; number of individuals caught; crayfish' European status (native or exotic); the entocytherid species recorded in the locality with the corresponding crayfish species and the protocol used to remove entocytherids from crayfish. In the protocol column, we indicate the time during which crayfish was immersed in the anaesthetic product used. We utilised carbonated water (CW) or chlorobutanol (CB). Alternatively, crayfishes were fixed in ethanol (see Mestre et al., 2011, for more detailed information about the protocols used).

Crayfish species	Locality code	Site description	Locality	State	Sampling date	Crayfishes sampled	Crayfish status	Entocytherid species	Protocol
Astacus astacus	LOC087	River	Mreznica	Croatia	25/07/11	6	Native	No entocytherids	5 min CW
A. astacus	LOC088	Stream	Kapela Korenicka	Croatia	07/08/11	7	Native	No entocytherids	5 min CW
A. astacus	LOC089	Stream	Zaklopaca	Croatia	08/08/11	10	Native	No entocytherids	5 min CW
A. astacus	LOC076	Flooded quarry	Mačkov	Czech Rep.	22/06/11	10	Native	No entocytherids	5 min CW
A. astacus	LOC090	Pond	Babylon	Czech Rep.	23/06/11	10	Native	No entocytherids	5 min CW
A. astacus	LOC091	Pond	Hrabice	Czech Rep.	05/10/11	10	Native	No entocytherids	5 min CW
Astacus leptodactylus	LOC075	River	Duga resa	Croatia	22/07/11	11	Native	No entocytherids	5 min CW
A. leptodactylus	LOC076	Flooded quarry	Mačkov	Czech Rep.	22/06/11	5	Native	No entocytherids	5 min CW
A. leptodactylus	LOC077	Lake	Mayaky	Ukraine	08/09/11	20	Native	No entocytherids	5 min CW
A. leptodactylus	LOC078	Lake	Stanislav	Ukraine	07/09/11	20	Native	No entocytherids	5 min CW
A. leptodactylus	LOC079	River	Komyshuvatka	Ukraine	01/09/11	19	Native	No entocytherids	5 min CW
A. leptodactylus	LOC080	Lake	Mayaky	Ukraine	08/09/11	20	Native	No entocytherids	5 min CW
A. leptodactylus	LOC081	Reservoir	Kerkrade	Netherlands	05/11/10	7	Native	No entocytherids	Alcohol
A. leptodactylus	LOC082	Lake	Karaburun	Turkey	06/07/11	10	Native	No entocytherids	5 min CW
A. leptodactylus	LOC083	Lake	Durusu	Turkey	06/07/11	10	Native	No entocytherids	5 min CW
A. leptodactylus	LOC084	Lake	Çakirli	Turkey	22/07/11	10	Native	No entocytherids	5 min CW
A. leptodactylus	LOC085	Lake	Boyalica	Turkey	22/07/11	10	Native	No entocytherids	5 min CW
Astacus sp.	LOC086	River	Generalski Stol	Croatia	23/07/11	10	Native	No entocytherids	5 min CW

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Crayfish species	Locality code	Site description	Locality	State	Sampling date	Crayfishes sampled	Crayfish status	Entocytherid species	Protocol
Austropotamobius pallipes	LOC070	Stream	Mora de Rubielos	Spain	05/03/10	9	Native	No entocytherids	10 min CB
A. pallipes	LOC071	Stream	Suera	Spain	27/07/11	22	Native	No entocytherids	7 min CB
A. pallipes	LOC072	Pond	El Hontanar	Spain	03/08/11	20	Native	No entocytherids	7 min CB
A. pallipes	LOC073	Pond	Bellestar	Spain	09/08/11	16	Native	No entocytherids	7 min CB
A. pallipes	LOC074	Crayfish farm	Rillo de Gallo	Spain	03/11/11	20	Native	No entocytherids	15 min CW
Austropotamobius torrentium	LOC092	Stream	Prijeboj	Croatia	08/08/11	5	Native	No entocytherids	5 min CW
A. torrentium	LOC093	Brook	Nova Kubice	Czech Rep.	23/06/11	10	Native	No entocytherids	4 min CW
Cherax quadricarinatus	LOC069	City pond	Wageningen	Netherlands	02/10/07	1	Exotic	No entocytherids	Live inspection
C. quadricarinatus	LOC094	Pet shop	Valencia	Spain	11/02/09	6	Exotic	Indeterminate	15 min CW
Cherax destructor	LOC068	Pond	Bagüés	Spain	01/06/10	7	Exotic	No entocytherids	15 min CW
Orconectes limosus	LOC053	Pond	Tutegny	France	01/10/10	33	Exotic	No entocytherids	Alcohol
O. limosus	LOC059	Dam	Lipno	Czech Rep.	19/10/11	10	Exotic	No entocytherids	5 min CW
O. limosus	LOC060	Ditch	Gorinxem	Netherlands	20/05/12	20	Exotic	No entocytherids	Alcohol
O. limosus	LOC061	Stream	Wageningen	Netherlands	07/10/12	40	Exotic	No entocytherids	Alcohol
Orconectes virilis	LOC062	River	London	England	01/09/11	6	Exotic	No entocytherids	15 min CW
O. virilis	LOC063	River	London	England	08/09/11	10	Exotic	No entocytherids	15 min CW
O. virilis	LOC064	Ditch	Boven-Hardinxveld	Netherlands	20/05/12	2	Exotic	No entocytherids	Alcohol
O. virilis	LOC065	Ditch	Oukoop	Netherlands	28/09/12	10	Exotic	No entocytherids	Alcohol
O. virilis	LOC066	Ditch	Oukoop	Netherlands	14/09/12	10	Exotic	No entocytherids	Alcohol
O. virilis	LOC067	Ditch	Kanis	Netherlands	28/10/12	10	Exotic	No entocytherids	Alcohol
Pacifastacus leniusculus	LOC039	Irrigation pond	Undués de Lerda	Spain	01/06/10	3	Exotic	*Uncinocythere occidentalis*	15 min CW

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Crayfish species	Locality code	Site description	Locality	State	Sampling date	Crayfishes sampled	Crayfish status	Entocytherid species	Protocol
P. leniusculus	LOC040	River	Cimballa	Spain	17/06/10	1	Exotic	*U. occidentalis*	Alcohol
P. leniusculus	LOC041	River	Ademuz	Spain	03/08/11	20	Exotic	*U. occidentalis*	15 min CW
P. leniusculus	LOC042	River	Covanera	Spain	28/04/09	1	Exotic	*U. occidentalis*	Alcohol
P. leniusculus	LOC043	River	Río Cuervo	Spain	25/09/10	15	Exotic	*U. occidentalis*	15 min CW
P. leniusculus	LOC044	River	London	England	20/09/11	10	Exotic	*U. occidentalis*	15 min CW
P. leniusculus	LOC045	Stream	Tilburg	Netherlands	14/11/12	35	Exotic	*U. occidentalis*	Alcohol
P. leniusculus	LOC046	River	Sant-Jean-du-Gard	France	28/08/10	3	Exotic	*U. occidentalis*	Alcohol
P. leniusculus	LOC047	Stream	Baden-Baden	Germany	20/07/05	30	Exotic	*U. occidentalis*	Alcohol
P. leniusculus	LOC048	Stream	Ponts	Spain	03/06/10	4	Exotic	Indeterminate sp.	15 min CW
P. leniusculus	LOC049	River	Sta Cruz de Campezo	Spain	09/06/10	7	Exotic	Indeterminate sp.	Alcohol
P. leniusculus	LOC050	Stream	Los Pintanos	Spain	01/06/10	10	Exotic	No entocytherids	15 min CW
P. leniusculus	LOC034	River	Cimballa	Spain	17/06/10	1	Exotic	Indeterminate sp.	Alcohol
P. leniusculus	LOC051	River	Oliana	Spain	26/05/10	5	Exotic	No entocytherids	Alcohol
P. leniusculus	LOC052	River	Sta Cruz de Campezo	Spain	09/06/10	5	Exotic	No entocytherids	Alcohol
P. leniusculus	LOC053	Pond	Tutegny	France	01/09/10	19	Exotic	No entocytherids	Alcohol
P. leniusculus	LOC054	Brook	Babačka brook	Czech Rep.	20/11/11	10	Exotic	No entocytherids	5 min CW
P. leniusculus	LOC055	Stream	Losser	Netherlands	06/02/07	4	Exotic	No entocytherids	Alcohol
Procambarus acutus	LOC057	Ditch	Boven-Hardinxveld	Netherlands	20/05/12	20	Exotic	No entocytherids	Alcohol
P. acutus	LOC058	Ditch	Giessenburg	Netherlands	20/05/12	20	Exotic	No entocytherids	Alcohol
Procambarus clarkii	LOC001	Irrigation channel	Oliva	Spain	28/04/10	15	Exotic	*Ankylocythere sinuosa*	15 min CW
P. clarkii	LOC002	Irrigation channel	Puçol	Spain	30/04/10	20	Exotic	*A. sinuosa*	15 min CW

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Crayfish species	Locality code	Site description	Locality	State	Sampling date	Crayfishes sampled	Crayfish status	Entocytherid species	Protocol
P. clarkii	LOC003	Reservoir	Valparaíso	Spain	06/05/10	9	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC004	Lake	Carucedo	Spain	07/05/10	15	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC005	Lake	Chozas de Arriba	Spain	07/05/10	11	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC006	Irrigation channel	Vivares	Spain	15/05/10	20	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC008	Stream	Rosalejo	Spain	15/05/10	5	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC009	Stream	Sarrià de Ter	Spain	20/05/10	20	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC010	Stream	Navarcles	Spain	20/05/10	10	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC011	River	Granollers	Spain	21/05/10	8	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC012	River	Quero	Spain	26/05/10	20	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC013	River	Socuéllamos	Spain	27/05/10	4	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC014	Reservoir	Mequinenza	Spain	02/06/10	20	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC015	Irrigation pond	Alpicat	Spain	03/06/10	20	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC016	Reservoir	Sòller	Spain	09/06/10	11	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC017	Irrigation channel	Puerto Real	Spain	16/06/10	20	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC018	Irrigation channel	Palacios	Spain	16/06/10	20	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC019	Irrigation channel	Padul	Spain	18/06/10	16	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC020	Reservoir	Cubillas	Spain	18/06/10	20	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC021	Lake	Robledo	Spain	23/06/10	11	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC022	Stream	Alcaraz	Spain	23/06/10	1	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC023	Pond	Valencia	Spain	20/04/11	20	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC024	Irrigation channel	Torreblanca	Spain	19/07/11	13	Exotic	*A. sinuosa*	15 min CW

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Crayfish species	Locality code	Site description	Locality	State	Sampling date	Crayfishes sampled	Crayfish status	Entocytherid species	Protocol
P. clarkii	LOC025	Irrigation channel	Torreblanca	Spain	19/07/11	20	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC026	Pond	Monroy	Spain	24/09/11	3	Exotic	*A. sinuosa*	15 min CW
P. clarkii	LOC027	Exotic pet shop	Vodnany	Czech Rep.	17/06/11	33	Exotic	*A. sinuosa*	5 min CW
P. clarkii	LOC028	Wetland	Delta Ebre	Spain	10/09/08	12	Exotic	Indeterminate sp.	15 min CW
P. clarkii	LOC029	Irrigation channel	Alberic	Spain	28/04/10	1	Exotic	No entocytherids	Alcohol
P. clarkii	LOC030	Irrigation channel	Sa Pobla	Spain	09/06/10	4	Exotic	No entocytherids	15 min CW
P. clarkii	LOC031	Stream	Canyamel	Spain	09/06/10	1	Exotic	No entocytherids	Alcohol
P. clarkii	LOC032	Irrigation channel	Sa Pobla	Spain	10/06/10	6	Exotic	No entocytherids	15 min CW
P. clarkii	LOC033	Irrigation channel	Balazote	Spain	23/06/10	1	Exotic	Indeterminate sp.	15 min CW
P. clarkii	LOC034	River	Cimballa	Spain	17/06/10	2	Exotic	Indeterminate sp.	Alcohol
P. clarkii	LOC035	Wetland	Torreblanca	Spain	20/06/10	1	Exotic	No entocytherids	Alcohol
P. clarkii	LOC036	River	Bicorp	Spain	18/11/07	4	Exotic	No entocytherids	15 min CW
P. clarkii	LOC037	City water	Den Haag	Netherlands	07/08/12	21	Exotic	No entocytherids	Alcohol
P. clarkii	LOC038	Stream	Schijndel	Netherlands	14/10/12	30	Exotic	No entocytherids	Alcohol
P. clarkii	LOC039	Irrigation pond	Undués de Lerda	Spain	01/06/10	10	Exotic	*A. sinuosa* and *U. occidentalis*	15 min CW
P. clarkii	LOC007	Lake	Valdebótoa	Spain	15/05/10	17	Exotic	*A. sinuosa*	15 min CW
Procambarus fallax	LOC056	Exotic pet shop	Wageningen	Netherlands	05/10/07	4	Exotic	No entocytherids	Alcohol

Table S2. Results of the generalized linear models analysing the effects of climatic predictors on the probability of species presence for *Ankylocythere sinuosa*, *Uncinocythere occidentalis*, *Procambarus clarkii* and *Pacifastacus leniusculus*. The climatic predictors were the maximum temperature of the warmest month (MaxT), the minimum temperature of the coldest month (MinT), annual precipitation (AnPrec) and precipitation seasonality (coefficient of variation, PrecSeas). SE is the standard error. Significant P values (< 0.05) are marked with the an asterisk.

Model	Predictor	Estimate	SE	Z Value	P
Ankylocythere sinuosa	Intercept	-4.437	1.358	-3.266	< 0.005*
	MaxT	0.028	0.004	6.905	< 0.001*
	MinT	0.006	0.002	2.422	< 0.05*
	AnPrec	-0.001	0.000	-2.038	< 0.05*
	PrecSeas	-0.093	0.009	-10.299	< 0.001*
Uncinocythere occidentalis	Intercept	-1.314	1.301	-1.010	0.312
	MaxT	0.003	0.004	0.958	0.338
	MinT	-0.005	0.002	-2.171	< 0.05*
	AnPrec	-0.000	0.000	-0.643	0.520
	PrecSeas	0.005	0.007	0.747	0.455
Procambarus clarkii	Intercept	-3.981	0.775	-5.133	< 0.001*
	MaxT	0.005	0.002	2.106	< 0.05*
	MinT	-0.012	0.002	-8.067	< 0.001*
	AnPrec	0.001	0.000	3.521	< 0.001*
	PrecSeas	0.036	0.004	8.436	< 0.001*
Pacifastacus leniusculus	Intercept	5.149	0.642	8.025	< 0.001*
	MaxT	-0.011	0.002	-4.839	< 0.001*
	MinT	0.010	0.001	7.582	< 0.001*
	AnPrec	-0.001	0.000	-2.996	< 0.005*
	PrecSeas	-0.033	0.004	-7.926	< 0.001*

Table S3. Results of the generalized linear models used to assess the effect of algorithm on the area under the curve (AUC) parameter of the 800 individual ecological niche models for *Ankylocythere sinuosa*, *Uncinocythere occidentalis*, *Procambarus clarkii* and *Pacifastacus leniusculus*. The algorithms used for modelling were: artificial neural network (ANN), generalized linear model (GLM), generalized additive model (GAM), generalized boosting model (GBM), classification tree analysis (CTA), random forest (RF), flexible discriminant analysis (FDA) and multiple adaptive regression splines (MARS). SE is the standard error. Significant *P* (< 0.05) are marked with an asterisk.

Model	Algorithm	Estimate	SE	*Z* Value	*P*
Ankylocythere sinuosa	Intercept	-0.048	0.005	-10.625	< 0.001*
	ANN	0.000	—	—	—
	GLM	0.027	0.006	4.225	< 0.001∗
	GAM	0.037	0.006	5.808	< 0.001*
	GBM	0.023	0.006	3.593	< 0.001*
	CTA	-0.011	0.006	-1.720	0.085
	RF	0.031	0.006	4.883	< 0.001*
	FDA	0.026	0.006	4.037	< 0.001*
	MARS	0.029	0.006	4.503	< 0.001*
Uncinocythere occidentalis	Intercept	-0.127	0.005	-27.405	< 0.001*
	ANN	0.000	—	—	—
	GLM	0.033	0.007	5.116	< 0.001*
	GAM	0.100	0.006	15.521	< 0.001*
	GBM	0.040	0.007	6.109	< 0.001*
	CTA	-0.046	0.007	-6.994	< 0.001*
	RF	0.068	0.006	10.436	< 0.001*
	FDA	0.033	0.007	5.028	< 0.001*
	MARS	0.029	0.007	4.434	< 0.001*
Procambarus clarkii	Intercept	-0.127	0.005	-27.401	< 0.001*
	ANN	0.000	—	—	—
	GLM	0.042	0.006	6.440	< 0.001*
	GAM	0.075	0.006	11.648	< 0.001*
	GBM	0.059	0.006	9.102	< 0.001*
	CTA	0.002	0.007	0.278	0.781
	RF	0.073	0.006	11.250	< 0.001*
	FDA	0.046	0.006	7.141	< 0.001*
	MARS	0.044	0.006	6.730	< 0.001*
Pacifastacus leniusculus	Intercept	-0.054	0.005	-11.987	< 0.001*
	ANN	0.000	—	—	—
	GLM	0.020	0.006	3.115	< 0.005*
	GAM	0.038	0.006	5.935	< 0.001*
	GBM	0.028	0.006	4.420	< 0.001*
	CTA	-0.017	0.006	-2.700	< 0.01*
	RF	0.036	0.006	5.701	< 0.001*
	FDA	0.024	0.006	3.781	< 0.001*
	MARS	0.027	0.006	4.170	< 0.001*

CAPÍTOL VII

Experimental spillover of an exotic ectosymbiont on a naive endangered crayfish

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In prep.

Experimental spillover of an exotic ectosymbiont on a naive endangered crayfish

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April 17, 2014

Abstract

1. Spillover, i.e. symbiont transmission from exotic to native species, represents a potential threat for the conservation of biodiversity. European native crayfish have been threatened for the last decades by exotic crayfish species introduced in Europe, due to competition and transmission of diseases. Recently, a new group of exotic ectosymbionts (Ostracoda, Entocytheridae) was recorded in Europe associated with American crayfish invaders.
2. Our main aim was to experimentally testing the transmission of an exotic entocytherid species (*Ankylocythere sinuosa*) from a crayfish invader (*Procambarus clarkii*) to a native endangered crayfish (*Austropotamobius italicus*). The experiment consisted in placing both crayfish transmitter (i.e. a crayfish *P. clarkii* with symbionts) and receiver (i.e. crayfish without symbionts) in the same container during four days. Three experimental treatments (with 25 replicates) were designed: (1) crayfish transmitter alone; (2) cleaned *P. clarkii* (i.e. with symbionts removed) as crayfish receiver; (3) *A. italicus* as crayfish receiver. After the experiment, we individually checked for entocytherid presence both in crayfish and off-host environment to test for symbiont transmission.
3. We observed a highly effective symbiont transmission onto native crayfish *A. italicus*, with 100 % of individuals reached. The transmission was via dispersing symbionts from off-host environment, emitted by crayfish transmitter. Symbiont emission rates were higher in treatments with presence of crayfish receiver. Transmission rates were higher in bigger crayfish receivers and in *A. italicus*. All eight entocytherid growth stages were able to be transmitted although younger and adult ones showed higher dispersal trends.
4. This study advances a new potential threat for crayfish conservation: spillover of exotic entocytherids into wild populations of native crayfish is possible, as evidenced here under experimental conditions. We cannot predict the effects of a symbiont on a novel host which, a priori, is not adapted to harbour this kind of organisms. Moreover, in case of spillover success, the eradication of exotic symbionts from a wild crayfish population would probably be impracticable. Then, we recommend a cautionary strategy based on two points: (1) Further experimental research focused on potential effects of hypothetical spillover; (2) preventive measures against spillover.

Keywords— Astacidae, cross-species transmission, Europe, host detection, invasion biology, ostracoda, species conservation, symbiont dispersal

Introduction

In invasion biology, the term "spillover" refers to the process of symbiont transmission from an exotic species to a native one. The process may facilitate the invasion success and represents a potential threat for the conservation of native species (Roy & Handley, 2012; Strauss et al., 2012). An exotic parasitic symbiont transmitted to a native species might severely impact the novel host due to lack of history-evolved resistance (Mastitsky et al., 2010). The phenomenon is called "naive host syndrome" and it can help the host invader to exclude potential competitors. An example of well-documented dramatic impact of spillover on the conservation of a native species is the transmission of the oomycete *Aphanomyces astaci* from American exotic to European native crayfish, responsible of the aphanomycosis (crayfish plague) that seriously endangered native crayfish populations in Europe (Alderman, 1996).

The European native crayfish belong to the family Astacidae, and are constituted by five or six species belonging to two genera (*Astacus* and *Austropotamobius*) (Holdich, 2003). Three of them (i.e. *Astacus astacus*, *Austropotamobius torrentium* and *Austropotamobius pallipes*) are protected by European legislation (Taylor, 2002). Recent research on crayfish molecular phylogenies supports the separation of *Austropotamobius pallipes* into two species, *A. pallipes* and *A. italicus* (Fratini et al., 2005; Bertocchi et al., 2008) and, therefore, in case of general acceptance, the new *A. italicus* should be included in future updates of European regulation of endangered species. In this survey, we will adopt the new proposed taxonomy. Additionally to habitat modification and pollution, exotic crayfish species introduced in Europe during the last 50 years constitute a main threat to European native crayfish through competition and transmission of diseases, notably aphanomycosis (Holdich, 2002). Nowadays, at least six exotic crayfish species (from America and Australia) have established wild populations in Europe (Holdich, 2003; Mestre et al., 2013).

Entocytherids are an ostracod family of 220 species entirely constituted by ectosymbionts on other crustaceans (Mestre et al., 2014). Both main entocytherid subfamilies, Entocytherinae and Notocytherinae, inhabit crayfish hosts. Entocytherinae (183 species) live on American Cambaridae and Astacidae, and Notocytherinae (26 species) inhabit Parastacidae species from Oceania. European native crayfishes do not host their own entocytherids. However, two exotic entocytherid species have been recently detected in Europe widely established and associated with two crayfish invaders: *Procambarus clarkii* (Cambaridae) and *Pacifastacus leniusculus* (American Astacidae) (Mestre et al., 2013). One of them, *Ankylocythere sinuosa*, was found widely distributed throughout the Iberian Peninsula in *P. clarkii* populations, showing high levels of host occupancy (i.e. prevalence) (Aguilar-Alberola et al., 2012). This entocytherid species has been cited in its native range in association with 47 host species (Mestre et al., 2014). Entocytherids are characterized by low degrees of host specificity (Mestre et al., 2014) and Young (1971) experimentally demonstrated the horizontal transmission of entocytherids between *Procambarus simulans* crayfish hosts within few days and without the need of direct physical contact between hosts. Both ideas suggest the possibility of entocytherid spillover into native European crayfish, even though no previous information on entocytherid transmission between different crayfish species is known. Studies of experimental cross-species transmissions in the frame of invasion biology have been useful to assess potential spillover or spillback (i.e. implying native symbionts) events, in terms of probability of transmission success (e.g. Vignon et al., 2009) and differences in symbiont growth and maturity (e.g. Paterson et al., 2013) between native and invader hosts. Other cross-species transmission experiments have been focused on differential effects of symbionts on native and invader hosts (e.g. Shirakashi & El-Matbouli, 2009).

The *Austropotamobius pallipes* species complex, still considered a unique species in the European legislation, represents the most endangered crayfish taxon in Europe, which suffered drastic reductions of their populations during recent decades as a consequence of water pollution, habitat alteration and invasive crayfish. It is considered endangered in the red list of the IUCN (2013), and listed as protected in Appendix III of the 1979 Bern Convention. Moreover, it is the only crayfish taxon that appears in the Appendix II of the EU Habitats Directive (Taylor, 2002). At least 12 European countries contain wild populations of the *A. pallipes* species complex (Holdich, 2002). *Austropotamobius pallipes* has a N European distribution (France, Switzerland, Austria, Great Britain and Ireland), whereas *A. italicus* is distributed along southern regions (Spain, Italy, the Balkans and Switzerland). Following the Habitats Directive, some countries have been involved in population monitoring and reintroduction programs to recover and assess both species (Füreder et al., 2010).

The main aim of the present study is to test the cross-species transmission of symbiont *A. sinuosa* from the crayfish invader *P. clarkii* to the native *A. italicus* under experimental conditions. With this, we pretend to obtain valuable information to answer the following question: is the entocytherid spillover from exotic species to European native crayfish possible? This is a crucial question in order to know whether or not we should take into account a new potential source of threats for crayfish conservation.

Methods

Crayfish collection, transport and maintenance

Crayfish individuals of *P. clarkii* were collected during autumn 2011 from a population harbouring *A. sinuosa* located in Pego-Oliva's wetland (E Iberian Peninsula; 38° 53′ 16.66″ N; 0° 4′ 7.74″ W) (Castillo-Escrivà et al., 2013). Two months before the experiment we collected a group of *P. clarkii* that was subjected to a cleaning protocol before the experiment to establish a negative control group of symbiont-free crayfish (see next subsection). In addition, one week before the experiment, another group of *P. clarkii* was sampled to be used as symbiont transmitters (donors) for the experiment. *Austropotamobius italicus* specimens were obtained from the astaciculture center Río de Gallo (Guadalajara, Spain; 40° 52′ 2.60″ N; 1° 56′ 4.66″ W), four days before the experiment. After their collection, all crayfish groups were transported to the lab wrapped in wet tissues. Once there, they were maintained until the beginning of the experiment in 200-L aquaria within a climatic chamber at 19 °C, with aerated conditions, photoperiod 12:12 L:D and without contact between experimental groups. The aquaria were previously cleaned through a one-day bleach treatment. Crayfishes were fed with commercial fish food during the previous phases before the experiment. We also added PVC tubes into the aquaria as shelter to avoid stress and cannibalism.

Cleanning protocol for the *P. clarkii* negative control group

The cleaning protocol to remove symbionts from the *P. clarkii* negative control group consisted on periodical submersion (every three days) of individuals into 20-L aquaria with 0.5 % chlorobutanol for 10 min, under aerated conditions (Mestre et al., 2011). After treatment, crayfishes were transferred to 200-L aquaria previously cleaned through a bleach treatment. We monitored symbiont presence by filtering the chlorobutanol solution after each cleaning treatment with a 63-µm size mesh, checking the retained content under a stereomicroscope. After 10 sessions, as we did not observe symbionts in the checked samples, we finished the cleaning treatment (one week before the experiment).

Experimental design

Basically, the experiment to test for symbiont transmission consisted on placing both the crayfish transmitter (i.e. a crayfish with symbionts) and receiver (i.e. crayfish without symbionts) within the same container (20 cm × 13.5 cm × 8 cm) filled with water, in coexistence during four days. Each crayfish was physically isolated from the other through a double layer of a 5-mm sized plastic mesh located in the center of the container, in vertical position, delimiting two equal compartments.

There was a small space between both mesh layers to avoid crayfish contact across the mesh. Using the same material, we added a mesh cover on top of the container to avoid crayfish getaways. All bottoms of the containers were previously sanded with an extra-fine sandpaper to allow symbiont off-host displacements (based on pers. obs.).

All the crayfishes used in the experimental treatments had adult sizes (weight *P. clarkii*: $\mu = 14.81$ g; SD = 3.99; N = 100, *A. italicus*: $\mu = 34.54$ g; SD = 8.19; N = 25). Prior to the experiment, we selected 25 *P. clarkii* ($\mu = 15.80$ g; SD = 3.78) from the cleaned group and 20 *A. italicus* ($\mu = 25.45$ g; SD = 6.68) as negative control groups (C–), to check for symbiont absences, as these crayfish groups would eventually act as receivers in the experiment. We applied to each individual from group C– a standardised protocol of entocytherid removal consisting on crayfish submersion into a 1-L container filled with commercial Fuente Primavera^{TM} carbonated water during 15 min (Mestre et al., 2011). Then, we filtered the carbonated water from each container through a 63-µm sized filter to isolate the symbionts. The filter was preserved in 96 % alcohol. We performed three experimental treatments, with 25 replicates each (Fig. 1):

1. The positive control (C+) consisted of a *P. clarkii* individual acting as transmitter (i.e. with symbionts) alone in a container;
2. The treatment with a *P. clarkii* receiver (CRT) consisted of a container with mesh barriers separating a *P. clarkii* transmitter in a compartment and a *P. clarkii* receiver (i.e. from the cleaned group) in the other;
3. Finally, the third treatment was the same as the previous one but with *A. italicus* as crayfish receiver (IRT) instead of another *P. clarkii* individual.

The experiment was done in the same climatic chamber used for crayfish stocking, and we used the same conditions for the experiment (i.e. water cond = 1.27 mS; T = 19 °C; photoperiod 12:12 L:D). During the four days of the experiment, every morning we measured the oxygen saturation of several containers (belonging to C+ and IRT) and aerated all the containers until we re-established the 100 % oxygen saturation.

After four days, each individual crayfish was exposed to the symbiont removal protocol of 15-min carbonated water submersion, followed by a filtering and preservation of its content in 96 % ethanol. We also filtered the water from each container to collect the symbionts present in the off-host environment. To improve the last process, after a first filtering we partially refilled the container with carbonated water and filtered it again. With this procedure, we separately isolated three symbiont groups:

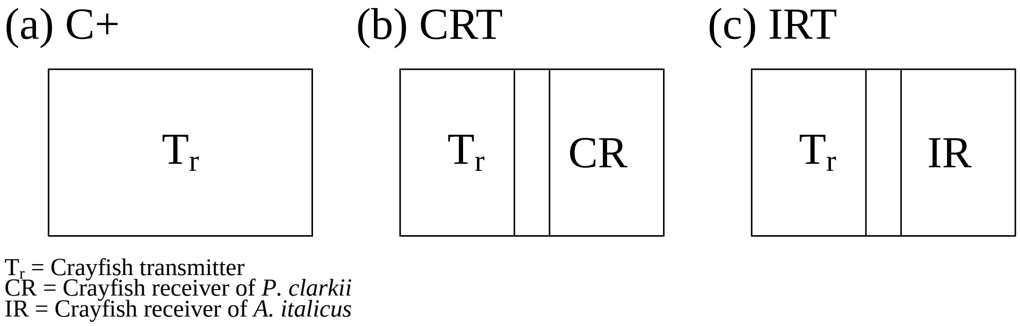

Figure 1. Experimental treatments to test symbiont transmission from a crayfish with symbionts (crayfish transmitter) to another free of symbionts (crayfish receiver). The experiment had three treatments, with 25 replicates each: **(a)** The positive control (C+) was a crayfish transmitter (i.e. a *Procambarus clarkii* with symbionts) standing alone in a water container without mesh barriers. **(b)** The treatment with *P. clarkii* receiver (CRT), was a container with mesh barriers in the middle, separating a *P. clarkii* transmitter in a compartment from a symbiont-free *P. clarkii* receiver (i.e. from the cleaned group) in the other. **(c)** Finally, the IRT was the same as the previous one but with *Austropotamobius italicus* as crayfish receivers.

1. those that remained within the crayfish transmitter after the experiment;
2. those emitted by the crayfish transmitter that remained in the off-host environment;
3. the symbionts that got to colonize the crayfish receiver.

After this treatment, crayfishes were preserved in 96 % alcohol. All crayfishes were sexed and weighed after the experiment. Moreover, we also estimated the moult stage using a code based on body hardness (0 = crayfish body completely and extremely soft, including the chelas; 1 = cephalothorax sides soft and very elastic; 2 = cephalothorax sides hard but slightly elastic; 3 = cephalothorax sides hard and totally rigid; Castillo-Escrivà et al., 2013). Symbiont samples were individually checked under the stereomicroscope and we counted all the entocytherids, distinguishing between the eight different entocytherid stages (from the youngest A–7 to subadults A–1, plus adults) and sexing the A–1 and adult individuals. We also registered the number of mating pairs (Aguilar-Alberola et al., 2012).

Statistical analyses

Initial analyses focused on testing the effects of treatment, weight, sex and moult of crayfish transmitters on their original symbiont abundances (i.e. the total number of entocytherids found in a replicate). We also analysed differences in crayfish weight between treatments. The main aim of our work was to quantify successful symbiont transmission from crayfish transmitters to receivers, in both

CRT and IRT treatments. For this, we compared symbiont abundances of crayfish receivers in both treatments with those of their respective C– groups.

We defined symbiont emissions as the number of symbionts that had left the crayfish transmitter after the treatment (i.e. symbionts from off-host environment plus symbionts from crayfish receiver) and symbiont emission rates as their proportion. Then, we analysed the effects of crayfish weight (transmitted and receiver) and species of receiver (*P. clarkii* or *A. italicus*) on symbiont emission rates by considering those treatments with presence of crayfish receiver (i.e. CRT and IRT treatments). Moreover, we also analysed the effects of transmitter crayfish weight and treatment type by considering the three experimental treatments (i.e. C+, CRT and IRT). The transmission rate was defined as the proportion of emitted symbionts that colonized the crayfish receiver. Host transmission rates were modelled in relation to features of crayfish receiver (i.e. weight and species).

A symbiont infrapopulation is defined as all the individuals of a symbiont species (i.e. *A. sinuosa*) living within a host (Poulin, 2007). In our experiment, the original symbiont infrapopulations are the entocytherids that the crayfish transmitter harboured before starting the experiment, considered here as all the symbionts found in one replicate. We compared proportions of the eight symbiont growth stages between original symbiont infrapopulations and emitted symbionts. We also compared the age-structure between all the emitted symbionts and those that got to colonise the crayfish receiver (i.e. transmitted symbionts). Both analyses were performed by using multinomial baseline-category logit models fitted via neural networks. Moreover, we compared sex proportions between emitted symbionts and those that remained in the crayfish transmitter, and between emitted and transmitted symbionts. Finally, we checked for differences in number of symbiont mating pairs between crayfish transmitter, off-host environment and crayfish receiver.

In order to test for differences in crayfish weight between treatments and species we used generalised linear models (GLMs) of the gaussian family with identity link function, and we accounted for effects via unstandardised coefficients (*B*). For those analyses implying counts as a response variable, we used GLMs of the negative binomial family with log link function (count data were affected by overdispersion), and the assessment of effects was based on relative risk (RR). Those implying proportions were done through GLMs of the binomial family with logit link function, and the effects were assessed with the odds ratios (OR). All the statistical analyses were done with R software (R Core Team, 2013). Additionally to standard R packages, we used MASS for negative binomial GLMs and NNET for multinomial models (Venables & Ripley, 2002).

Results

Checking for experimental biases

The median value and quartiles of original abundances of crayfish transmitters were: Me = 368 symbionts; Q25 = 297.5; Q75 = 1471. We found a positive effect of host weight on symbiont original abundances in *P. clarkii* transmitters, with an expected 8 % increase per gram (RR = 1.076; df = 68; 95 % CI: $1.039 - 1.116$). Moreover, the original abundances were 25 % lower in CRT than C+ (RR = 0.755; df = 68; 95 % CI: $0.578 - 0.988$), while those of IRT did not differ from C+ (RR = 0.906; df = 68; 95 % CI: $0.684 - 1.205$). Crayfish moult stage and sex did not affect original abundances.

Crayfish weight of *P. clarkii* transmitters belonging to IRT did not differ from those of C+ (C+: $\mu = 13.27$ g, IRT: $\mu = 14.28$ g; $B = 0.76$; $df = 72$; $t = 0.85$; $P > 0.05$), while those of CRT were approx. 2 g heavier (CRT: $\mu = 15.45$ g; $B = 2.18$; $df = 72$; $t = 2.45$; $P < 0.05$). Regarding crayfish receivers, those of IRT, i.e. belonging to *A. italicus*, were approx. 18 g heavier than *P. clarkii* individuals of CRT (CRT: $\mu = 16.48$ g, IRT: $\mu = 34.54$ g; $B = 18.06$; $df = 48$; $t = 9.20$; $P < 0.01$).

Symbiont transmission success

The cleaning treatment was found to be successful as, among those *P. clarkii* individuals composing the C– group (N = 25), only one crayfish harboured a unique entocytherid individual. Moreover, the C– for *A. italicus* (N = 25) was completely free of entocytherids, confirming that they come from a population without the exotic symbiont. The transmission was confirmed for both crayfish species, as crayfish receivers of both CRT and IRT showed a high host occupancy after the experiment (92 % for *P. clarkii* and 100 % for *A. italicus*).

Symbiont emission rates

The mean emission rate for C+ was 2.56 %. Both treatments with the presence of crayfish receiver showed around 2.5 times higher odds of symbiont emission than C+ (CRT: OR = 2.330; df = 71; 95 % CI: $2.036 - 2.671$, IRT: OR = 2.720; df = 71; 95 % CI: $2.397 - 3.093$) and weight of crayfish transmitter appeared to have no effect (OR = 0.990; df = 71; 95 % CI: $0.976 - 1.004$; Model 1 in Table 1). Furthermore, considering only both CRT and IRT, symbiont emission rates showed an exclusive and negative dependence on weight of crayfish transmitter, with a 3 % decrease in odds per gram (OR = 0.971; df = 46; 95 % CI: $0.957 - 0.986$), without being affected by host features of crayfish receiver (i.e. weight and species; Model 2 in Table 1).

Table 1. Summary of the Generalized Linear Models (GLMs) used to analyse symbiont emission rates (i.e. proportion of symbionts that had left the crayfish transmitter at the end of the experimental treatment) by considering the three experimental treatments (C+, CRT and IRT; **Model 1**) or only those two treatments with presence of crayfish receiver (CRT and IRT; **Model 2**). The explanatory variables are weight (g) of crayfish transmitter and receiver (Weight T and Weight R), species of crayfish receiver (*Procambarus clarkii* or *Austrapotamobius italicus*) and treatment type (C+, CRT and IRT; see Fig. 1 for treatment description). For each fixed effect, we show the odds ratio (OR), the unstandardised coefficient (*B*) with its 95 % confidence interval (95 % CI), the z-statistic (*Z*) and p-value (*P*). The degrees of freedom were 71 for the model 1 and 46 for the model 2. *Significant *P* (< 0.05).

Model term	OR	*B*	95 % CI	*Z*	*P*
Model 1					
Intercept	—	-3.280	(-3.502, -3.0578)	-28.95	< 0.001*
Weight T	0.990	-0.010	(-0.025, 0.004)	-1.44	0.149
C+	1	0	—	—	—
CRT	2.330	0.846	(0.711, 0.982)	12.22	< 0.001*
IRT	2.720	1.001	(0.874, 1.129)	15.39	< 0.001*
Model 2					
Intercept	—	-2.133	(-2.429, -1.838)	-14.15	< 0.001*
Weight T	0.971	-0.029	(-0.044, -0.014,)	-3.78	< 0.001*
Weight R	1.000	0.000	(-0.008, 0.008)	0.05	0.963
P. clarkii	1	0	—	—	—
A. italicus	1.122	0.115	(-0.064, 0.294)	1.26	0.206

Symbiont transmission rates

The mean value of symbiont transmission rates in CRT was 38.48 %. The odds of symbiont transmission was 2.1 times higher in *A. italicus* receivers than in *P. clarkii* (OR = 2.127; df = 47; 95 % CI: 1.423 − 3.186), and bigger crayfish receivers showed increased odds of being colonised by symbionts from the off-host environment, with a 5 % increase per gram (OR = 1.054; df = 47; 95 % CI: 1.034 − 1.076; Table 2).

Age-structure in emitted and transmitted symbionts

We found individuals belonging to any of the eight entocytherid stages in both off-host environment and crayfish receiver. The multinomial model for emitted stages (Fig. 2a) showed an original age-structure of symbiont infrapopulations (see OR for intercept in Fig. 2a) characterized by a predominance of younger stages (up to A–5), with very low relative amounts of symbionts of the remaining stages, with an expected reduction of more than 50 % in respect to the reference stage, i.e A–7 (ORs < 0.5). The adults were an exception, having a moderate 20 %

Table 2. Summary of the GLM used to analyse factors affecting host transmission rates (i.e. proportion of emitted symbionts that colonized the crayfish receiver) by considering both experimental treatments with the presence of a crayfish receiver, i.e. CRT and IRT (see Fig. 1 for treatment description). The explanatory variables are weight (g; Weight R) and species (*Procambarus clarkii* or *Austropotamobius italicus*) of crayfish receiver. For each fixed effect, we show the odds ratio (OR), unstandardised coefficient (B) with its 95 % confidence interval (95 % CI), the z-statistic (Z) and p-value (P). The degrees of freedom were 47. *Significant P (< 0.05).

Model term	OR	*B*	95 % CI	*Z*	*P*
Intercept	—	-1.559	(-1.927, -1.198)	-8.387	< 0.001*
Weight R	1.054	0.053	(0.033, 0.073)	5.176	< 0.001*
P. clarkii	1	0	—	—	—
A. italicus	2.127	0.755	(0.353, 1.159)	3.674	< 0.001*

decrease in odds (OR = 0.805; $df = 71$; 95 % CI: $0.769 - 0.842$). In contrast with original abundances, the age-structure of emitted symbionts (see OR for emitted symbionts in Fig. 2a) showed increased odds in individuals belonging to A–6, A–5 and adult stages and decreased odds in intermediate stages between A–4 and A–1.

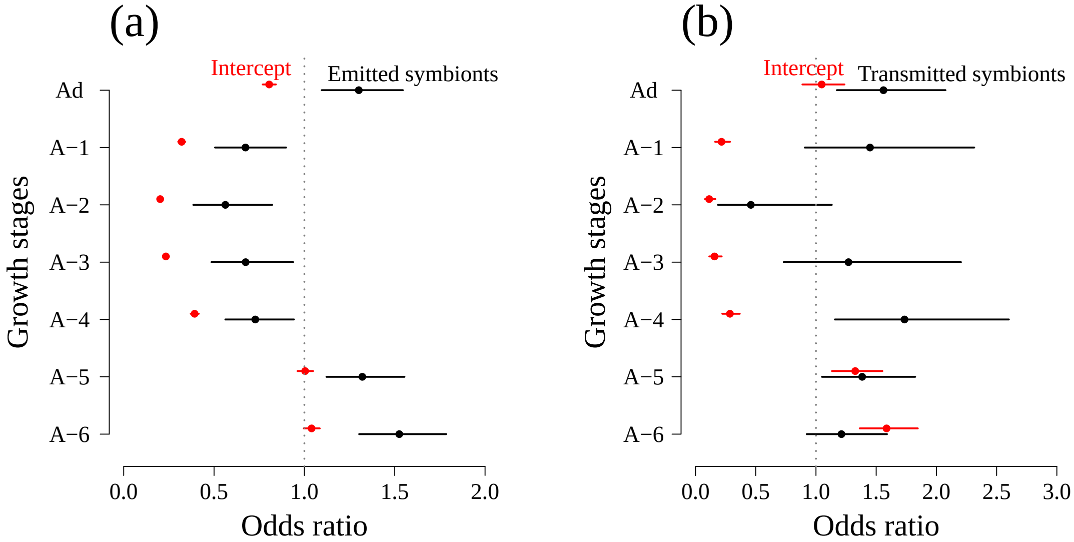

Figure 2. Odds ratios and their confidence intervals for terms of the multinomial model to compare the proportions of the eight symbiont growth stages between **(a)** the emitted symbionts (i.e. symbionts that left the crayfish transmitter) and the original symbiont infrapopulations, and **(b)** transmitted (i.e. those symbionts that got to colonise the crayfish receiver) versus emitted symbionts. We selected the youngest stage A–7 as the reference level of the response variable. The intercept terms (in red) represent **(a)** the age-structure of original symbiont infrapopulations and **(b)** the age-structure of the emitted symbionts. The terms in black represent the changes in age-structure of **(a)** emitted symbionts in respect to the original infrapopulations and **(b)** transmitted symbionts in respect to that of all emitted symbionts.

Accordingly, the model for transmitted stages (Fig. 2b) showed higher odds ratios in A–6, A–5 and adult stages for emitted symbionts (intercept in Fig. 2b) compared with the original age-structure (intercept in Fig. 2a). Moreover, when comparing age-structure of transmitted (i.e. symbionts that get to colonise the crayfish receiver) versus emitted symbionts, odds ratios appear with wider confidence intervals and the differences between stages are not as clear, although transmission of A-7 stages seem to be unfavoured, compared to other stages as adults, A-4 and A-5 (transmitted symbionts in Fig. 2b).

Regarding sex proportions, we did not find differences neither between emitted symbionts and those that remained within the crayfish transmitter (OR $= 0.916$; $df = 131$; 95 % CI: $0.722 - 1.161$) nor between emitted and transmitted symbionts (OR $= 1.043$; $df = 79$; 95 % CI: $0.669 - 1.627$). The median number of mating pairs within crayfish transmitters was: Me $= 2$; Q25 $= 1$; Q75 $= 4.5$. We observed symbiont mating pairs in the off-host environment, although their number showed a reduction of 91 % compared with those found within the transmitter crayfish (RR $= 0.094$; $df = 148$; 95 % CI: $0.055 - 0.153$). Finally, we did not find any mating pair in crayfish receivers.

Discussion

Successful symbiont spillover under experimental conditions

The main contribution of this survey was the success of exotic entocytherid transmission to a native endangered crayfish under experimental conditions. Moreover, symbionts showed a higher transmission efficacy onto native crayfish, with a 100 % of *A. italicus* reached. This finding open new perspectives in crayfish conservation, as entocytherid spillover would imply the emergence of new potential threats to European native crayfish. The generally accepted hypothesis about the symbiont-host relationship of entocytherids is commensalism (Hart & Hart Jr, 1974), but it is purely based on observational evidence, and it requires experimental evidence. In contrast to this general view, Cuéllar et al. (2002) suggested that symbiont ostracods (entocytherids according to the picture shown in their paper) had pathogenic effects on crayfish reproduction. Many symbioses do not clearly fit into the traditional classification of interactions, i.e. parasitism, commensalism and mutualism, so that those terms should be used with caution (Leung & Poulin, 2008). Interactions between crayfish and their ectosymbionts can be complex, as observed in branchiobdellidans, an obligate group of ectosymbiotic annelids that can act in a variety of ways from mutualistic cleaners to ectoparasites of crayfish depending on different factors such as branchiobdellidan species, abundance and ecological context (Skelton et al., 2013). On the other hand, we cannot predict the effects of a symbiont on a novel host which, a priori, is not adapted to harbour this kind of organisms. Furthermore, ostracods are known to function as intermediate hosts for some parasites (Moravec, 2004). Indeed, entocytherids also harbour their own associated biota (Aguilar-Alberola et al., 2012). Therefore, the transmission of pathogen symbionts through entocytherids acting as disease vectors is also possible.

Is symbiont dispersal stimulated by close presence of other potential hosts?

In agreement with Young (1971), our results show that direct contact between crayfish transmitter and receiver is not a requirement for symbiont transmission because crayfish transmitters lose a small proportion of their symbionts to the off-host environment, from where they are transferred to the crayfish receiver. Entocytherids are capable of surviving out of their hosts for long periods (i.e. several months; Baker, 1969; Young, 1971). However, they rarely appear in plankton and benthic samples of free-living microcrustaceans. The only entocytherid records from the off-host environment come from particular habitats tightly related to the crayfish, such as crayfish shelters (Hart & Hart Jr, 1974). This suggests that symbiont dispersal events are reduced in time and/or space. Entocytherids are tiny (less than 0.5 mm) and do not swim, but they move crawling through the sediment. So, their dispersal capacity seems to be limited. Therefore, adaptations to adjust their dispersal to increase the probability of new host encounters could be favoured to minimise symbiont losses, as observed in other ectosymbionts (Blanco & Frías, 2001). In this sense, our experiment showed an increase in symbiont dispersal under the presence of crayfish receiver compared to its absence. Moreover, when considering only those treatments with presence of crayfish receiver, a negative effect of crayfish weight on entocytherid dispersal appears, so that smaller crayfishes emit higher proportion of symbionts. A possible hypothesis to explain this is symbiont dispersal stimulation by the detection of the closer presence of a new host as an adaptation to improve the efficiency of symbiont transmission. Furthermore, the stimulation would be more favoured in smaller crayfishes because their ectosymbiont infrapopulations tend to be more unstable due to more reduced spatial resources and higher moult frequencies (Mori et al., 2001) if they have a chance to colonise a bigger host. However, we should be cautious about this hypothesis because alternative explanations might involve stimulation to dispersal triggered by oxygen drops during the experiment before the daily re-establishment of 100 % O_2 saturations, or by other not measured environmental factors such as concentration of host catabolic substances or secretions. So, new experiences aimed to test our proposed hypothesis would require a higher control of O_2 concentrations and other products.

Absence of species-specific host detection

Host specificity of a symbiont is controlled by filters that determine the range of host species that can be used by the symbiont. They can be divided into encounter and compatibility filters (Poulin, 2007). In this work, we experimentally set encounter rates between entocytherid vectors (exotic crayfish) and novel hosts to 100 %. However, in wild populations, encounters are driven by multiple factors such as host density and behaviour (Tompkins et al., 2011; Telfer & Bown, 2012). Once encounter filters are overcome, compatibility filters appear to act. Here, we focused our research on the transmission process in which filters related to host detection and attachment are acting on symbionts, as a part of the compatibility filters. We found higher probability of transmission in bigger hosts and in *A. italicus*, that is the novel host species belonging to a different family (Astacidae) from the original host (Cambaridae). Thus, the existence of chemosensory mechanisms of host detection specific for a given range of crayfish hosts seems to be improbable in our entocytherid species. Instead, entocytherids might base their host detection on generic cues of crayfish targets (perhaps even more general Decapoda or Crustacea chemical signals). Bigger hosts could imply higher host signal or higher probability of host contact due to an increase in host areas in contact with the substrate from where symbionts achieve the host. Our results support the increase in rates of symbiont acquisition with host size to explain the frequent pattern of positive effect of host size on symbiont abundances (Poulin, 2013). On the other hand, host bodies of *A. italicus* were apparently more exposed to symbionts than *P. clarkii*. This might be related to differences in host body structure (i.e. proportion of external surface areas in contact with substrate; Poulin, 2013) or behaviour (e.g. grooming activity; Jones & Lester, 1996; Stanko et al., 2006). Once we evidenced that host detection and attachment are not highly selective at host specific level, our findings open new potential research lines focused on other symbiont capabilities related to the compatibility filter (i.e. development and reproduction), which would allow us to know if entocytherids are able to establish viable infrapopulations in a novel host like native European crayfish.

Higher dispersal in youngest and adult stages

Our experiment demonstrates that all the entocytherid stages are potentially able to be transmitted. Accordingly, morphological descriptions of entocytherid stages do not reflect stage-specific adaptations to dispersal, host detection or attachment (Smith & Kamiya, 2005). This non-stage-specific dispersal may favour entocytherids to overcome the issue related to the fragility of symbiont infrapopulations due to crayfish ecdysis that provokes periodical symbiont losses (Castillo-Escrivà et al., 2013), because all the stages are capable of reaching a target host after being expelled through a moult. The age-structure of emitted symbionts was similar to that of original infrapopulations but with a notable increase in proportions of A–6 and A–5 and adults. A possible explanation for higher dispersal in younger stages may relate to their high abundances after hatching. In ectosymbionts with direct reproduction within a host such as entocytherids, stochastic reproductive events produce explosive increases in infrapopulation sizes, responsible of aggregation patterns in symbiont abundances (Matthee & Krasnov, 2009). As a consequence, younger stages become more abundant and, as they are expected to be less competitive than others due to their smaller sizes, under intraspecific competition pressures dispersal behaviour could be favoured in these symbiont stages. The higher emission rates in A–6 and A–5 compared with A–7 and the negative trend of dispersal A-7 to reach the host, compared with other stages, might be a result of dispersal limitations of A–7 due to their degree of immaturity (i.e. the first stage after the hatching), although apparently there are not morphological differences between A–7 and A–6 stages (Smith & Kamiya, 2005). Finally, the higher emission rates shown by adults would guarantee quick starting of new reproductive events in other hosts, an important advantage when the threat of losing the laid eggs through a crayfish moulting event is constant.

Future perspectives

In order to manage invasive species, we should take into account their associated symbiont biota. Here, understanding host-symbiont transmission dynamics is essential to predict which exotic symbionts are most likely to invade new native hosts through spillover (Telfer & Bown, 2012). The exotic entocytherid *Ankylocythere sinuosa* has features of a good symbiont invader (Taraschewski, 2006; Almberg et al., 2012), i.e. simple life cycle, high prevalences in exotic populations (Aguilar-Alberola et al., 2012), low host specificity (Mestre et al., 2014), hight transmissibility and, finally, high capability for cross-species transmission to European native crayfish, as demonstrated in this work. According to our results and given the wide presence of exotic entocytherids in Europe (Mestre et al., 2013), their spillover to wild populations of native crayfish has a high probability to occur. The ecological consequences of the process are unknown, but might have implications in conservation of crayfish biodiversity. Moreover, in case of spillover success, the eradication of exotic symbionts from wild crayfish populations would probably be impracticable. Then, given the irreversibility of the process and the potential underlying consequences, we consider appropriate to adopt a cautionary strategy based on two points: (1) Further experimental research focused on the potential effects of a hypothetical spillover; (2) preventive measures against spillover.

In our view, further research on entocytherids would be useful for conservation purposes, mainly focused on two important aspects. Firstly, experiments to check for successful development and reproduction of exotic entocytherids into European native crayfish would extend our knowledge about the viability of symbiont spillover for entocytherids (i.e. compatibility filters). A priory, if we take into account that the American native entocytherids (Entocytherinae) inhabit two crayfish families, i.e. Cambaridae and Astacidae (Hart & Hart Jr, 1974), and that the European crayfish belong to one of them, i.e. Astacidae, we could expect that they are able to successfully invade the native European crayfish. Secondly, experimental research aimed to test the effects of entocytherids on crayfish hosts in terms of growth, survival and reproduction rates would be valuable, specially in exotic entocytherids on novel European crayfish. At present, a significant effort is being done to establish conceptual models and conservation strategies for recovering and protecting native European crayfish (Kozák et al., 2011). Here, we present a new element to be considered in current discussions about crayfish conservation. In this sense, our findings provide additional support to the importance of maintaining "ark sites" (i.e. safe isolated sanctuaries) for native European crayfish conservation. To conclude, special attention should be paid not only to *P. clarkii* but also to *Pacifastacus leniusculus*, because it hosts its own entocytherid symbiont (Mestre et al., 2013) and has similar habitat preferences than autochthonous endangered crayfish species such as *A. pallipes* and *A. italicus* (Hiley, 2003; Vedia & Miranda, 2013). Therefore, *P. leniusculus* represents a clear spillover threat and measures to contain its expansion and reduce its distribution (i.e. eradication programs, monitoring watercourses and warning human populations about crayfish translocations; Hiley, 2003; Vedia & Miranda, 2013) would help to prevent its potential negative impacts.

Acknowledgements

We thank Pilar Ore, Gregorio Herrera, J. A. Gil-Delgado and the astaciculture center of Río de Gallo (Guadalajara, Spain) for their help in supply and transport of native crayfish specimens for the experiment; the governments of Castilla-La Mancha and Generalitat Valenciana provided us the permissions to manage native crayfish for this work; we want to thank Lucas Cabrera and Silvia Piñeiro for their technical support and Romina Liberto for her help during the experiment; the experiments were carried out in the Aquarium Plant of the Central Service for Experimental Research (SCSIE, University of Valencia); This research was funded by the Spanish Ministry of Science and Innovation Project ECOINVADER (CGL2008-01296/BOS) and the University of Valencia ("V-Segles" predoctoral grant to A. Mestre).

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Supporting Information

Table S1. Summary of the GLM used to analyse the effects of host features (i.e. size, moult stage and sex) and treatment group on symbiont original abundances of *P. clarkii* individuals (N = 75) used as symbiont transmitters for the experiment of this survey. We used the crayfish weight (g) as estimator of host size. The moult stage factor was composed by three levels (M1, M2 and M3, with higher numbers indicating older moult stage; see methods section for more details). The factor treatment had three levels representing the three experimental treatments: C+, CRT and IRT treatments (see Fig. 1 for treatment description). For each fixed effect, we show the relative risk (RR), the unstandardised coefficient (*B*) with its 95 % confidence interval (95 % CI), the z-statistic (*Z*) and p-value (*P*). The degrees of freedom were 68. The dispersion parameter of the model was $k = 5.49$. *Significant *P* (< 0.05).

Model term	RR	*B*	95 %	*Z*	*P*
Intercept	—	4.485	(3.572, 5.568)	9.12	<0.001*
Weight	1.076	0.073	(0.038, 0.110)	4.44	<0.001*
M1	1	0	—	—	—
M2	2.032	0.709	(-0.302, 1.511)	1.56	0.119
M3	2.031	0.708	(-0.299, 1.504)	1.57	0.116
Male	1	0	—	—	—
Female	0.840	-0.174	(-0.390, 0.043)	-1.54	0.124
C+	1	0	—	—	—
CRT	0.755	-0.281	(-0.548, -0.012)	-1.99	0.047*
IRT	0.906	-0.098	(-0.380, 0.187)	-0.68	0.495

Resum ampliat

El carranc roig americà *Procambarus clarkii* (Girard, 1852) fou introduït a la Península Ibèrica en 1973 amb propòsits comercials. Com a resultat de la seua expansió des de la Península Ibèrica i, probablement, també de posteriors introduccions en altres països europeus, actualment es troba amplament distribuït per gran part d'Europa. Els impactes ecològics d'aquest carranc invasor han rebut una atenció creixent, però no es coneixia rés sobre els ostracodes entocitèrids simbionts associats a *P. clarkii* fora del continent americà. En aquest estudi examinàrem més de 200 carrancs de riu *P. clarkii* provinents de 12 localitats distribuïdes al llarg d'una ampla àrea de l'Est i Sud de la Península Ibèrica (capítol I). Vam aplicar un protocol de mostreig d'entocitèrids individual per a cada carranc i els entocitèrids obtinguts foren identificats, recomptats, classificats en funció de l'estadi de desenvolupament i sexats. En totes les localitats mostrejades excepte una, vam registrar presència d'entocitèrids. L'espècie identificada fou la mateixa en tots els casos: *Ankylocythere sinuosa* (Rioja, 1942). Fotos de l'entocitèrid realitzades al microscopi electrònic mostraren la presència de ciliats epibionts (família Lagenophryidae) i bacteris associats al cos de l'entocitèrid. La taxa d'ocupació d'hostes fou del 100 % en totes les localitats excepte una. L'abundància d'entocitèrids varià notablement en relació a la grandària de l'hoste i el lloc de mostreig. L'efecte de la grandària pot estar relacionat amb la quantitat de recursos i la freqüència de muda. Els carrancs més grans ofereixen major espai i aliments i, a més, tenen una menor freqüència de muda i, per tant, com que la muda produeix pèrdues d'entocitèrids, les infrapoblacions de simbionts que mantenen romanen més estables. D'altra banda, la variació espacial de les abundàncies d'entocitèrids suggereixen l'existència de trets específics de cada lloc que afecten a les poblacions d'entocitèrids, bé de forma directa, o a través de l'efecte sobre la dinàmica poblacional de l'hoste. Aquest estudi representa la primera cita d'un entocitèrid exòtic a Europa i evidencia la seua ampla distribució en la Península Ibèrica, amb elevades taxes d'ocupació d'hostes. Amb aquest estudi es posava de manifest la necessitat d'investigar més sobre aquests nous organismes exòtics a Europa, ja que poden tenir implicacions en la conservació dels carrancs de riu europeus.

La creació de bases de dades de biodiversitat lliures per a la comunitat científica és una feina valuosa per estimular la investigació en biodiversitat. Entre altres repositoris, la Infraestructura Mundial d'Informació en Biodiversitat (GBIF) és un destacat recurs lliure i gratuït que proveeix accés a dades de biodiversitat de grups taxonòmics molt diversos (incloent els Ostracoda) d'ecosistemes terrestres i marins. Anteriorment ja s'havien construït bases de dades específiques per a dades georeferenciades d'ostracodes (e.g. NACODe[1], OMEGA[2]...), algunes d'accés lliure. Tanmateix, els entocitèrids, una família d'ostracodes comensals d'altres crustacis que, amb 220 espècies, representen la tercera família d'ostracodes no marins en nombre d'espècies vives, han sigut notablement subrepresentats. Amb la finalitat de cobrir aquesta carència, presentem una base de dades mundial de la família Entocytheridae, d'accés lliure, publicada en GBIF (capítol II). A més, revisem el coneixement actual del grup mitjançant l'aportació d'una llista actualitzada de les espècies conegudes d'Entocytheridae i una altra de la bibliografia del grup. També analitzem l'especificitat d'hoste dels entocitèrids i el patró latitudinal de la riquesa d'espècies d'Amèrica del Nord i Central (incloent 186 spp.). Com a resultat de la recerca bibliogràfica sobre els entocitèrids vam indexar un total de 215 publicacions que contenien referències directes als entocitèrids. La distribució temporal de les publicacions mostra que el principal esforç d'investigació sobre entocitèrids es produí durant els anys seixanta i principis del setanta. La base de dades que hem creat consta actualment de 3509 registres de 220 espècies, que contrasta amb els 44 únics registres georeferenciats prèviament publicats en GBIF. La llista taxonòmica actualitzada conté 43 espècies i 2 gèneres que no apareixien en la darrera revisió sobre els Entocytheridae publicada per Hart i Hart en 1974, així com 40 espècies no incloses en la llista de 2013 del reconegut Catàleg de la Vida. D'altra banda, mostrem que l'especialització en una única espècie d'hoste no sembla ser la regla en Entocytheridae, i descobrim un patró de riquesa inusual en els entocitèrids americans, probablement relacionat amb la història biogeogràfica dels seus hostes.

L'estudi dels ectosimbionts requereix de metodologies adequades per a extreure'ls dels seus hostes. Nosaltres vam avaluar l'efectivitat del clorobutanol i l'aigua carbonatada per desprendre els individus de l'entocitèrid *A. sinuosa* del seu hoste *P. clarkii* (capítol III). Per això, vam realitzar proves amb els dos productes i amb diferents temps d'exposició als tractaments, i vam avaluar els seus efectes sobre la supervivència del carranc i dels entocitèrids alliberats, i la quantitat de simbionts obtinguts. Els resultats de l'experiment mostren diferències entre tractaments quant a proporció d'entocitèrids alliberats de l'hoste i supervivències de carranc i entocitèrids després del tractament. La submersió del carranc en clorobutanol

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[1] North American Combined Ostracode Database

[2] Ostracod Metadatabase of Environmental and Geographical Attributes

en períodes curts és molt efectiva per extraure entocitèrids sense produir la mort del carranc hoste. En canvi, la submersió en aigua carbonatada és millor per extraure grans quantitats d'entocitèrids vius. Finalment, els dos tractaments són adequats per establir protocols estandarditzats d'extracció d'entocitèrids dels seus hostes per la realització d'estudis ecològics perquè, a partir d'un cert període d'exposició al tractament, les proporcions d'entocitèrids extrets romanen constants independentment de l'abundància d'entocitèrids de cada carranc.

Basant-nos en la troballa de l'entocitèrid *A. sinuosa* amplament distribuït en la Península Ibèrica associat al carranc de riu americà *P. clarkii* (capítol I), vam realitzar un estudi de seguiment mensual durant un any d'una població d'*A. sinuosa* i el seu hoste *P. clarkii* de la Marjal de Pego-Oliva (capítol IV), amb l'objectiu de descriure, per primera vegada, la dinàmica poblacional de l'entocitèrid exòtic fora del seu rang natiu. També vam analitzar el paper de variables ambientals (temperatura i conductivitat de l'aigua) i característiques de l'hoste (sexe, pes i muda) sobre els patrons demogràfics observats. El període amb majors abundàncies d'entocitèrids fou el comprés entre juliol i octubre, mentre que el període de novembre a febrer tingué els valors d'abundància més baixos. D'acord amb els resultats del primer estudi (capítol I), les abundàncies dels entocitèrids estigueren positivament correlacionades amb el pes del carranc. També foren afectades per la muda del carranc, de forma que carrancs d'estadis d'intermuda més avançats tingueren majors quantitats d'entocitèrids. La temperatura de l'aigua també mostrà un efecte positiu sobre les abundàncies dels entocitèrids. Les proporcions d'entocitèrids pertanyents a l'estadi més jove, un indicador de períodes recents d'eclosió del simbiont, també estigueren relacionades amb la temperatura i la conductivitat de l'aigua. D'acord amb els nostres resultats, els esdeveniments de muda del carranc de riu, els quals produeixen una pèrdua d'entoctèrids, junt amb la temperatura i les concentracions iòniques de l'aigua, són factors importants que influencien el cicle de vida de l'ectosimbiont exòtic *A. sinuosa*.

La simbiosi representa un estil de vida amplament estès i reeixit, essencial per comprendre les interaccions ecològiques i qüestions evolutives dels organismes, i el seu estudi també pot ajudar a resoldre problemes relacionats amb la conservació biològica. Malgrat això, la seua recerca ha estat principalment enfocada als paràsits. S'han proposat tres patrons generals en ecologia de paràsits: (1) Agregació; (2) estreta correlació positiva entre l'abundància[1] i la seua variància; (3) correlació positiva entre l'abundància i la taxa d'ocupació d'hostes[2] del simbiont. Els factors que afecten els paràmetres poblacionals d'una espècie de simbiont poden ser agrupats en característiques de l'hoste i condicions ambientals. La investigació

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[1] Nombre mitjà de simbionts per hoste en una població de simbionts (tenint en compte hostes lliures de simbionts)

[2] Percentatge d'hostes amb simbionts respecte al total d'hostes de la població

sobre ecologia de paràsits suggereix que les condicions ambientals juguen un paper minoritari. Nosaltres vam comprovar els tres patrons en un ectosimbiont d'aigua dolça no parasitari i vam analitzar els efectes de característiques de l'hoste (sexe, mida del cos i muda) i condicions ambientals (clima i química de l'aigua) sobre l'abundància i la taxa d'ocupació d'hostes del simbiont (capítol V). La nostra espècie model fou l'entocitèrid *A. sinuosa* en les poblacions europees del carranc invasor *P. clarkii*. Vam mostrejar 373 carrancs provinents de 26 localitats de la Península Ibèrica i les Illes Balears. Vam modelar l'abundància per hoste ocupat[1] (i.e. intensitat) i la taxa d'ocupació d'hostes (i.e. prevalència) fent ús de models "zero-alterats"[2] i una estratègia "backward"[3] de selecció del model òptim. El model òptim fou avaluat per mitjà de l'anàlisi gràfica dels residus de Pearson i la relació entre els valors observats i els predits pel model. Els resultats evidencien un valor global de taxa d'ocupació d'hostes del 91.15 %. Les abundàncies d'*A. sinuosa* entre els hostes ocupats mostraren un alt grau d'agregació, amb un 20 % dels hostes acumulant el 66.99 % dels entocitèrids. Hi hagué una correlació positiva entre el logaritme de l'abundància i el logaritme de la seua variància, amb un fort ajust ($r^2 = 0.92$). També s'observà una correlació positiva entre l'abundància per hoste ocupat i la taxa d'ocupació. El major efecte sobre les abundàncies d'*A. sinuosa* correspongué a la conductivitat elèctrica de l'aigua (amb efecte negatiu), seguit de la densitat (efecte positiu) i la muda (efecte negatiu) de l'hoste, i de variables relacionades amb la química de l'aigua, i.e. concentració de NH_4^+ i proporcions alcalinitat/($Cl^- + SO_4^{2-}$) i Cl^-/SO_4^{2-}, amb efectes moderats. La taxa d'ocupació d'hostes fou afectada principalment per la densitat de carrancs (efecte positiu), i també per les condicions climàtiques (i.e. T del mes més càlid, precipitació anual i estacionalitat de les precipitacions). Els nostres resultats suggereixen que algunes regles observades en ecologia de paràsits podrien ser realment regles més generals de simbionts. A més, el nostre model de simbiont es veié marcadament afectat per les condicions climàtiques actuant sobre la taxa d'ocupació d'hostes a escala regional, i per la química de l'aigua actuant sobre les abundàncies del simbiont a nivell més local. Aquest resultat recolza la hipòtesi del paper destacat de les condicions ambientals com a moduladores dels paràmetres poblacionals d'ectosimbionts no parasitaris d'aigua dolça. Les toleràncies fisiològiques a factors ambientals altament inestables característics dels sistemes d'aigua dolça, junt amb la dependència de fonts d'aliment també molt variables són, probablement, les raons de l'alta sensibilitat d'aquest tipus de simbionts a les condicions ambientals.

En biologia d'invasions, els factors abiòtics i biòtics són considerats essencials. Tot i això, habitualment, els factors biòtics no són considerats a l'hora de modelar

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[1] Nombre mitjà de simbionts per hoste, considerant sols els hostes ocupats (i.e. amb simbionts)

[2] Models estadístics dissenyats per l'anàlisi de variables resposta de distribució Poisson amb excés de zeros

[3] "Cap enrere", estratègia de selecció que parteix d'un model complet (i.e. amb totes les variables explicatòries)

les àrees potencials de propagació de les espècies exòtiques. En el marc de la teoria de conjunts, les interaccions entre factors biòtics (B), abiòtics (A) i de moviment o dispersius (M) en l'espai geogràfic poden ser representades per una adaptació dels diagrames de Venn i provades mitjançant l'ús de models de nínxol ecològic[1] (MNEs) per estimar les àrees A i B. El principal objectiu del següent estudi (capítol VI) fou l'avaluació de les interaccions entre factors abiòtics (i.e. climàtics) i biòtics (i.e. disponibilitat d'hoste) en l'espai geogràfic de simbionts exòtics, fent ús de tècniques d'MNEs combinades amb un marc basat en la teoria de conjunts, i emprant als entocitèrids exòtics trobats en Europa com a organismes model. Prèviament, vam dur a terme un mostreig extensiu per avaluar la distribució d'entocitèrids en carrancs de riu a Europa que inclogué 94 localitats europees i 12 espècies de carranc de riu (exòtics i natius) mostrejades. Com a resultat del mostreig vam trobar dues espècies d'entocitèrid: l'anteriorment descobert *A. sinuosa* (capítol I) i una nova espècie per a Europa anomenada *Uncinocythere occidentalis* (Kozloff i Whitman, 1954). Les dos aparegueren amplament distribuïdes en l'Oest d'Europa en coexistència amb carrancs americans invasors *P. clarkii*, en el cas d'*A. sinuosa*, i *Pacifastacus leniusculus* (Dana, 1852) en el d'*U. occidentalis*. Cap altre entocitèrid fou detectat en la resta d'espècies de carranc mostrejades. D'acord amb els nostres models, l'àrea climàticament apropiada per *A. sinuosa* fou restringida per les seues pròpies limitacions fisiològiques a les temperatures mínimes de l'Oest i el Nord d'Europa, i l'estacionalitat de les precipitacions en àrees circummediterrànies. Per la seua banda, *U. occidentalis* mostrà restriccions per disponibilitat d'hoste en les regions circummediterrànies, degudes a les limitacions de *P. leniusculus* a l'elevada estacionalitat de les precipitacions i les temperatures màximes. La combinació d'MNEs amb la teoria de conjunts permet l'estudi de la biologia de les invasions dels simbionts i aporta informació important sobre l'existència de barreres biogeogràfiques relacionades amb factors abiòtics o biòtics que limiten l'expansió del simbiont en diferents regions del seu rang invasor. Amb això, podem avaluar la relativa importància dels factors abiòtics i biòtics en l'espai geogràfic i aplicar-ho als plans de conservació de les espècies afectades. L'aproximació metodològica que presentem en aquest treball pot ser implementada en altres sistemes on l'espècie objecte d'estudi presenta una forta interacció amb altres.

El concepte "spillover", utilitzat en biologia de les invasions, fa referència a la transmissió de simbionts d'una espècie exòtica a una nativa. El procés representa una amenaça potencial per a la conservació de la biodiversitat. El carranc natiu europeu ha estat amenaçat les darreres dècades per carrancs exòtics introduïts a Europa, degut a fenòmens d'exclusió competitiva i transmissió de malalties. D'entre les espècies més afectades es troba *Austropotamobius italicus*, un dels carrancs natius més amenaçats d'Europa. Recentment, un nou grup d'ectosimbionts

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[1] Models predictius de distribució de les espècies

exòtics (Ostracoda, Entocytheridae) ha sigut registrat en Europa associat a carrancs invasors provinents d'Amèrica (capitols I i VI). L'objectiu principal d'aquest darrer estudi (capítol VII) fou testar la transmissió de l'entocitèrid exòtic *A. sinuosa* des del carranc de riu invasor *P. clarkii* al carranc natiu *A. italicus*. Bàsicament, l'experiment consistí a posar en coexistència un carranc transmissor (un carranc *P. clarkii* amb simbionts) i un receptor (un carranc sense simbionts) durant quatre dies. Vam dissenyar tres tractaments experimentals, amb 25 rèpliques per cada un: (1) carranc de riu transmissor sol (sense carranc receptor); (2) *P. clarkii* netejat (és a dir, amb els simbionts eliminats) com a carranc receptor; (3) *A. italicus* com a carranc receptor. Després de l'experiment, vam comprovar la presència i abundància de simbionts de cada carranc i del medi extern als hostes mitjançant l'aplicació de protocols estandarditzats d'extracció de simbionts prèviament avaluats en el capítol III. Com a resultat, vam evidenciar una transmissió altament efectiva del simbiont al carranc natiu, amb un 100 % dels carrancs receptors d'*A. italicus* amb simbionts després de l'experiment. La transmissió fou a través de simbionts dispersants en el medi extern, prèviament emesos pel carranc transmissor. Les taxes d'emissió de simbionts per part del carranc transmissor foren majors en els tractaments amb presència de carranc receptor. Les taxes de transmissió per part dels simbionts dispersants foren més elevades en carrancs receptors de majors dimensions, i en els individus de l'espècie nativa *A. italicus*. Vam observar que tots els estadis de creixement del simbiont (un total de huit estadis) foren capaços de transmetre's a l'hoste receptor, encara que els més joves i els adults mostraren una major tendència dispersiva. Aquest estudi avança una nova amenaça potencial per la conservació del carranc de riu: la transmissió o "spillover" dels entocitèrids exòtics a poblacions salvatges de carranc natiu europeu és possible, com hem evidenciat ací sota condicions experimentals. No podem predir els efectes d'un simbiont sobre un nou hoste que, a priori, no està evolutivament adaptat a suportar aquest tipus d'organismes. També hem de tenir en compte que, en cas de produir-se un esdeveniment de transmissió, l'eradicació del simbiont exòtic d'una població salvatge de carranc probablement resultaria una tasca impracticable. Per tant, nosaltres recomanem l'adopció d'una estratègia preventiva basada en dos punts: (1) Continuar l'esforç de recerca experimental, amb la finalitat d'analitzar els efectes potencials d'un hipotètic "spillover" sobre el carranc natiu i (2) l'aplicació de mesures preventives per evitar el contacte de poblacions salvatges de carrancs autòctons amb individus de carranc exòtic.

Discussió global i perspectives

Bases bibliogràfiques i metodològiques per l'estudi dels entocitèrids

En primer lloc, amb les nostres aportacions de caire bibliogràfic i metodològic en forma de base de dades biogeogràfica d'Entocytheridae lliure i gratuïta i l'actualització dels llistats d'espècies i referències bibliogràfiques (capítol II), junt a la informació útil per la creació de protocols estandarditzats d'extracció d'entocitèrids dels seus hostes (capítol III), hem contribuït a establir unes bases per a què es puga fer ús dels entocitèrids com a models per a l'estudi dels simbionts metazous. Les bases de dades georeferenciades permeten investigar patrons biogeogràfics a gran escala i comprovar la generalitat de les regles establertes en biogeografia, com per exemple, la disminució de la riquesa d'espècies amb la latitud (Hillebrand, 2004), o la regla de Rapoport (Lomolino et al., 2010). Sobretot, pot resultar interessant l'ús de models d'organismes atípics, com els simbionts. Així, nosaltres hem observat un patró de variació de riquesa amb la latitud en els entocitèrids americans que no s'ajusta a la regla general (capítol II) el qual, probablement, és un reflex del mateix patró observat en el seu principal grup d'hostes, els carrancs de riu americans (France, 1992). L'estret vincle dels entocitèrids amb els seus hostes pot haver tingut implicacions sobre la seua història biogeogràfica. L'existència de bases de dades biogeogràfiques d'accés lliure i gratuït, com la que hem presentat, obri les portes a futurs estudis en aquesta línia i, a més, són ferramentes dinàmiques, les quals poden anar sent actualitzades a mesura que s'aporten noves dades.

Ecologia de poblacions dels entocitèrids

S'han fet grans esforços per buscar patrons generals en ecologia de poblacions de simbionts metazous, amb un enfocament preferent cap als paràsits (Shaw &

Dobson, 1995; Poulin, 2007; Matthee & Krasnov, 2009; Poulin, 2013). És clar que els paràsits, a diferència d'altres grups de simbionts, poden regular les poblacions dels seus hostes a través del seu efecte nociu sobre aquests i, probablement, sofreixen més l'efecte dels mecanismes defensius de l'hoste, com el sistema immunològic (Stanko et al., 2006). Però, podrien aquestes diferències veure's reflectides en l'existència de patrons poblacionals exclusius de simbionts parasitaris? Fruit de la present investigació, hem pogut comprovar que l'ostracode *Ankylocythere sinuosa*, un ectosimbiont d'aigua dolça no parasitari, segueix tres patrons generals establerts en ecologia de paràsits (Poulin, 2007; Matthee & Krasnov, 2009; Poulin, 2013): alts nivells d'agregació, estreta correlació positiva entre abundància mitjana del simbiont i la seua variància i correlació positiva entre taxa d'ocupació d'hostes i abundància per hoste ocupat (capítol V). Els resultats obtinguts suggereixen que les tres "regles de paràsits" podrien ser, en realitat, regles més generals de simbionts metazous.

Les investigacions sobre ecologia de simbionts metazous, com hem dit, principalment enfocades als paràsits de vertebrats, suggereixen que les condicions ambientals juguen un paper irrellevant comparades amb les característiques de l'hoste i del propi simbiont (Krasnov et al., 2005; Poulin, 2006). En contraposició amb aquesta visió, nosaltres hem pogut evidenciar com un ectosimbiont d'aigua dolça no parasitari, associat a un hoste crustaci, es veu marcadament afectat per les condicions climàtiques, tant a nivell temporal (capítol IV) com espacial (capítols V i VI), i considerant diferents escales espacials (capítols IV, V i VI). A més, els efectes s'observaren tant sobre la seua distribució geogràfica (capítol VI), com la taxa d'ocupació d'hostes entre distintes localitats (capítol V) o l'abundància d'entocitèrids entre distints mesos de l'any (capítol IV). En referència al darrer fet, vam constatar l'efecte de dilució de les abundàncies del simbiont en hivern, com a conseqüència de la colonització de la nova cohort de carrancs de l'any (Young, 1971). No obstant, l'efecte de dilució, fenomen observat en altres grups de simbionts (Krasnov et al., 2007), no es véu reflectida a nivell espacial (capítol V), suggerint que l'entocytherid estudiat, *A. sinuosa*, restableix les seues infrapoblacions la primavera següent, i l'efecte de dilució és solament estacional. D'altra banda, les abundàncies d'*A. sinuosa* també es veieren afectades per la química de l'aigua, tant a nivell temporal (capítol IV) com espacial (capítol V). Per tant, la importància de les condicions ambientals depèn del sistema de simbiont considerat, de manera que alguns grups particulars de simbionts podrien ser especialment sensibles a la variabilitat ambiental, per exemple els ectosimbionts d'aigua dolça no parasitaris com els entocitèrids. La recerca enfocada en simbionts no parasitaris i una visió més ampla de l'ecologia de simbionts metazous, no exclusivament enfocada als paràsits, contribuiria a discernir entre les implicacions generals de l'estil de vida simbiont i aquelles específiques del tipus de relació simbiont-hoste (parasitària, comensal o mutualista), en relació a aspectes ecològics com l'existència de patrons característics en els paràmetres poblacionals, el grau d'independència de l'ambient extern a l'hoste o els tipus de mecanismes reguladors de les abundàncies dels simbionts.

Simbionts i invasions: el cas dels entocitèrids

Les troballes recents d'espècies exòtiques d'entocitèrids a Europa (capítols I i VI) ens han permès utilitzar-los com a model de simbiont invasor (capítol VI), per avaluar la importància de factors abiòtics i biòtics com a limitants de l'expansió del simbiont. Tot i que les interaccions biòtiques juguen un paper destacat en els processos d'invasió (Peterson et al., 2011; Wisz et al., 2013), generalment han sigut desconsiderades degut a la seua gran complexitat, que dificulta el desenvolupament de metodologies i tècniques estadístiques i d'anàlisi espacial amb capacitat d'incorporar factors biòtics (Elith & Leathwick, 2009). Tanmateix, s'estan aconseguint avanços (Kissling et al., 2012). Des d'aquest punt de vista, els models de simbionts, que impliquen l'existència d'interaccions biòtiques molt fortes, com és la necessitat de presència de l'hoste per poder subsistir, tal vegada podrien ajudar a abordar els factors biòtics en les invasions. En relació al model dels simbionts en biologia de les invasions, existeix un filtre en els passos preliminars d'un procés d'invasió que selecciona tan sols un subgrup de simbionts que aconsegueixen arribar a l'àrea envaïda junt amb l'hoste invasor (Torchin et al., 2003). Però, què determina quines espècies de simbionts acompanyen a l'hoste invasor? En el cas dels entocitèrids, de les 183 espècies que conviuen amb carrancs en Nord Amèrica, només hem detectat dues espècies exòtiques a Europa (capítol I, VI). Evidentment, el fet d'estar associades a una de les espècies de carranc amb potencial invasor és un requisit previ, i el procés va associat a un important component d'atzar. Però, a més, la baixa especificitat d'hoste pot ajudar a un simbiont a encertar amb l'hoste adequat. Malgrat que, en general, la baixa especificitat està ben establerta en els entocitèrids (capítol II), amb aprox. un 64 % de les espècies observades en associació amb més d'una espècie d'hoste, hi han diferències marcades entre espècies. De fet, sols 4 espècies arriben a superar els 40 hostes. Curiosament, *A. sinuosa*, amb 47 espècies d'hoste registrades, forma part d'aquest grup privilegiat. En el cas d'*A. sinuosa*, també observàrem, en general, altes taxes d'ocupació d'hostes en les poblacions exòtiques europees analitzades (capítol I, IV i V). Una alta taxa d'ocupació d'hostes augmenta la probabilitat d'estar present en els hostes invasors fundadors, i disminueix la probabilitat d'extinció global en les àrees envaïdes (Bush & Kennedy, 1994). Ambdues característiques, i.e. baixa especificitat d'hoste i elevades taxes d'ocupació, poden haver contribuït a l'èxit invasor d'*A. sinuosa*. Aquelles investigacions que aprofundiren en l'estudi de les característiques dels simbionts que els converteixen en invasors reeixits aportarien llum a la comprensió del mecanisme de filtre de simbionts evidenciat per Torchin et al. (2003).

Però, una vegada establert el simbiont, és capaç d'acompanyar al seu hoste invasor allà on vaja? O, per contra, poden existir altres filtres de caire espacial en etapes més avançades de la invasió? Els models que desenvolupem en el capítol VI suggereixen que pot ocórrer el segon cas, és a dir, un simbiont, *A. sinuosa* en el nostre cas, pot veure's limitat per les seues pròpies toleràncies fisiològiques a les condicions climàtiques en determinades regions geogràfiques susceptibles de ser envaïdes pel seu hoste, *P. clarkii*. Segons les nostres prediccions, l'arribada de l'hoste invasor a aquestes regions amb restriccions climàtiques per al simbiont, suposarien un alliberament, per part de l'hoste, de la seua interacció simbiòtica, fenomen que podria tenir efectes sobre la seua capacitat invasora, ja que el simbiont pot interferir en el procés d'invasió, directament a través del seu efecte sobre l'hoste, o bé a través de la interacció amb altres hostes natius de l'àrea envaïda (Prenter et al., 2004; Roy & Handley, 2012). Les restriccions climàtiques del simbiont a què hem fet referència serien especialment proclius a existir en models de simbiont especialment sensibles a les condicions ambientals externes a l'hoste, com hem vist que ocorre amb els entocitèrids (capítols IV i V). Un aspecte important de la biologia de les invasions que no hem tractat és l'anàlisi de l'estructura genètica de les poblacions invasores, tant del simbiont com de l'hoste, comparada amb les poblacions del rang natiu. Això ens permetria detectar possibles efectes de coll de botella, o de múltiples introduccions o translocacions, sobre l'estructura genètica poblacional dels simbionts invasors i els seus hostes i, a més, podria donar pistes sobre l'origen de les poblacions invasores (Kelly et al., 2006). Futurs estudis experimentals de tolerància dels entocitèrids a diferents rangs de condicions abiòtiques, com s'ha fet per a altres grups d'ostracodes (Mezquita et al., 1999; Aguilar-Alberola & Mesquita-Joanes, 2011), contribuirien a establir de manera més precisa els seus límits fisiològics, informació valuosa alhora d'interpretar i millorar els nostres models d'anàlisi dels paràmetres poblacionals dels entocitèrids (capítols IV i V) i d'avaluació dels factors limitants de l'expansió d'entocitèrids exòtics en l'espai geogràfic (capítol VI).

Entocitèrids exòtics i el carranc de riu natiu europeu

Amb l'experiment del capítol VII, hem demostrat la transmissió horitzontal d'un entocitèrid exòtic a un carranc natiu europeu. A més, l'eficàcia de transmissió fou molt elevada, i tots els huit estadis de creixement de l'entocitèrid *A. sinuosa* foren capaços de colonitzar el nou hoste de forma activa (a través de la dispersió al medi extern i posterior contacte amb el nou hoste). Això concorda amb els experiments de Young (1971) i amb les altes taxes d'ocupació d'hostes que mostraren les poblacions exòtiques europees d'*A. sinuosa* (capítols I, IV i V). En suma, els resultats evidencien que els entocitèrids mostren una alta transmissibilitat que, en part, explicaria, d'una banda, el fet que les taxes d'ocupació d'hostes es mantinguen elevades al llarg de l'any (capítol IV) i, d'altra banda, l'efecte positiu de la densitat poblacional de l'hoste sobre la taxa d'ocupació d'hostes del simbiont, dues característiques que indiquen que els nous reclutaments d'hostes són eficaçment colonitzats pel simbiont (Stanko et al., 2006). La transmissió d'*A. sinuosa* a un carranc natiu europeu sota condicions experimentals (capítol VII), junt amb l'observació de l'ampla presència d'entocitèrids exòtics a Europa (capítol VI), evidencien el risc de la seua transmissió a poblacions salvatges de carranc natiu. Les conseqüències ecològiques del procés són desconegudes, però podrien tenir efectes perniciosos sobre la conservació dels carrancs de riu. La hipòtesi establerta sobre la relació dels entocitèrids amb els seus hostes és el comensalisme (Hart & Hart Jr, 1974). No obstant, no està fonamentada en una base experimental. En simbionts similars suposadament no parasitaris, com els branquiobdèlids, s'ha vist que les relacions simbiont-hoste poden ser molt complexes i sofrir variacions entre el mutualisme i el parasitisme en funció de diferents factors, com l'espècie de simbiont, la seua abundància o el context ecològic (Skelton et al., 2013). A més, no podem predir els efectes d'un simbiont sobre un hoste novell que, a priori, no estaria evolutivament adaptat al nou simbiont (Mastitsky et al., 2010). D'altra banda, els ostracodes poden ser utilitzats com a hostes intermediaris d'alguns paràsits (Moravec, 2004). De fet, els entocitèrids també tenen la seua pròpia biota associada (capítol I). Per tant, les transmissions de simbionts patògens a través dels entocitèrids és una possibilitat a considerar.

Des del nostre punt de vista, futures investigacions sobre els entocitèrids ajudarien a aclarir el grau d'amenaça real que suposen els entocitèrids exòtics per a la conservació de la biodiversitat. En primer lloc, la realització d'experiments per comprovar l'èxit reproductiu dels entocitèrids exòtics en el carranc natiu europeu ens permetrien avaluar la viabilitat de la transmissió. En segon lloc, l'experimentació per analitzar els efectes dels entocitèrids sobre els seus hostes en termes de creixement, supervivència o taxes de reproducció seria valuosa, ja fora aplicada a la relació dels entocitèrids amb els seus hostes natius, com a la dels entocitèrids exòtics amb hostes novells, com ara el cas del carranc natiu europeu. El carranc exòtic *P. leniusculus* hauria de rebre especial atenció, ja que allotja entocitèrids exòtics (capítol VI) i mostra preferències d'hàbitat similars a les d'espècies de carranc autòcton amenaçades, com *A. pallipes* i *A. italicus* (Hiley, 2003; Vedia & Miranda, 2013). Finalment, la nostra investigació sobre els entocitèrids afegeix suport a la idea de protegir els santuaris aïllats que mantenen poblacions salvatges de carranc natiu estables i lliures de contacte amb carrancs exòtics (Kozák et al., 2011).

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Conclusions

1. Hem pogut constatar, per primera vegada, l'ampla distribució de dues espècies d'ostracodes entocitèrids exòtics a Europa, associats a carrancs invasors americans. En canvi, no s'ha pogut trobar cap espècie d'entocitèrid nativa associada a carrancs autòctons a Europa, una absència notable des del punt de vista biogeogràfic.
2. Hem contribuït a establir unes bases bibliogràfiques per a l'estudi dels entocitèrids, a través de l'aportació d'una base de dades georeferenciada d'escala global, i els llistats actualitzats de les espècies i la bibliografia del grup.
3. Hem confirmat que la baixa especificitat d'hoste està ben establerta en els entocitèrids, amb aproximadament un 64 % de les espècies, observades en associació amb més d'una espècie d'hoste.
4. L'anàlisi biogeogràfica de la base de dades també mostra que els entocitèrids americans (186 spp.) tenen un patró atípic de variació de riquesa amb la latitud, sense un augment monotònic de la biodiversitat en reduir-se la latitud, com seria esperable de patrons generals latitudinals. Probablement siga degut, en gran part, al seu lligam estret amb la història biogeogràfica dels seus hostes, els carrancs de riu americans.
5. La comparació de l'eficiència en l'extracció d'entocitèrids utilitzant clorobutanol i aigua carbonatada en diferents temps d'immersió ens ha permès decidir-nos pel mètode més barat i de fàcil obtenció de l'aigua carbonatada (5–15 min d'exposició) per a estudis ecològics posteriors, excepte en casos en què es requeria la supervivència del carranc, on el clorobutanol es mostrà més adequat.
6. Hem evidenciat el compliment de tres patrons generals establerts en ecologia de poblacions de paràsits en un ectosimbiont no parasitari, l'ostracode comensal *Ankylocythere sinuosa*. Això suggereix que algunes "regles de paràsits" podrien ser, en realitat, regles més generals dels simbionts.
7. Hem corroborat l'"efecte de dilució" de les abundàncies d'*A. sinuosa*, observat

per Young (1971), com a conseqüència de la colonització de la nova cohort d'hostes joves de l'any. Tanmateix, el simbiont restableix les seves infrapoblacions la primavera següent, i l'efecte de dilució és solament estacional.

8. D'acord amb els nostres models, les poblacions de l'entocitèrid exòtic *A. sinuosa* es veuen marcadament afectades per les condicions climàtiques: (1) a escala europea, a través de limitacions en la seua distribució, (2) a escala regional, a través de la generació de variabilitat interpoblacional de les taxes d'ocupació d'hoste, i (3) a escala local, en forma de variació estacional de les abundàncies del simbiont.

9. Les poblacions d'*A. sinuosa* també es veuen afectades per variables relacionades amb la química de l'aigua, principalment la conductivitat (contingut en sals de l'aigua), les quals estan implicades en la variació inter- i intrapoblacional de les abundàncies del simbiont, en el darrer cas, reflectida en l'existència de fluctuacions estacionals.

10. Els efectes ambientals (climàtics i limnològics) significatius suggereixen que, malgrat que l'estil de vida simbiont ofereix una certa estabilitat ambiental, el grau d'independència front a les condicions ambientals externes a l'hoste depèn del sistema simbiont considerat, de manera que alguns grups particulars de simbionts podrien ser especialment sensibles a la variabilitat ambiental, per exemple els ectosimbionts d'aigua dolça no parasitaris, com els entocitèrids.

11. L'escassa riquesa d'entocitèrids dels carrancs exòtics a Europa comparada amb la dels seus rangs natius afegeix suport a l'existència del filtre de simbionts en les etapes inicials del procés d'invasió evidenciat per Torchin et al. (2003).

12. Les altes taxes d'ocupació d'hostes, per dalt del 90 %, i la baixa especificitat d'hoste (47 hostes) d'*A. sinuosa*, poden ser algunes de les característiques relacionades amb l'èxit invasor de l'entocitèrid.

13. La combinació d'un marc teòric basat en la teoria de conjunts amb l'aplicació de models de nínxol ecològic (MNEs) poden servir per analitzar el paper de factors abiòtics, biòtics i dispersius com a elements limitants de l'expansió d'una espècie invasora, com hem mostrat basant-nos en un model de simbiont invasor.

14. Els nostres models d'avaluació de les restriccions geogràfiques a l'expansió dels entocitèrids exòtics suggereixen la possibilitat d'existència de filtres espacials deguts a limitacions climàtiques del simbiont, durant l'etapa d'expansió de l'hoste invasor, que implicarien la pèrdua del simbiont en aquelles àrees envaïdes per l'hoste afectades pels filtres.

15. Hem comprovat l'èxit en la transmissió d'un entocitèrid exòtic, *A. sinuosa*, a un carranc de riu natiu europeu, sota condicions experimentals. Això, junt

amb l'ampla presència d'entocitèrids exòtics a Europa, fa patent una certa probabilitat de transmissió d'entocitèrids a poblacions salvatges de carranc natiu. Per tant, s'hauria d'avaluar experimentalment l'amenaça real que suposaria l'esdeveniment per a la conservació.

Conclusions

1. We observed, for the first time, two exotic entocytherid species to be widely distributed in Europe, associated with American invasive crayfish. However, we did not find any native entocytherid species inhabiting autochthonous European crayfish, a remarkable absence from a biogeographic point of view.
2. We contributed to establish a bibliographic base for research on entocytherids, by providing a global-scale georeferenced database, and updated bibliographic and species checklists of the group.
3. We evidenced that a low host specificity is well established in Entocytheridae, with approx. 64 % of species registered in association with more than one host species.
4. The biogeographic analysis of our database showed that the American entocytherids (186 spp.) have an unusual latitudinal pattern of species richness, not showing the expected monotonic decrease in richness with latitude according to general latitudinal patterns. Probably, this is mainly due to their tight link with the biogeographic history of their hosts, the American crayfish.
5. The comparison of the efficiency in entocytherid removal from its host by using chlorobutanol or carbonated water, with different submersion times, allowed us to choose the cheapest and readily available method of carbonated water (5–15 min of exposure) for subsequent ecological surveys, with exception of those that required the crayfish survival, in which chlorobutanol showed a better adequacy.
6. We evidenced the accomplishment of three general patterns established in population ecology of parasites in an non-parasitic ectosymbiont, the commensal ostracod *Ankylocythere sinuosa*. This suggests that some "parasite rules" could be, actually, more general rules for symbionts.
7. We found support for the "dilution effect" of *A. sinuosa* abundances, observed by Young (1971), as a consequence of the colonization of the new recruited host cohort of the year. However, the symbiont recovers its infrapopulations

the following Spring, so that the dilution effect is only seasonal.

8. According to our models, the exotic populations of *A. sinuosa* were markedly affected by climatic conditions: (1) at the European scale, through limitations to geographical distribution, (2) at a regional scale, through the generation of interpopulation variablility of host occupancy, and (3) at a local scale, as seasonal variation of symbiont abundances.

9. The *A. sinuosa* European populations were also affected by variables related to water chemistry, mainly conductivity (water salt content), which were involved on inter- and intrapopulation variation in symbiont abundances, in the last case, reflected in the existence of seasonal fluctuations.

10. The significant environmental effects (climatic and limnological) suggest that, although symbiont life style offers certain environmental stability, the degree of independence from off-host environmental conditions depends on the symbiont system considered, so that some particular symbiont groups could be specially sensitive to environmental variability, for example non-parasitic freshwater ectosymbionts, as the entocytherids.

11. The poor entocytherid species richness found in exotic crayfish in Europe compared to the native range adds support to the existence of filters acting against symbionts in the initial steps of an invasion process, evidenced by Torchin et al. (2003).

12. The high host occupancy, above 90 %, and the low host specificity (47 hosts) of *A. sinuosa*, can be some of the features related to the invasion success of the exotic entocytherid.

13. The combination of a theoretical frame based on set theory with the application of ecological niche models (ENMs) can serve to analyse the role of abiotic, biotic and dispersal factors as restrictive elements for the geographical spread of an invasive species, as we showed based on a model of a symbiont invader.

14. Our models for assessing geographical restrictions to the spread of exotic entocytherids suggest the existence of spatial filters as a consequence of symbiont climatic limitations, which would imply the symbiont loss in those areas invaded by the host and affected by the filters.

15. We evidenced the transmission success of an exotic entocytherid, *A. sinuosa*, onto a European native crayfish, under experimental conditions. This, together with the wide presence of exotic entocytherids in Europe, highlight a certain probability of entocytherid transmission to wild populations of native crayfish. Thus, it should be convenient to experimentally assess the actual degree of threat that would mean such an event for crayfish conservation.

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